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

Michael E. Shea, Oscar Juárez, Jonathan Cho, and Blanca Barquera

From the Department of Biology and Center of Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 121801

The Na\(^{+}\)-pumping NADH:ubiquinone oxidoreductase complex is found in *Vibrio cholerae* and other marine and pathogenic bacteria. NADH:ubiquinone oxidoreductase oxidizes NADH and reduces ubiquinone, using the free energy released by this reaction to pump sodium ions across the cell membrane. In a previous report, a conserved aspartic acid residue in the NqrB subunit at position 397, located in the cytosolic face of this protein, was proposed to be involved in the capture of sodium. Here, we studied the role of this residue through the characterization of mutant enzymes in which this aspartic acid was substituted by other residues that change charge and size, such as arginine, serine, lysine, glutamic acid, and cysteine. Our results indicate that NqrB-Asp-397 forms part of one of the at least two sodium-binding sites and that both size and charge at this position are critical for the function of the enzyme. Moreover, we demonstrate that this residue is involved in cation selectivity, has a critical role in the communication between sodium-binding sites, by promoting cooperativity, and controls the electron transfer step involved in sodium uptake (2Fe-2S → FMNC).

The Na\(^{+}\)-pumping NADH:ubiquinone oxidoreductase (Na\(^{+}\)-NQR) is a membrane-bound complex present in various marine and pathogenic bacteria, including *Vibrio cholerae* (1–3). This enzyme is the entry site of electrons into the aerobic respiratory chain, catalyzing the electron transfer from NADH to ubiquinone, which is coupled to the pumping of sodium ions across the membrane. The sodium gradient produced by Na\(^{+}\)-NQR is used by the cell for ATP synthesis, transport of nutrients, rotation of the flagellum, among other processes (4–7).

Na\(^{+}\)-NQR is a 200-kDa protein complex of six subunits, corresponding to the six open reading frames of the *nqr* operon. Subunits NqrB, NqrC, NqrD, NqrE, and NqrF contain one or more transmembrane helices, whereas subunit A is hydrophilic and located in the cytosol (8).

The electrons move through the different redox centers in enzyme in a linear pathway. During the first step of electron transfer, the noncovalently bound FAD accepts two electrons from NADH (9). Subsequently, the electrons are transferred stepwise by passing to the 2Fe-2S center, the two FMN molecules covalently attached to NqrC and NqrB (in this order), riboflavin, and finally to ubiquinone-8 (10). Our results showed that the one-electron reduction of FMN in NqrC (FMNC) is the step involved in sodium uptake and that the reduction of riboflavin is involved in sodium translocation (11). The data indicate that the coupling mechanism of the enzyme is mediated by conformational changes, which are energized by the different redox reactions of the enzyme. Indeed, Neehaul et al. (12) demonstrated that electrons move downhill in the electron transfer chain and that the midpoint potential of FMNC increases in the presence of sodium, suggesting that the electron transfer is thermodynamically regulated by this ion, although most of the control is exerted at the kinetic level, probably through conformational changes.

We have identified a series of acidic residues within the transmembrane helices of the subunits NqrB, NqrD, and NqrE that possibly form part of the structures involved in sodium transport. Seven of these 17 acidic residues are essential for the enzyme activity, and four of these, located on the cytosolic face of the plasma membrane, seem to have an important role in the sodium uptake mechanism (13). In particular, aspartic acid 397 in NqrB is especially important for enzyme catalysis. The mutant in which the acidic residue was substituted to an aliphatic group (NqrB-D397A) becomes insensitive to sodium, following an unsaturable behavior, with a $K_{m(app)}$ at least 2 orders of magnitude larger compared with wild type Na\(^{+}\)-NQR. This observation suggests that this residue could be part of one or more sodium-binding sites or that it could be involved in sodium uptake by the enzyme, forming part of vestibules or gates that guide sodium to the binding sites that control its entry. In this study we have examined the role of the NqrB-Asp-397 residue by making substitutions that have the poten-
NqrB-Asp-397 Role in a Sodium-binding Site of Na\textsuperscript{+}-NQR

