The Kinetic Reaction Mechanism of the *Vibrio cholerae* Sodium-dependent NADH Dehydrogenase**

The sodium-dependent NADH dehydrogenase (Na⁺-NQR) is the main ion transporter in *Vibrio cholerae*. Its activity is linked to the operation of the respiratory chain and is essential for the development of the pathogenic phenotype. Previous studies have described different aspects of the enzyme, including the electron transfer pathways, sodium pumping structures, cofactor and subunit composition, among others. However, the mechanism of the enzyme remains to be completely elucidated. In this work, we have studied the kinetic mechanism of Na⁺-NQR with the use of steady state kinetics and stopped flow analysis. Na⁺-NQR follows a hexa-uniping-pong mechanism, in which two redox states participate in the catalytic cycle. This conformation, the enzyme is able to capture two sodium ions and transport them to the external side of the membrane. In the last step, ubiquinone is bound and reduced, and ubiquinol is released. Our data also demonstrate that the catalytic cycle involves two redox states, the three- and five-electron reduced forms. A model that gathers all available information is proposed to explain the kinetic mechanism of Na⁺-NQR. This model provides a background to understand the current structural and functional information.

The sodium-dependent NADH dehydrogenase (Na⁺-NQR) is the main ion transporter in *Vibrio cholerae*, and it is the first enzyme in the respiratory chain (1, 2). Na⁺-NQR transfers electrons from the cytosolically produced NADH to ubiquinone (3–5), which is a hydrophobic molecule located in the plasma membrane, thus feeding the lower part of the respiratory chain. This redox reaction releases a large amount of energy, which is used to pump ions. Hence, Na⁺-NQR fulfills a similar role as complex I, but in contrast with this enzyme, which is a proton pump, Na⁺-NQR pumps specifically sodium (6). These two complexes are not related, and their subunit and cofactor composition and their reaction mechanisms are completely different (7).

Na⁺-NQR is a common component of the respiratory chain of pathogenic bacteria, such as most γ-Proteobacteria, Bacteroidetes, and Chlamydiae (8). Its activity seems to be essential in many of these microorganisms, sustaining different processes such as pH regulation, the transport of nutrients, the synthesis of ATP (driven by ΔΨ), the elimination of drugs, and the secretion of virulence factors (2, 8–10).

Na⁺-NQR is composed of six subunits and five redox cofactors (3–5). The NqrA subunit is cytosolic, does not contain any transmembrane helices or cofactors (11, 12), and seems to fulfill structural roles (5). The NqrB subunit contains the attachment site for one covalently bound FMN molecule (13), as well some of the structures involved in the transport of sodium, including one of the at least two sodium-binding sites (14–17). Indeed, the mutation of aspartate residue 397 affects the positive cooperativity of the sites (15), changes the cation selectivity, and produces an uncoupling effect in the presence of potassium (17). Recent crystallographic data have shown that NqrB contains 10 transmembrane helices (12), with an organization that allows the transport of sodium. Previous studies suggested that NqrA might be able to bind up to two ubiquinone molecules (18). However, our data have demonstrated that the native complex binds only one ubiquinone molecule and that the ubiquinone binding to the isolated NqrA seems to be nonspecific, because relatively low amounts of detergent prevent the interaction (19). Moreover, the tridimensional structure shows that NqrA, isolated or in the complex, is devoid of ubiquinone and lacks a pocket that can accommodate this substrate (12), although it is still possible that NqrA might bind the noncatalytic tightly bound ubiquinone molecule (19). In contrast, it has been shown that the catalytic binding site for ubiquinone is located in NqrB, in which glycine residues 140 and 141, located in transmembrane helix III of NqrB, control the conformational changes that allow the binding of this substrate (19, 20).

---

**Background:** Na⁺-NQR is the main sodium pump in *Vibrio cholerae*.

**Results:** Na⁺-NQR follows a hexa-uniping-pong mechanism, in which two redox states participate in the catalytic cycle.

**Conclusion:** A comprehensive kinetic model of the reaction catalyzed by Na⁺-NQR is proposed, covering pre-steady and steady conditions.

**Significance:** The kinetic model proposed provides a background to understand the structural and functional data.
Kinetic Mechanism of \( \text{Na}^{+} \)-NQR

The location of these residues with respect to other cofactors seems to favor this role (12). The crystallographic structure shows that the NqrC subunit contains only one transmembrane segment and a large hydrophilic domain that faces the extracellular side of the membrane (12), contrasting with previous topological models (11). The soluble domain contains the binding site of the second covariantly bound FMN molecule (13). The subunits NqrD and NqrE are homologous transmembrane proteins (11, 12) that show an antiparallel arrangement of the transmembrane domains. Together with NqrB, these subunits participate in the transport of sodium, due to the presence of several conserved acid residues, whose mutants block specifically the sodium sensitive activity (14). The crystal structure indicates that these subunits might also bear a sixth cofactor, a single iron atom coordinated through four conserved cysteine residues (12). However, the evidence obtained from different methodologies, including EPR, does not support the presence of the iron center in the enzyme (see “Discussion”) (21–25). The NqrF subunit is a monotopic membrane protein with a cytosolic domain that faces the internal side of the membrane (11, 12), which bears the binding sites for the substrate NADH and for the cofactors FAD and the 2Fe–2S center (26). \( \text{Na}^{+} \)-NQR is the only reported enzyme, and perhaps one of the few in nature, that uses riboflavin as a cofactor (27, 28). Although the crystallographic data offers new clues about the location of this cofactor, its binding site remains unidentified.