TABLE 1

| Mutation   | Sequence of forward primer |
|------------|---------------------------|
| NqrB- D397K | GCGAACCTTTTTCGCCCTTGGTTTTAAAAGTTGGTGTTAGAGAGAAATATCA |
| NqrB- D397E | GCGAACCTTTTTCGCCCTTGGTTTTAAAAGTTGGTGTTAGAGAGAAATATCA |
| NqrB- D397N | GCGAACCTTTTTCGCCCTTGGTTTTAAAAGTTGGTGTTAGAGAGAAATATCA |
| NqrB- D397S | GCGAACCTTTTTCGCCCTTGGTTTTAAAAGTTGGTGTTAGAGAGAAATATCA |
| NqrB- D397A | GCGAACCTTTTTCGCCCTTGGTTTTAAAAGTTGGTGTTAGAGAGAAATATCA |
| NqrB- D397C | GCGAACCTTTTTCGCCCTTGGTTTTAAAAGTTGGTGTTAGAGAGAAATATCA |

ential to be functionally viable instead of the aliphatic groups that may have a more general effect on the structure of the enzyme and the properties of the resulting mutants studied by kinetic methods.

Our results show that both size and charge are important for proper functioning of the enzyme and that NqrB-Asp-397 is an essential component in one of the sodium-binding sites, participating in cation selectivity. The data reported here are key to the understanding of the catalytic mechanism of Na\textsuperscript{+}-NQR. Here, we show that the structural and functional role of NqrB-Asp-397 is finely tuned, and any substitution has a significant effect in the cooperativity between cation-binding sites, which demonstrates that the complex relationship between these sites is essential for sodium transport.

Moreover, we show that the enzyme is partially functional with only one sodium-binding site, which indicates that the sites are connected to independent and probably parallel sodium pumping pathways.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—V. cholerae wild type Na\textsuperscript{+}-NQR and mutant strains were grown in 30-liter fermentors in LB (Luria Bertani) medium in the presence of 100 μg/ml ampicillin, 50 μg/ml streptomycin, under constant aeration and agitation at 37 °C. Arabinose was added to induce the expression of Na\textsuperscript{+}-NQR, as reported previously (1).

Mutant Construction—Mutagenesis reactions were performed as reported previously (14) with the Quikchange site-directed mutagenesis kit (Stratagene), using as template the wild type nqr operon cloned into a pBAD expression vector. Primers designed to mutate the NqrB-Asp-397 to lysine, glutamic acid, asparagine, serine, and cysteine are listed in Table 1. Directed mutagenesis kit (Stratagene), using as template the nqr operon cloned into a pBAD expression vector. Primers designed to mutate the NqrB-Asp-397 to lysine, glutamic acid, asparagine, serine, and cysteine are listed in Table 1.

A V. cholerae deletion strain lacking the genomic nqr operon (Δnqr) was used to express the mutant gene copies. Mutations were verified by DNA sequencing.

Enzyme Purification—Cells were lysed using a microfluidizer, and membranes were recovered as described previously (1, 13). Wild type Na\textsuperscript{+}-NQR and mutant enzymes were solubilized using n-dodecyl β-D-maltoside and purified using nickel-nitriotriacetic acid affinity chromatography, as reported before. Samples were concentrated in Millipore spin devices and kept in liquid nitrogen until used.

Steady State Activity—NADH dehydrogenase and ubiquinone reductase (CoQ\textsubscript{red}) activities of the wild type enzyme, together with those of the six NqrB-Asp-397 mutants used in this study, in the absence and in the presence of 100 mM NaCl or LiCl. In the wild type enzyme, the CoQ\textsubscript{red} (the physiologic activity of the enzyme) is stimulated 8-fold in the presence of saturating amounts of sodium. In the case NqrB-D397K, the introduction of a positive charge abates completely the stimulatory effect of sodium, demonstrating that the presence of the positive charge in the binding site is not the factor that increases electron flow per se. The mutations that eliminate the negative charge, but that introduce polar residues with a partial negative charge such as asparagine and serine, were stimulated two times by sodium. The activity for the semiconservative mutant NqrB-D397E, which contains a carboxylate but increases the size of the residue, was stimulated by sodium to almost the same extent as the activities of NqrB-D397S and NqrB-D397N.

RESULTS

Cation Effect on Enzyme Activity—in our earlier examination of conserved acidic residues in the membrane-spanning helices of Na\textsuperscript{+}-NQR, we showed that NqrB-Asp-397 has an especially important role in the sodium translocation process (13). When this residue was replaced by alanine, the enzyme turnover was severely inhibited, apparently due to the impairment in sodium uptake. In wild type Na\textsuperscript{+}-NQR, the sodium dependence of the steady state turnover follows a Michaelis-Menten behavior, with a K\textsubscript{m(app)} of 1–2 mM (13). In contrast, the kinetics of the NqrB-D397A mutant show an unsaturable behavior for sodium. This suggests that either this residue forms part of the sodium-binding sites or that it is part of a vestibule or gateway through which the cation has access to its binding site. To address these two possibilities, the cation selectivity was studied in mutants at this position in which size and charge were modified.

Table 2 shows the NADH dehydrogenase (NADH\textsubscript{DHS}) and ubiquinone reductase (CoQ\textsubscript{red}) activities of the wild type enzyme, together with those of the six NqrB-Asp-397 mutants used in this study, in the absence and in the presence of 100 mM NaCl or LiCl. In the wild type enzyme, the CoQ\textsubscript{red} (the physiologic activity of the enzyme) is stimulated 8-fold in the presence of saturating amounts of sodium. In the case NqrB-D397K, the introduction of a positive charge abates completely the stimulatory effect of sodium, demonstrating that the presence of the positive charge in the binding site is not the factor that increases electron flow per se. The mutations that eliminate the negative charge, but that introduce polar residues with a partial negative charge such as asparagine and serine, were stimulated two times by sodium. The activity for the semiconservative mutant NqrB-D397E, which contains a carboxylate but increases the size of the residue, was stimulated by sodium to almost the same extent as the activities of NqrB-D397S and NqrB-D397N.
We also introduced a cysteine residue at this position, which has a similar size compared with aspartate, and at pH 8.0, ~50% of this residue was charged negatively. In this mutant, sodium has a stimulatory effect of 2.6 times, the highest found in the mutants studied. These results show that the role played by NqrB-Asp-397 is highly specific in terms of both charge and size. Altering the charge (NqrB-D397N and NqrB-D397C) or the size (NqrB-D397E) at this sequence position is sufficient to dramatically change the sensitivity for Na⁺ of the CoQred activity.

It must be pointed out that the NADH dehydrogenase activity (NADHₜₐₜ) is largely unaffected in all the mutants, which demonstrates that the general structure of these proteins is not altered by the mutation. Indeed, Neehau et al. (17) using deuterium exchange experiments recently demonstrated that the mutant NqrB-D397E contains approximately the same conformational flexibility as the wild type enzyme and is able to undergo a series of conformational changes induced by the redox reaction and by the addition of different cations. We have shown that this activity represents the total rate at which the electrons enter the enzyme and that the main effect of sodium occurs at the level of electron branching between two separate activities, the NADH oxidase and the ubiquinone reductase activities (10). In the wild type enzyme exposed to sodium, ~90% of the electrons flow to ubiquinone, although in its absence they are directed to oxygen. In these mutants, a similar effect was observed as follows: the NADHₜₐₜ was unaltered, and the CoQred activity was diminished, in agreement with the specific effect of the mutations on the sodium-dependent activity.