\( \text{Na}^{+} \)-NQR belongs to a family of membrane-bound redox-driven pumps that has evolved independently from the main families of respiratory enzymes and ion pumps (8), and it shows novel characteristics, such as distinctive folding patterns in some of its subunits (12), the use of riboflavin as a cofactor (27), and mechanisms of ion translocation that are very different compared with other respiratory enzymes (30). The studies that have been performed on this enzyme have clarified the stoichiometry of the reactions, the types of side reactions catalyzed by the enzyme, most substrate- and cofactor-binding sites, the pathway of electron transfer, the physicochemical properties of the cofactors, and some of the structures involved in sodium transport (4, 5). However, the kinetic reaction mechanism of the enzyme remains unclear. In this work, the pathways used by \( \text{Na}^{+} \)-NQR to react with the redox substrates and transport sodium were studied. The data show that the enzyme follows a hexa-uni ping-pong mechanism, in which NADH reduces the enzyme and NAD leaves the binding site. This step is followed by the binding of two intracellular sodium ions, which are subsequently transported to the extracellular side of the membrane. In the last step, ubiquinone is bound, and ubiquinol is released before the next NADH molecule can react with the enzyme. Moreover, the data shown in this report allows the identification of the catalytically active redox states of \( \text{Na}^{+} \)-NQR. The enzyme can adopt multiple redox states, due to the presence of the five cofactors (many of which can accommodate one or two electrons). To fully understand its reaction mechanism, we followed the pre-steady state pathways of electron transfer in the enzyme exposed to the three substrates. According to our data, the oxidized form of \( \text{Na}^{+} \)-NQR (which carries one unpaired electron in riboflavin) is not active and reacts with one NADH molecule, producing the three-electron reduced form, which enters the catalytic cycle. The enzyme is then reduced by a second NADH molecule producing a transient five-electron reduced state, which is able to bind sodium. The one-electron reduction of the FMN cofactor in NqrC triggers the closing of the intracellular gates and promotes the transport of sodium. In the last step of the cycle, ubiquinone oxidizes the five-electron reduced form, producing the three-electron reduced state, which can continue the catalytic cycle. The data presented here are essential to understand the mechanism of the enzyme and to clarify different aspects of the current crystallographic structure, such as the number and properties of the conformational states.

Experimental Procedures

Elimination of the Genomic nqr Operon—In these experiments, the full-length \( \text{Na}^{+} \)-NQR complex produced in its natural host \( V. \text{cholerae} \) was characterized. The nqr operon was eliminated by homologous recombination, following the protocol described by Marvig and Blokesch (31). O395N1 \( V. \text{cholerae} \) cells were grown in M9 medium supplemented with 32 mM MgCl₂. The cells were harvested, washed, resuspended, and incubated for 20 h with 50 mg of chitin flakes. Previous studies have demonstrated that the exposure to chitin produces naturally competent \( V. \text{cholerae} \) cells that are able to uptake external DNA (32). The cells were washed one more time and incubated for 24 h with the donor DNA, consisting of the chloramphenicol resistance cassette and enhanced GFP sequence, flanked by 150 bases of the nqr operon (see below). The cells were plated, and the chloramphenicol-resistant fluorescent colonies were selected. The elimination of the operon was confirmed by PCR.

To eliminate the nqr operon by homologous recombination, two regions of 150 bp flanking the operon were cloned and added to the 5’ and 3’ ends of the chloramphenicol resistance cassette and enhanced GFP sequence. The recombinant DNA was assembled together by Gibson cloning and inserted in pUC19 using the primers shown in Table 1. \( V. \text{cholerae} \) genomic DNA, pDK3 and pLND-GFP plasmids were used as templates, respectively. The recombinant DNA construct was amplified by PCR and used in the transformation.

nqr Operon Cloning—\( V. \text{cholerae} \) O395N1 cells were grown in LB medium for 12 h. The cells were harvested, and the total genomic DNA was obtained, using the GeneJet Genomic DNA purification kit (Thermo Scientific). The entire nqr operon was cloned by PCR and inserted in-frame into the 5’-XhoI and 3’-KpnI restriction enzyme sites of pBAD/HisB, using the primers listed in Table 1. The forward primer contained a stop codon to eliminate the histidine tag from the N terminus of the resulting protein and a ribosome-binding site. The reverse primer contained a spacer of four glycine residues followed by six histidine residues and a stop codon. The sequence of the cloned operon was confirmed by sequencing.

\( \text{Na}^{+} \)-NQR Expression and Purification—\( V. \text{cholerae} \) O395N1 Δnqr cells harboring the nqr operon cloned in the pBAD/HisB vector were grown at 37 °C in LB medium and supplemented with 50 mM glucose, in 2-liter culture flasks, under constant agitation. The cells were harvested by centrifugation and
The interaction of each substrate will be perturbed by the inhibitor or the product, providing indications of the order in which the substrates react or are bound. The data obtained with HQNO and CoQH$_2$-1 were globally fitted to the equations describing the behavior of competitive (Equation 4), uncompetitive (Equation 5), and mixed inhibitors (Equation 6).

The inhibitor is represented by I, with $K_i$ as the kinetic inhibition constant. In the case of mixed inhibition, the two $K_i$ values describing the two components of the inhibition are $K_{i,c}$ (competitive component) and $K_{i,u}$ (uncompetitive).

**Fast Kinetics Experiments**—Stopped flow kinetics experiments were performed to understand the pathways of electron flow in wild-type Na$^+$-NQR in the presence of the three substrates. The purified enzyme (20 µM) was suspended in reaction buffer containing 200 µM CoQ-1, 100 µM NaCl, and 0.1% dodecyl maltoside. This solution was mixed (1:1) with buffer containing 600 µM NADH. Data analysis and spectral component assignments were performed as described previously (24, 27).

### Pre-steady State Membrane Formation

The fluorescent indicator RH421 was used to measure the formation of membrane potential. This dye has a very fast response to the changes in the membrane potential and has been used to study the pre-steady state kinetics of the Na$^+$-K$^+$-ATPase (35–39). The purified Na$^+$-NQR was reconstituted into proteoliposomes, following the protocol described before (14). RH421 (100 nM) was added to the reconstituted enzyme, and the suspension was mixed (1:1) with buffer containing 600 µM NADH, 100 µM CoQ-1, and 100 mM NaCl. The fluorescence was measured in the SX20 stopped flow (Applied Photophysics), using a filter of 600 nm. To eliminate the residual contributions of the flavins,
the fluorescence of a sample that did not contain the dye was subtracted from the raw data.