We have previously reported that Na⁺-NQR is able to translocate lithium, as well as sodium, and that these two cations compete for the binding sites in the enzyme (16). Table 2 shows that the CoQred activity of the wild type enzyme is stimulated three times by lithium and that the mutants NqrB-D397A and NqrB-D397K, which are completely insensitive to sodium, are also not stimulated by lithium. However, the activities of the mutants NqrB-D397C, NqrB-D397E, NqrB-D397N, and NqrB-D397S were stimulated by lithium, and interestingly, the fraction of stimulation was greater for lithium compared with sodium. The CoQred activity of NqrB-D397C was stimulated 1.9 times by lithium and 2.6 times by sodium. The stimulation by lithium was 65% (1.9:2.9) of what it was in the wild type enzyme, whereas stimulation by sodium was only 33% (2.6:8.0) as much. In the case of NqrB-D397N, NqrB-D397E, and NqrB-D397S, this ratio was 1.6, 1.5, and 1.4, respectively. Thus, mutations at this position have a more severe effect on the sodium-dependent activity compared with the activity dependent on lithium. These results strongly suggest that NqrB-Asp-397 has a role in the cation selectivity filter of the enzyme.

**Kinetic Properties of the Mutants**—To further investigate the effects of mutations at position NqrB-Asp-397 on the interactions of the enzyme with sodium and lithium, the kinetic properties of mutants were studied (Table 2). In all cases, the uncoupled activity (U) (basal activity independent of the cation) was unaltered (60 s⁻¹), compared with the wild type enzyme activity. The ratio of k₉cat (maximum turnover rate of the enzyme) with respect to the uncoupled activity indicates the cation sensitivity of the enzyme, which in all cases was similar to the stimulation with saturating concentrations of sodium, as found in Table 3.

All the mutations have a large effect on the k₉cat obtained with sodium as substrate, reducing the rate to 20–25% of the wild type activity (Table 3). This effect is more evident by comparing the “coupled” turnover rate (k₉cat), which is the rate involving exclusively the pumping activity, with respect to the uncharged activity indicates the cation sensitivity of the enzyme, which in all cases was similar to the stimulation with saturating concentrations of sodium, as found in Table 3.

Model A describes a mechanism in which the two binding sites work in sequence as follows: the binding of the first sodium ion triggers the opening of the second binding site, and only the enzyme with the two occupied sites can be active. Model B describes a two-component Michaelis-Menten system, which mechanistically can be attributed to an enzyme with two sodi-
um-binding sites with exit pathways that work independently, in other words to two independent and parallel sodium-pumping sites.

The best fit for the data of NqrB-D397C was obtained with model B, which allowed us to calculate the kinetic parameters for two different sodium-pumping sites, for sites I and II. As shown in Table 3, the coupled turnover rate \( k_{\text{catC}} \), the ratio \( k_{\text{catC}}/K_{\text{m(app)}} \) and the stimulation by sodium \( k_{\text{catC}}/UC \) of site I in the NqrB-D397C mutant are similar to the data found in the other mutants analyzed. This suggests that in the mutants NqrB-D397E, NqrB-D397N, and NqrB-D397S only, sodium-binding site I is active, although site II is inactive, which accounts for the effect on turnover rate and essentially no perturbation on \( K_{\text{m(app)}} \).

According to our data, sodium-binding site II might be located in NqrB and aspartate 397 participates directly in this binding site. Moreover, both size and charge in this position are critical for its function, which explains why NqrB-D397E, NqrB-D397N, and NqrB-D397S are inactive. In the case of NqrB-D397C, the cysteine substitution produces an enzyme...
mutants, although the $K_m$ rate of inactivation were calculated by fitting the data to a single exponential decay and were plotted against the sodium concentration used in each experiment. The rates of inactivation were calculated by fitting the data to a single exponential decay. The experiment was performed with different concentrations of sodium, and as observed in Fig. 2, increasing concentrations of the cation protected the mutant against iodoacetamide inactivation, with a pseudo-first order rate of inactivation calculated by fitting the data to a single exponential decay. The experiment was performed by iodoacetamide at these concentrations. This demonstrates the thiol group that is specifically modified in these conditions.

To corroborate that NqrB-Asp-397 forms part of a sodium-binding site, we tested the effect of the cation concentration on the inactivation of the NqrB-D397C mutant by the thiol-modifying agent iodoacetamide. The inactivation kinetics were followed using a constant concentration of the inhibitor, and pseudo-first order rate of inactivation was calculated by fitting the data to a single exponential decay. The experiment was performed with different concentrations of sodium, and as observed in Fig. 2, increasing concentrations of the cation protected the mutant against iodoacetamide inactivation, with a $K_m$ of 80 mM. In contrast, wild type Na$^+$-NQR is not inhibited by iodoacetamide at these concentrations. This demonstrates the thiol group that is specifically modified in these conditions is the cysteine residue at position NqrB-397.

Data in Table 3 confirm that the lithium-dependent activity is not affected as much as the sodium-dependent activity in these mutants, because the lithium stimulation ($k_{catC}/UC$) is 60% compared with wild type Na$^+$-NQR. Results in Table 3 also demonstrate that the effect of the mutation(s) is primarily on the $k_{catC}$ value, which is reduced to 32–43% in all the mutants, although $k_{cat(app)}$ remained completely unaffected. These data suggest that in all mutants, both sites might be functional with lithium and have the same apparent affinity. This also suggests that NqrB-Asp-397 has an important role in the selectivity filter of the enzyme by determining the size of the cation(s) that can go into the binding site. Lithium is smaller than sodium and requires five instead of six ligands (19), which explains the less severe effect on the lithium dependent activity. However, all the mutants affected $k_{catC}$, indicating that this residue might be also involved in other steps in the transport process. It is clear that NqrB-Asp-397 residue forms part of at least one sodium-binding site (site II) in NqrB, determining to a large extent the size of the site. Thus, this residue is involved in the cation selectivity filter and affects the cooperativity between the two binding sites.

**Fast Kinetics of Electron Transfer**—Fast kinetic measurements were performed to understand the effect of two mutants, NqrB-D397E and NqrB-D397C, on the internal electron transfer reactions. Fig. 3 shows the different spectral components of the reaction and the kinetics of the enzyme reduction at 450 nm in three conditions as follows: with no cation present, 100 mM NaCl, and 100 mM LiCl. The first component in the two mutants corresponds to the two-electron reduction of FAD (FAD $\rightarrow$ FADH$_2$), which is cation-independent and the first reaction in the reduction process (10). The difference spectra have minima at 390 and 460 nm and maxima at 525 nm, with a rate constant similar to the one obtained for the wild type (Table 4) (10). The second phase of reduction in wild type Na$^+$-NQR is stimulated by sodium and lithium and is the one-electron reduction of riboflavin (RibH$^+$ $\rightarrow$ RibH$_2$). We have shown previously that the real sodium-dependent step is the one-electron reduction of FMN$_{C2}$, but due to the rates of the subsequent steps in the reaction, this intermediate cannot be detected, i.e. once that the electron passes the bottle neck at FMN$_{C2}$, it is rapidly transferred to FMN$_B$, and then to riboflavin (11). For NqrB-D397E and NqrB-D397C, the second phase of reduction contained two transitions: RibH$^+$ $\rightarrow$ RibH$_2$, as in wild type, and the 2(FMN $\rightarrow$ FMN$^+$), which is not catalytically relevant and is found in the third phase of reduction in the wild type enzyme. The rate of reduction of riboflavin was stimulated by the presence of sodium and lithium, which roughly corresponds to the stimulation of the steady state activity in these two mutants (50% stimulation approximately).
To corroborate that the mutations at position NqrB-Asp-397 affect exclusively the sodium-dependent step of the reaction, the reduction kinetics in the double mutant NqrB-D397C/NqrB-T236Y were also studied (Fig. 4). The mutation at position NqrB-Thr-236 eliminates the site for the covalent attachment of the FMN cofactor in this subunit (14), and thus it allows us to study the first three steps of the reaction (NADH → FAD → 2Fe-2S → FMNC). As observed in Fig. 4, the reduction process occurred in three steps. In the first step, the reduction of FAD (FAD → FADH2) was unaffected by the mutation (at around 320 s⁻¹) (Table 4). The reductions of FMNC and the 2Fe-2S center (FMN → FMN⁺ and 2Fe-2SOX → 2Fe-2SRED) were observed in the second phase, with a rate dependent on sodium concentration, as shown in Fig. 4G, inset. The third step corresponds to the full reduction of FMNC⁺ (FMNC⁺ → FMN⁻H₂), which is not catalytically relevant, as reported before. The rate of the second step was stimulated 50% by sodium, which confirms the effect of the mutation on the sodium-sensitive step of the reaction. The fast kinetics analysis of NqrB-D397C and Nqr-D397E corroborates that the Na⁺ uptake step is controlled by the electron transfer step from the 2Fe-2S center to FMNC⁺.