**Results**

**Initial Velocity Plots at Different Substrate Concentrations**

To understand the kinetic mechanism of Na\(^+\)-NQR, we performed a characterization of the enzyme activity under steady state conditions. In this protocol, the initial activity of the enzyme was measured at variable concentrations of one of the substrates, assaying different fixed-variable concentrations of a second substrate at a saturating concentration of the third one (40). For instance, Fig. 1A shows the activity obtained at different concentrations of NADH, using several fixed-variable concentrations of NaCl as follows: 1 mM (○), 2 mM (□), 5 mM (■), 10 mM (▲), 20 mM (●), and 50 mM (△). The double-reciprocal plot is shown in B. C, NADH concentration was varied; the concentration of NaCl was saturating (50 mM), and the following concentrations of CoQ-1 were fixed variables: 1 μM (○), 2 μM (□), 5 μM (■), 10 μM (▲), 20 μM (●), and 50 μM (△). The double-reciprocal plot is shown in D. E, CoQ-1 concentration was varied at saturating concentrations of NADH (250 μM), and at fixed variable concentrations of NaCl as follows: 1 mM (○), 2 mM (□), 5 mM (■), 10 mM (▲), 20 mM (●), and 50 mM (△). The double-reciprocal plot is shown in F.
kinetic mechanisms. The best fit was obtained with the ping-pong mechanism equation, in which a ternary complex of the enzyme with the two substrates bound is not formed. Instead, the enzyme reacts with one substrate, and the first product leaves before the second substrate is able to react. A characteristic feature of the ping-pong mechanism is a pattern of parallel lines in the double-reciprocal plots, contrasting with the intercepting pattern obtained with random and ordered mechanisms (40). Fig. 1B shows the Lineweaver-Burk plot of this data set, with the parallel line behavior, supporting the ping-pong mechanism of NADH versus NaCl. This analysis was performed with the other two substrate pairs, NADH versus CoQ-1 and CoQ-1 versus NaCl. Fig. 1C shows the activity of the enzyme at different concentrations of NADH, as in the previous case, but under different fixed-variable concentrations of CoQ-1, at saturating concentrations of NaCl (50 mM). Fig. 1E shows the activity of the enzyme at different concentrations of CoQ-1, at different fixed-variable concentrations of NaCl, and at saturating concentrations of NADH (250 µM). As in the previous cases, the data were globally fitted to the equations of several kinetic models, and the best fitting was obtained with the equation of the ping-pong mechanism. To corroborate this behavior, the data were re-plotted as double reciprocals, and parallel line patterns were obtained (Fig. 1, D and F), strongly supporting a ping-pong mechanism for the three pairs of substrates. The kinetic constants obtained from the fitting of the data are found in Table 2. The \( k_{\text{cat}} \) and \( K_m \) values are very similar to the values found previously for \( V.\ choleræ \) Na\(^+\)-NQR (6, 14).

Substrate Reaction Order and Inhibitor and Product Effects—The kinetic pattern obtained with the three substrate pairs demonstrates that Na\(^+\)-NQR follows a hexa-uni ping-pong mechanism, in which the substrates react with the enzyme and each of the products is released before the next substrate can be bound. However, the data do not provide indications about the order in which the substrates react (or are transported, in the case of sodium). Studies using inhibitors and products of the reaction were performed to address this question. The inhibitor and product interaction with the enzyme can provide indications of whether they compete with a specific substrate or whether the substrate and the inhibitor can be bound simultaneously to the enzyme, producing mixed or uncompetitive inhibition, and thus can help to understand the way in which substrates react (40).

Fig. 2 shows the activity of Na\(^+\)-NQR with different concentrations of NADH (Fig. 2A), CoQ-1 (Fig. 2C), and NaCl (Fig. 2E), in the presence of variable concentrations of the inhibitor HQNO (34), at fixed saturating concentrations of the other two substrates. The data were globally fitted to the different inhibition mechanisms discussed under “Experimental Procedures,” and the assignments of the type of inhibitory effect were corroborated by the analysis of the double-reciprocal plots. HQNO behaves as a mixed inhibitor against NADH as shown by the global analysis, with double-reciprocal plots intercepting in the abscissa (Fig. 2B), which indicates that the competitive and uncompetitive components are of equal magnitude (\( K_{i,c} = K_{i,u} \)) (Table 2). In contrast with previous analyses that have proposed that the enzyme contains several binding sites for ubiquinone and its analogs (41), we have demonstrated that the stoichiometry of binding of HQNO to the active complex is 1:1 (19). Thus, this inhibition pattern indicates that HQNO can interact with two different forms of the enzyme, as an analog of both the substrate (CoQ-1) and the product, ubiquinol (CoQH\(_2\)-1). Fig. 3 shows the proposed mechanism of inhibition of HQNO. The inhibitor acts as a ubiquinol analog, competing with NADH for the free form of the enzyme (\( E \)), and as a ubiquinone analog, competing for its binding site in the G form, explaining the competitive and uncompetitive components of the inhibition toward NADH. In contrast, HQNO behaves as a mixed inhibitor against ubiquinone (Fig. 2D), which corroborates that it is able to interact with the form of the enzyme that naturally binds ubiquinone and with the free form of the enzyme, explaining both inhibition components. Finally, HQNO behaves as a purely uncompetitive inhibitor against sodium, as demonstrated by the global fitting and the parallel double-reciprocal plots (Fig. 2F), which indicates that it does not compete with sodium for any form. As a whole, the data are consistent with the kinetic mechanism proposed in Fig. 3. According to this mechanism, the NADH molecule reduces Na\(^+\)-NQR and leaves as NAD. Consistent with the stoichiometry of sodium pumped per electron, as reported previously (4), two intracellular sodium ions are then bound by the enzyme and transported to the extracellular side of the membrane. In the last step, ubiquinone is bound and reduced, and the ubiquinol molecule is released. An important characteristic of this mechanism is that no tertiary or quaternary complexes were found, such as enzyme-NADH-Na\(^+\) or enzyme-NADH-Na\(^+\)-CoQ-1. It should be pointed out that the reactions shown in Fig. 3 correspond exclusively to sequence of events found under steady state conditions (for a comprehensive view of the catalytic mechanism, covering pre-steady and steady state, see Fig. 7).

To confirm this interpretation of the kinetic experiments, the inhibitory effects of CoQH\(_2\)-1, one of the products of the reaction, were tested. These experiments were necessary, especially because the inhibition pattern found with HQNO was identical with NADH and CoQ-1. We have reported previously that CoQH\(_2\)-1 is able to reduce the enzyme in the millimolar range (27), indicating that it is a suitable candidate for inhibition experiments. Fig. 4 shows the inhibition patterns obtained with CoQH\(_2\)-1 against the three substrates of the enzyme. CoQH\(_2\)-1 behaves as a purely competitive inhibitor toward NADH (Fig. 4, A and B), with a \( K_i \) of 3.1 mM (Table 2), which indicates that NADH is the first substrate to react and CoQH\(_2\)-1 is the last product to be released, both interacting with the free form of the enzyme (Fig. 3). CoQH\(_2\)-1 is a purely uncompetitive inhibitor toward sodium (Fig. 4, E and F), which

### TABLE 2

| Kinetic constant | Ligand | Value |
|-----------------|--------|-------|
| \( k_{\text{cat}} \) | CoQ-1  | 490 ± 25 s\(^{-1}\) |
| \( K_m \)      | NADH   | 240 ± 15 µM |
| \( K_i,c \)    | NaCl   | 2.4 ± 0.6 mM |
| \( K_i,u \)    | HQNO   | 0.22 ± 0.04 µM |
| \( K_i,u \)    | CoQH\(_2\)-1 | 3.1 ± 0.6 mM |

**Kinetic Mechanism of Na\(^+\)-NQR**

Based on the analysis presented, the kinetic mechanism of Na\(^+\)-NQR can be described as follows: NADH is the first substrate to react, followed by CoQ-1, and finally NaCl. The enzyme contains binding sites for ubiquinone and its analogs, allowing for competitive and uncompetitive interactions. The inhibition pattern observed with HQNO supports the ping-pong mechanism, which is characterized by parallel lines in the double-reciprocal plots. The kinetic constants for Na\(^+\)-NQR are detailed in Table 2, with values very similar to previous findings for \( V.\ choleræ \) Na\(^+\)-NQR.
indicates that its binding and reduction occurs after the cation has been transported, and suggests that sodium is the second substrate in the sequence. Finally, CoQH$_2$1 behaves as an uncompetitive inhibitor against CoQ-1 (Fig. 4, C and D), which indicates that only the E form is able to bind it and that the G form is specific for ubiquinone (Fig. 3). Although the incubation of the enzyme with ubiquinol can drive the reverse electron reaction, only one cofactor is reduced (riboflavin) (27), and thus the G form is not reached, accounting for the purely uncompetitive behavior, rather than a mixed behavior.

Catalytically Active Redox States of Na$^+$_NQR—Na$^+$_NQR contains five cofactors, which can accept up to nine electrons, producing a multiplicity of redox forms that the enzyme can
adopt, especially considering that several of these cofactors can accept one or two electrons (24, 25, 29, 42). Barquera et al. (25, 29) have established that Na\textsuperscript{+}-NQR contains unpaired electrons in all accessible redox forms, including the fully oxidized form, in which riboflavin is found as a neutral semiquinone radical, and the fully reduced form, which contains the signals of the 2Fe-2S center, and an anionic semiquinone radical in the covalently bound FMN cofactor found in the NqrB subunit. As shown in previous studies, the electrons from NADH follow a linear pathway, in which two redox equivalents are delivered to the FAD cofactor. The electron pair is then split, and the electrons move, one by one, to the 2Fe-2S center, to the covalently bound FMN cofactors in the NqrC and NqrB subunits, and riboflavin, and then they are finally delivered to CoQ-1 (24, 27). These studies have also shown that not all redox forms of Na\textsuperscript{+}-NQR are active or participate in the reaction cycle. Thus, a key aspect to understand the mechanism of Na\textsuperscript{+}-NQR is the characterization of the redox forms that participate in the catalytic cycle.

To perform this study, we identified the cofactors involved in the pre-steady state redox reactions and the rates of electron transfer between the cofactors, in the presence of the substrates of the reaction.

The kinetics of reduction of the wild-type enzyme at 450 and 550 nm are shown in Fig. 5, A and B. At 450 nm, the FAD and FMN cofactors have the largest contribution, and at 550 nm, the riboflavin neutral radical signal is more prominent (24, 27). The wild-type enzyme exposed to NADH and NaCl is reduced in three steps, with the rate constants shown in Table 3, similar to values reported previously (24). The first step of the process involves the two-electron reduction of the FAD cofactor (FAD $\rightarrow$ FADH\textsubscript{2}), with a difference spectrum with valleys at 390 and 460 nm, and the one-electron reduction of the riboflavin neutral radical (RibH$^+$ $\rightarrow$ RibH\textsubscript{2}), with a difference spectrum with valleys at 525 and 575. These two reactions are evident in the initial phase of the reaction, as sharp decreases in the absorbance at 450 and 550 nm (Fig. 5, A and B). These two processes happen almost simultaneously in the presence of sodium (Fig. 5C), with similar rate constants (270 and 155 s\textsuperscript{-1}, Table 3), but in its absence they can be clearly separated (24). The second step corresponds to the one-electron reduction of the two covalently bound FMN cofactors (Fig. 5E), with a difference spectrum showing a single valley at 460 nm and a shoulder at 390 nm. The third step of the process is the full reduction of the covalently bound FMN cofactor attached to NqrC, with a spectrum with wide valley from 470 to 510 nm (Fig. 5F). It has been previously described that the reduction of the 2Fe-2S center is difficult to track in the wild-type enzyme, because the UV-visible signal of this center is substantially weaker compared with the flavin signals (24). The rate of reduction of the 2Fe-2S center was re-evaluated in the current analysis by subtracting the spectral components corresponding to the FAD $\rightarrow$ FADH\textsubscript{2} and RibH$^+$ $\rightarrow$ RibH\textsubscript{2} transitions from the data matrix. The spectrum of the FAD $\rightarrow$ FADH\textsubscript{2} was obtained from the mutant NrqF-C276S, which lacks the 2Fe-2S center and is only able to accept electrons at the FAD cofactor (24, 26). The spectrum of the RibH$^+$ $\rightarrow$ RibH\textsubscript{2} transition was obtained from the reduction of wild-type Na\textsuperscript{+}-NQR with ubiquinol, which reduces exclusively the riboflavin cofactor (27). This method allowed us to identify a kinetic component, with valleys at 400 and 420 (Fig. 5D), corresponding to the spectrum of the 2Fe-2S center, found in NqrF (43). The reduction of the 2Fe-2S center has a rate constant of 255 s\textsuperscript{-1} and seems to occur simultaneously with the reduction of FAD, indicating the presence of a three-electron reduced form and the electron split, as reported previously (4).