The study of mutations at this site not only confirms that Na⁺-NQR uses at least two Na⁺-binding sites for the uptake of the ion but demonstrates that a functional relationship between these two sites is required to perform the sodium translocation. Most mutations at NqrB-Asp-397 avert site II, disrupting the cooperative behavior between the two Na⁺-binding sites.

DISCUSSION

We have demonstrated, using ²²Na⁺ equilibrium binding titrations and activity measurements, that Na⁺-NQR contains up to three functional sodium-binding sites (16). At least two of these sites participate in catalysis and exhibit cooperative behavior, in which the binding of sodium to one site increases the affinity of the second site (or third site). These binding sites are specific for sodium and are unable to bind large cations such as potassium and rubidium, but they are able to use lithium as
NqrB-Asp-397 Role in a Sodium-binding Site of Na\textsuperscript{+} -NQR

substrate, possibly because lithium has a smaller ionic radius (19), a common feature among sodium-binding proteins. Previously, a number of conserved acidic residues, located in the transmembrane helices of NqrB, NqrD, and NqrE, were shown to have an important role in sodium transport. Aspartic acid 397 in NqrB is one of the essential parts for sodium transport, because the mutation to an aliphatic residue (NqrB-D397A) decreases the apparent affinity of the enzyme for sodium in more than 2 orders of magnitude (13). Moreover, this residue is absolutely conserved in all the members of the Na\textsuperscript{+} -NQR family and in the related RNF family, which are also sodium-pumping enzymes (13, 18). This suggests that this residue could have a participation in the sodium transport mechanism of the enzyme. It is possible that NqrB-Asp-397 could be part of one or more sodium-binding sites or that it might form part of the vestibule or other hydrophilic cavities, guiding sodium to its binding sites. The data in this report allow us to clearly distinguish between these two possibilities, which ultimately clarifies the role, location, and interactions of sodium-binding site II.

The data in this report demonstrate that NqrB-Asp-397 forms part of a sodium-binding site (site II), because any alterations in size or charge at this position disrupts completely the sodium-dependent activity of the enzyme. If this residue would form part of a hydrophilic pocket, like a vestibule or an entry gateway, it might not be absolutely conserved in both the Na\textsuperscript{+} -NQR and the RNF proteins, and the changes to other residues should have been tolerated by the enzyme, especially the semi-conservative substitution to glutamic acid. This absolute requirement of aspartate acid at this position suggests that sodium-binding site II has a specific functional structure and that aspartate is the only residue, with the proper size and charge, to allow the site to be functional.