The wild-type enzyme exposed to the three substrates at saturating concentrations showed a single phase of reduction. In this phase, the reduction of the FAD can be observed together with a reduction of ~60% of the riboflavin cofactor, with characteristic signals at 390, 460, and 520–600 nm and similar rates compared with the absence of CoQ-1 (Table 3 and Fig. 5G). As in the previous case, the components corresponding to the FAD $\rightarrow$ FADH\textsubscript{2} and RibH$^+$ $\rightarrow$ RibH\textsubscript{2} were subtracted from the data matrix, to study the signal of the 2Fe-2S center (Fig. 5H), which is reduced at a rate of 170 s\textsuperscript{-1} (Table 3). As can be observed from the kinetic traces, the enzyme is not completely reduced in the presence of the three substrates (Fig. 5, A and B), and after 20 ms the steady state level is reached, corresponding to the five-electron reduced form (Fig. 7). The presence of the three- and five-electron reduced forms indicate that during the catalytic cycle Na\textsuperscript{+}-NQR is reduced twice by NADH. Also, the incomplete reduction of riboflavin indicates that once the electrons have reached this cofactor, they can be delivered to ubiquinone, because riboflavin is the last carrier in the electron transfer sequence (27).

**Pre-steady State Membrane Potential Formation**—To corroborate that the three- and five-electron reduced forms are catalytically active, the pre-steady state formation of membrane potential was measured in a preparation of Na\textsuperscript{+}-NQR reconstituted in phospholipid vesicles, using the fluorescent indicator RH421. This dye is membrane potential-sensitive and has a very fast response, and it has been used to study the pre-steady state kinetics of ion pumping by the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (35–39), among other transporters. Fig. 6 shows the fluorescence of the indicator in the first 50 ms of the reaction (green line), com-

---

**FIGURE 3. Kinetic reaction mechanism of Na\textsuperscript{+}-NQR.** The reaction mechanism follows a hexa-unit ping-pong system, in which NADH is the first substrate; sodium is the second, and CoQ is the third. The inhibitory mechanisms of HQNO and CoQH\textsubscript{2}-1 are shown in the lower part of the figure. The reactions shown in the figure correspond strictly to the events occurring under steady state conditions. Reactions occurring under pre-steady state are shown in Fig. 7.
pared to the concentrations of several intermediates of the reaction, including the oxidized form (A, black line), the three-electron reduced form (FADH<sub>2</sub>, 2Fe-2S<sub>red</sub>, and RibH<sub>x</sub>), and the five-electron reduced form (FADH<sub>2</sub>, 2Fe-2S<sub>red</sub>, and RibH<sub>x</sub>). As can be observed, the increase in the fluorescence of RH421 correlates with the rise in the concentration of the five-electron reduced state of Na<sup>+</sup>-NQR, corroborating the kinetic and stopped flow data. Following the burst in membrane potential generation, the fluorescence increased for several seconds (data not shown) at a steady state rate, indicating that the vesicles are

FIGURE 4. Inhibition patterns produced by CoQH<sub>2</sub>-2. The concentrations of CoQH<sub>2</sub>-1 used in these experiments are as follows: no CoQH<sub>2</sub>-1 ( ), 0.2 mM ( ■ ), 0.5 mM ( □ ), 1 mM ( ▲ ), and 2 mM ( △ ). A, concentration of NADH was varied at saturating concentrations of CoQ-1 (50 μM) and NaCl (50 mM). The Lineweaver-Burk plot of this data set is shown in B. C, concentration of CoQ-1 was varied at saturating concentrations of NADH (250 μM) and NaCl (50 mM). The Lineweaver-Burk plot of this data set is shown in D. E, concentration of NaCl was varied at saturating concentrations of NADH (250 μM) and CoQ-1 (50 μM). The Lineweaver-Burk plot of this data set is shown in F.
not leaky and have the ability to support a substantial membrane potential (>100 mV) (14, 30).

Discussion

Kinetic Mechanism of Na\textsuperscript{+}-NQR—This work demonstrates that Na\textsuperscript{+}-NQR follows a hexa-uni ping-pong mechanism that allows each of the substrates to react, or be transported, independently at different stages of the catalytic cycle. This type of mechanism has been described in other enzymes, such as the bc\textsubscript{1} complex (44), pyruvate dehydrogenase (45), and oxoglutarate dehydrogenase (46), among others. These enzymes are multimeric complexes that are organized in modules that can act independently, allowing the operation of the ping-pong mechanism. This type of module organization is also found in Na\textsuperscript{+}-NQR, with the NADH dehydrogenase module located in the NqrF subunit (26, 43), CoQ-1 reduction in the NqrB subunit (19, 20), and sodium transport in the NqrB and NqrD/NqrE subunits (14, 15, 17, 30). The hexa-uni ping-pong mechanism is conformational in character, because the individual binding sites of the substrates are not preformed (or are inaccessible in certain states), which confirms our previous hypothesis that the enzyme undergoes multiple conformational changes (5, 16, 19, 30). This type of mechanism would be advantageous to harness and transduce the energy, because the energy is released gradually as the redox steps take place in the different domains of the enzyme, coupling the electron transfer through the redox centers to the conformational changes, thus driving sodium pumping.

The data reported here also clarify the role of the different redox states of Na\textsuperscript{+}-NQR in the catalytic cycle, a key aspect to
fully understand its kinetic mechanism. The evidence demonstrates that the oxidized form of the enzyme is inactive and that the three- and five-electron reduced forms participate in the catalytic cycle. Fig. 7 shows the proposed general catalytic model for Na⁺-NQR, which takes into account the current data and all previously published aspects of the enzyme. The first step of the cycle begins with the two-electron reduction of the FAD molecule, which is the first carrier in the electron sequence, accepting electrons from NADH (Fig. 7A) (24) producing the three-electron reduced state, because the oxidized form contains an unpaired electron in riboflavin (25). This reduction step is very fast and probably “primes” the enzyme and allows a proper conformational state that brings the redox centers closer and allows electron transfer (Fig. 7B). In the next step, the three-electron reduced state is further reduced by a second NADH molecule and enters the catalytic cycle, following the hexa-uni ping-pong mechanism. Bogachev et al. (47) have previously described that the binding of NADH to the three-electron reduced state splits the electrons, producing the reduced 2Fe-2S center and a short lived FAD neutral radical (FADH⁺). Our analysis shows that the 2Fe-2S center is reduced concomitantly with the FAD cofactor, which corroborates the results found by Bogachev et al. (Fig. 7C). The evidence obtained with different mutants indicates that once one electron has reduced the 2Fe-2S center, Na⁺-NQR is able to bind sodium (30), opening one of the at least two sodium-binding sites (Fig. 7C). This sodium-binding site could be found in NqrE/NqrD subunits (15). Once the first sodium is bound, the accessibility of the second binding site increases, producing positive cooperativity between the sites (Fig. 7D), as have been demonstrated before (6, 15). The second binding site is located in NqrB, in which residue NqrB Asp-397 plays an important role in the cooperative behavior and in determining the size of the cation that can be bound (15). Although the operation of the two sites is important for the optimal work of Na⁺-NQR, the enzyme can be functional with only one sodium-binding site, possibly the one located in NqrD/NqrE (15). Once the two sites are filled, the intracellular gates for the cation are closed, which is probably driven by electron transfer from the FMN cofactor located in NqrC to FMN in NqrB (Fig. 7E). As proposed previously, the electron transfer from the FMN cofactor in NqrB to riboflavin opens the extracellular gates, releasing sodium to the positive side of the membrane (Fig. 7G) (30). In the last step, a single ubiquinone molecule is bound, as indicated by the stoichiometry measurements (19), probably in NqrB (19, 20, 34), and the two electrons are delivered to this substrate, producing the three-electron reduced form that can continue the cycle (Fig. 7G). The kinetic model presented in this report does not take into account the sixth redox cofactor found in the crystal structure of Na⁺-NQR, a single iron atom, coordinated

![FIGURE 7. Model of the kinetic mechanism of Na⁺-NQR. The redox states of the cofactors are shown as red circles. Open circle, oxidized state; half-circle, one-electron reduced; full circle, two-electron reduced. The predicted conformational changes of different structures involved in the catalytic cycle are highlighted in color. The oxidized form of the enzyme, which is noncatalytic is shown in gray.](image-url)
through four cysteine residues in subunits NqrD and NqrE (12), due to the lack of experimental evidence supporting its presence. Fadeeva et al. (21) have demonstrated that the characteristic EPR signal of a single iron center is missing completely in Na\textsuperscript{+}-NQR and that the mutants of the conserved cysteine residues in NqrD and NqrE affect the assembly of the enzyme and the incorporation of the flavin cofactors, rather than the content of iron or the EPR signals. Moreover, the spin concentration of the complex, which accounts for the amount of radical signals, in the fully reduced form of the enzyme is 2 (22, 23), corresponding to the unpaired electron in the 2Fe-2S center and the anionic radical found in the covalently bound FMN cofactor of NqrB (24, 25). Thus, the presence of the sixth cofactor seems controversial at this point.

Implications for Interpretation of the Crystal Structure—The model proposed here sheds light onto the available structural data of Na\textsuperscript{+}-NQR. We have demonstrated that the electrons follow a linear pathway from NADH to CoQ-1 (24). This functional characterization has been corroborated through redox titrations (22, 23, 42), showing that the electrons move downhill in most of these transitions. However, the structural data obtained at low resolution shows that most of these centers are too far apart to sustain physiological rates of electron transfer (12). The present study shows that the oxidized form of the enzyme, which corresponds to the crystallized structure, does not participate in the catalytic cycle, explaining the long distances that separate the cofactors. As discussed here, the two-electron reduction of the FAD cofactor probably primes the enzyme, rearranging different domains, and bringing cofactors to a distance that can allow electron transfer. Nonetheless, as predicted by our model, the oxidized form of the enzyme can still perform the two-electron reduction and the electron split between the FAD and 2Fe-2S center, due to the close distance between the NADH-binding site and these cofactors (12, 43).

An important aspect predicted from our kinetic model is that the substrate-binding sites should be empty or missing in the structure of the oxidized enzyme. In a ping-pong model, the substrate-binding sites are not pre-formed (or are inaccessible to the substrates, due to gating mechanisms), and they exist transiently in some of the conformations of the enzyme. This predicted behavior can be observed in the structure of the enzyme and is particularly evident in the case of the ubiquinone-binding site. The current tridimensional structure of Na\textsuperscript{+}-NQR lacks ubiquinone and a pocket that can accommodate this substrate. Surprisingly, the structure also lacks the noncatalytic tightly bound ubiquinone molecule, which has been reported in most preparations (48–50). The predictions of our model can also be extended to the sodium-binding sites. A motif with a octahedral array of six negatively charged ligands, with the ability to bind sodium is not found within the structure (12). Moreover, several of the acid residues that have been demonstrated to be involved in the sodium-binding sites and in the transport of the cation (14, 15) seem to be missing in the channel that has been proposed for sodium transport. Furthermore, careful analysis of the functional data available indicates that it is highly unlikely that the active forms of the enzyme contain a channel that can traverse entirely the membrane. This channel might allow the pumping (or even the simple diffusion) of protons, or other small ions, instead of sodium. Our model predicts that the accessibility of the structures involved in sodium pumping should be tightly controlled by the enzyme, to guarantee ion specificity and the unidirectionality of the process. Interestingly, the crystallographic structure is now allowing the corroboration of one part of the evolutionary pathway suggested for the Na\textsuperscript{+}-NQR complex. It was proposed that the complex evolved in the Chlorobi/Bacteroides phyla of bacteria, through the duplication of the homologous complex RNF (a cation-pumping NADH:ferredoxin oxidoreductase) (8). The origin of the NqrA has been under debate, due to the low similarity of this protein with its closest homolog, the RnfC subunit. However, based on the organization of the operon and on a small but significant similitude, we proposed that NqrA evolved from the subunit RnfC, eventually losing the binding sites for FMN and the Fe-S centers found in RnfC. The crystal structure of the complex and of the isolated NqrA shows clearly a Rossman motif for the binding of NADH and the motifs for the binding of the Fe-S clusters and FMN (12), confirming the evolutionary path proposed.