The effects observed on the kinetic parameters for all the mutants in this site can be explained by the model shown in Fig. 5. According to this model, Na\textsuperscript{+} -NQR should present at least two sodium-pumping sites, one in NqrB (site II) and another (site I) in NqrD and/or NqrE, which have a limited accessibility to the aqueous environment in the oxidized form of the enzyme. The reduction of the enzyme by two NADH molecules produces a four two-electron-reduced FAD, a reduced 2Fe-2S center. This triggers the one electron transfer from the 2Fe-2S center to FMN\textsubscript{C}, which is the redox step involved in sodium capture (11). In this redox state, one sodium-binding site is open and filled with the cation. This produces an inter-subunit communication that opens the second sodium-binding site, increasing its apparent affinity, which in turn produces the cooperative behavior observed in the wild type enzyme (16). According to this model, the sodium-binding site located in NqrB (site II) is disrupted in the NqrB-D397E, NqrB-D397S, and NqrB-D397N mutants, due mainly to lack of proper size and charge of these residues. Site I is partially active and responsible for the activity observed in these mutants. The fact that site II is inactive explains the dramatic decrease in the $k_{cat}$ and $k_{cat}/K_{m(app)}$ ratio, although $K_{m(app)}$ remained constant. In NqrB-D397C, site I is fully functional and site II is partially active, exhibiting a low affinity for sodium. Cysteine seems to be the only residue capable of partially replacing aspartic acid, because it is the closest residue in terms of size and charge. Further evidence supporting the ability of cysteine to partially replace the aspartate residue in binding site II comes from the sodium protection experiments. As mentioned before, the mutant NqrB-D397C is specifically inhibited by the thiol-modifying reagent iodoacetamide. The addition of sodium to the inactivation buffer protects the enzyme, demonstrating that the cysteine residue interacts directly with the cation. The large difference in the kinetic properties between the two sodium-binding sites in this mutant is therefore responsible for the apparent negative cooperativity observed. In contrast to the wild type enzyme, in which the two binding sites work sequentially, in NqrB-D397C they operate independently. A key finding of this work is the fact that the enzyme can function with only one binding site, which demonstrates that the sites are not connected and work independently.

Interestingly, NqrB-Asp-397 seems to have a much more important role in the binding of sodium, compared with the binding of lithium, because mutations at this site are less
disruptive in lithium-dependent activity. All the mutants are able to bind lithium with a similar apparent affinity, likely because it has a smaller ionic radius compared with sodium and can use only five ligands instead of the six ligands needed for sodium ligation (19), explaining the less disruptive effect of the mutations on the lithium dependent activity. Thus, it seems that NqrB-Asp-397 participates in the cation selectivity filter, by determining the size of the ion that the enzyme can bind.

We previously used an FTIR electrochemical method to study changes that take place upon oxidation and reduction of Na⁺-NQR (17). The results demonstrate that the enzyme undergoes large structural rearrangements that are triggered by the reduction process and by the binding of sodium and lithium, which confirms our hypothesis that indicates that the sodium pumping mechanism of the enzyme is driven by conformational changes. Signals in the amide I and II regions suggest that these movements involve α-helices, β-sheet, and β-turn structures. The data also provide structural confirmation demonstrating that both the oxidized and reduced forms of the enzyme can interact with sodium, which was previously shown by our group (16), and that sodium is bound by a monodentate carboxylate in the oxidized form and bidentate carboxylate in the reduced form, with peaks at 1370 and 1410 cm⁻¹.

The mutant NqrB-D397E also undergoes a structural rearrangement upon reduction and after the cation uptake but not to the same extent that the wild type Na⁺-NQR does, which agrees with our model in which only one sodium-binding site may be active in this mutant. The mutant spectra also show a peak at 1714 cm⁻¹, a position typical of C=O modes of protonated aspartate and glutamic acids, which is absent in wild type Na⁺-NQR. Although further studies are necessary to elucidate the identity of this acidic group, it can be envisioned that it could be part of sodium-binding site II, which is disrupted in the mutant. The mutation may produce a major change in this site, altering the hydrophobicity, in which the protonated carboxylate could be more stable. In NqrB-D397E, the peaks corresponding to the monodentate and bidentate carboxylate are inverted with respect to the wild type enzyme. This indicates that the mutation causes a significant change in the structural interaction of the pumped ions with the binding site(s) and is consistent with the weaker binding of both Na⁺ and Li⁺ observed in steady state kinetic measurement on the mutant enzyme. Finally, this study opens the possibility to