Finally, previous functional characterizations challenge the assignment for the localization of the riboflavin cofactor. The tridimensional structure suggests that the binding site of this cofactor could be located in the interface of NqrB and NqrE, facing the cytoplasm (12). However, previous studies support the idea that riboflavin is deeply embedded in the transmembrane helices, due to the remarkable stability of the neutral radical (25, 29). Moreover, the redox potential of this cofactor is completely independent of the pH (22), strongly suggesting that riboflavin is not accessible to the aqueous environment. Barquera et al. (25) showed that the elimination of the FMN cofactor in NqrB affected substantially the ENDOR spectra of the riboflavin neutral semiquinone radical, indicating that these cofactors are in proximity, most likely in NqrB. This hypothesis was subsequently corroborated by Casutt et al. (51), describing that the elimination of the NqrB subunit removed the riboflavin cofactor from the complex. Interestingly, the later study also showed that the elimination of NqrE did not produce a change in the content of riboflavin in Na\textsuperscript{+}-NQR, which challenges the proposed localization of riboflavin.

Conclusion—The kinetic model presented in this work is a powerful tool that explains the current structural data and predicts the minimal number of conformations and structural changes that can be expected in the catalytic cycle. This model also conciliates seemingly contradictory structural and functional data and predicts a strong influence of the redox state on the structure of this sodium pump, part of which has been corroborated experimentally (16, 19). The model also helps to understand the effects of mutations in the catalytic mechanism of the enzyme and in the structural reorganizations of the enzyme.

References

1. Steuber, J., Halang, P., Vorburger, T., Steffen, W., Vohl, G., and Fritz, G. (2014) Central role of the Na\textsuperscript{+} -translocating NADH:quinone oxidoreductase (Na\textsuperscript{+}-NQR) in sodium bioenergetics of Vibrio cholerae. Biol. Chem. 395, 1389–1399
2. Häse, C. C., and Barquera, B. (2001) Role of sodium bioenergetics in Vibrio cholerae. Biochim. Biophys. Acta 1505, 169–178
3. Hayashi, M., Nakayama, Y., and Unemoto, T. (2001) Recent progress in
the Na⁺-translocating NADH:quinone reductase from the marine Vibrio alginolyticus. Biochim. Biophys. Acta 1505, 37–44
4. Verkhovsky, M. I., and Bogachev, A. V. (2010) Sodium-translocating NADH:quinone oxidoreductase as a redox-driven ion pump. Biochim. Biophys. Acta 1797, 738–746
5. Juárez, O., and Barquera, B. (2012) Insights into the mechanism of electron transfer and sodium translocation of the Na⁺-pumping NADH:quinone oxidoreductase. Biochim. Biophys. Acta 1817, 1823–1832
6. Juárez, O., Shea, M. E., Makhatadze, G. I., and Barquera, B. (2011) The role and specificity of the catalytic and regulatory cation-binding sites of the Na⁺-pumping NADH:quinone oxidoreductase from Vibrio cholerae. J. Biol. Chem. 286, 26383–26390
7. Yagi, T. (1991) Bacterial NADH-quinone oxidoreductases. J. Bioenerg. Biomembr. 23, 211–225
8. Reyes-Prieto, A., Barquera, B., and Juárez, O. (2014) Origin and evolution of the sodium-pumping NADH: Ubiquinone oxidoreductase. PLoS One 9, e96696
9. Häse, C. C., Fedorova, N. D., Galperin, M. Y., and Dibrov, P. A. (2001) Sodium ion cycle in bacterial pathogens: evidence from cross-genome comparisons. Microbiol. Mol. Biol. Rev. 65, 353–370
10. Skulachev, V. P. (1989) The sodium cycle: a novel type of bacterial energy pathway. Science 243, 516–525
11. Duffy, E. B., and Barquera, B. (2006) Membrane topology mapping of the Na⁺-pumping NADH: quinone oxidoreductase from Vibrio cholerae by PhoA-green fluorescent protein fusion analysis. J. Bacteriol. 188, 8343–8351
12. Steuber, J., Vohl, G., Casutt, M. S., Diederichs, K., and Fritz, G. (2014) Structure of the V. cholerae Na⁺-pumping NADH-quinone oxidoreductase. Nature 516, 62–67
13. Hayashi, M., Nakayama, Y., Yasui, M., Maeda, M., Furuishi, K., and Unemoto, T. (2001) FMN is covalently attached to a threonine residue in the NqrD subunit of the Na⁺-translocating NADH: quinone oxidoreductase from Vibrio alginolyticus. FEBS Lett. 488, 5–8
14. Juárez, O., Atthearn, K., Gillespie, P., and Barquera, B. (2009) Acid residues in the transmembrane helices of the Na⁺-pumping NADH:quinone oxidoreductase from Vibrio cholerae involved in sodium translocation. Biochemistry 48, 9516–9524
15. Shea, M. E., Juárez, O., Cho, J., and Barquera, B. (2013) Aspartic acid 397 in subunit B of the Na⁺-pumping NADH:quinone oxidoreductase from Vibrio cholerae forms part of a sodium-binding site, is involved in cation selectivity, and affects cation-binding site cooperativity. J. Biol. Chem. 288, 31241–31249
16. Neehaul, Y., Juárez, O., Barquera, B., and Hellwig, P. (2013) Infrared spectroscopic evidence of a redox-dependent conformational change involving binding residue NqrB-D397 in the Na⁺-pumping NADH:quinone oxidoreductase from Vibrio cholerae. Biochemistry 52, 3085–3093
17. Shea, M. E., Meibom, K. L., Dolganov, N. A., Wu, C.-Y., and Schoolnik, G. K. (2005) Chitin induces natural competence in Vibrio cholerae. Science 310, 1824–1827
18. Minagawa, K., and Edwards, C. A. (1979) Purification of a reductively active iron-sulfur protein (oxidation factor) from succinate. cytochrome c reductase complex of bovine heart mitochondria. J. Biol. Chem. 254, 8697–8706
19. Nakayama, Y., Hayashi, M., Yoshikawa, K., Mochida, K., and Unemoto, T. (1999) Inhibitor studies of a new antibiotic, kororimicin, 2-(heptyl-4-hydroxyquinolinel N-oxide and Ag⁺ toward the Na⁺-translocating NADH-quinone reductase from the marine Vibrio alginolyticus. Biol. Pharm. Bull. 22, 1064–1067
20. Pratap, P. R., Robinson, J. D., and Steinberg, M. I. (1991) The reaction sequence of the Na⁺/K⁺-ATPase: rapid kinetic measurements distinguish between alternative schemes. Biochim. Biophys. Acta 1069, 288–298
21. Pratap, P. R., and Robinson, J. D. (1993) Rapid kinetic analyses of the Na⁺/K⁺-ATPase distinguish among different criteria for conformational change. Biochim. Biophys. Acta 1151, 89–98
22. Bühler, R., Stürmer, W., Apell, H. J., and Läuger, P. (1991) Charge translocation by the Na,K-pump. 1. Kinetics of local field changes studied by time-resolved fluorescence measurements. J. Membr. Biol. 121, 141–161
23. Stürmer, W., Bühler, R., Apell, H. J., and Läuger, P. (1991) Charge translocation by the Na,K-pump. II. Ion binding and release at the extracellular face. J. Membr. Biol. 121, 163–176
24. Clarke, R. J., Kane, D. J., Apell, H. J., Roudna, M., and Bamberg, E. (1998) Redox properties of the prosthetic groups of the Na⁺-translocating NADH:quinone oxidoreductase. 1. Electron paramagnetic resonance study of the enzyme. Biochemistry 46, 6291–6298
binding site of the Na\textsuperscript{+}-translocating NADH:quinone oxidoreductase from \textit{Vibrio cholerae}. J. Biol. Chem. \textbf{288}, 30597–30606

42. Neehaul, Y., Juárez, O., Barquera, B., and Hellwig, P. (2012) Thermodynamic contribution to the regulation of electron transfer in the Na\textsuperscript{+}-pumping NADH:quinone oxidoreductase from \textit{Vibrio cholerae}. Biochemistry \textbf{51}, 4072–4077

43. Türk, K., Puhar, A., Neese, F., Bill, E., Fritz, G., and Steuber, J. (2004) NADH oxidation by the Na\textsuperscript{+}-translocating NADH:quinone oxidoreductase from \textit{Vibrio cholerae}: functional role of the NqrF subunit. J. Biol. Chem. \textbf{279}, 21349–21355

44. Kubota, T., Yoshikawa, S., and Matsubara, H. (1992) Kinetic mechanism of beef heart ubiquinol:cytochrome \textit{c} oxidoreductase. \textit{J. Biochem.} \textbf{111}, 91–98

45. Nigo, T. T., and Barbeau, A. (1978) Steady state kinetics of rat brain pyruvate dehydrogenase multienzyme complex. \textit{J. Neurochem.} \textbf{31}, 69–75

46. McMinn, C. L., and Ottaway, J. H. (1977) Studies on the mechanism and kinetics of the 2-oxoglutarate dehydrogenase system from pig heart. \textit{Biochem. J.} \textbf{161}, 569–581

47. Bogachev, A. V., Belevich, N. P., Bertsova, Y. V., and Verkhovsky, M. I. (2009) Primary steps of the Na\textsuperscript{+}-translocating NADH:ubiquinone oxidoreductase catalytic cycle resolved by the ultrafast freeze-quench approach. \textit{J. Biol. Chem.} \textbf{284}, 5533–5538

48. Barquera, B., Hellwig, P., Zhou, W., Morgan, J. E., Hase, C. C., Gosink, K. K., Nilges, M., Bruesehoff, P. J., Roth, A., Lancaster, C. R., and Gennis, R. B. (2002) Purification and characterization of the recombinant Na\textsuperscript{+}-translocating NADH:quinone oxidoreductase from \textit{Vibrio cholerae}. Biochemistry \textbf{41}, 3781–3789

49. Zhou, W., Bertsova, Y. V., Feng, B., Tsatsos, P., Verkhovskaya, M. L., Gennis, R. B., Bogachev, A. V., and Barquera, B. (1999) Sequencing and preliminary characterization of the Na\textsuperscript{+}-translocating NADH:ubiquinone oxidoreductase from \textit{Vibrio harveyi}. Biochemistry \textbf{38}, 16246–16252

50. Pfenninger-Li, X. D., Albracht, S. P., van Belzen, R., and Dimroth, P. (1996) NADH:ubiquinone oxidoreductase of \textit{Vibrio alginolyticus}: purification, properties, and reconstitution of the Na\textsuperscript{+} pump. Biochemistry \textbf{35}, 6233–6242

51. Casutt, M. S., Huber, T., Brunisholz, R., Tao, M., Fritz, G., and Steuber, J. (2010) Localization and function of the membrane-bound riboflavin in the Na\textsuperscript{+}-translocating \textit{NADH:quinone oxidoreductase} (Na\textsuperscript{+}-NQR) from \textit{Vibrio cholerae}. J. Biol. Chem. \textbf{285}, 27088–27099