FIGURE 5. Model of sodium-binding site cooperativity in Na⁺-NQR. The model describes two sodium-pumping sites in NqrB (site II) and in NqrD/E (site I). In the oxidized form of the enzyme, the two sites are preformed (A) and sodium exchange may be slow. The one-electron reduction of FMNC increases the accessibility of sodium to one site (B and C) and upon binding to this site, an inter-subunit interaction occurs, which opens the second site (D) producing a fully active enzyme. E represents a partially active form, where site II is disrupted by mutations at NqrB-Asp-397. Disruption of site II interrupts the interaction with site I, eliminating the cooperative behavior of these two sites.
discriminate between Na\(^+\)-binding sites and characterize them independently.

REFERENCES

1. Barquera, B., Hellwig, P., Zhou, W., Morgan, J. E., Häse, 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\(^+\)-translocating NADH:quinone oxidoreductase from *Vibrio cholerae*. *Biochemistry* **41**, 3781–3789

2. 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

3. Bogachev, A. V., and Verkhovsky, M. I. (2005) Na\(^+\)-translocating NADH:quinone oxidoreductase: progress achieved and prospects of investigation. *Biochemistry* **70**, 143–149

4. Steuber, J., Krebs, W., and Dimroth, P. (1997) The Na\(^+\)-coupled oxidative phosphorylation in *Vibrio alginolyticus*-redox states of the FAD prosthetic group and mechanism of Ag\(^+\) inhibition. *Eur. J. Biochem.* **249**, 770–776

5. Dibrov, P. A., Lazarova, R. L., Skulachev, V. P., and Verkhovsky, M. L. (1986) The sodium cycle. II. Na\(^+\)-coupled oxidative phosphorylation in *Vibrio alginolyticus* cells. *Biochim. Biophys. Acta* **850**, 458–465

6. Dashper, S. G., Brownfield, L., Slakesi, N., Zilm, P. S., Rogers, A. H., and Reynolds, E. C. (2001) Sodium ion-driven serine/threonine transport in *Porphyromonas gingivalis*. *J. Bacteriol.* **183**, 4142–4148

7. Kojima, S., Yamamoto, K., Kawagishi, I., and Homma, M. (1999) The polar flagellar motor of *Vibrio cholerae* is driven by an Na\(^+\) motive force. *J. Bacteriol.* **181**, 1927–1930

8. 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

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

10. Júarez, O., Morgan, J. E., and Barquera, B. (2009) The electron transfer pathway of the Na\(^+\)-pumping NADH:quinone oxidoreductase from *Vibrio cholerae*. *J. Biol. Chem.* **284**, 8963–8972

11. Júarez, O., Morgan, J. E., Nilges, M. L., and Barquera, B. (2010) The energy transducing redox steps of the Na\(^+\)-pumping NADH:quinone oxidoreductase from *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 12505–12510

12. Neehaul, Y., Júarez, O., Barquera, B., and Hellwig, P. (2012) Thermodynamic contribution to the regulation of electron transfer in the Na\(^+\)-pumping NADH:quinone oxidoreductase from *Vibrio cholerae*. *Biochemistry* **51**, 4072–4077

13. Júarez, O., Athearn, K., Gillespie, P., and Barquera, B. (2009) Acid residues in the transmembrane helices of the Na\(^+\)-pumping NADH:quinone oxidoreductase (Na\(^+\)-NQR) from *Vibrio cholerae* involved in sodium translocation. *Biochemistry* **48**, 9516–9524

14. Barquera, B., Nilges, M. J., Morgan, J. E., Ramirez-Silva, L., Zhou, W., and Gennis, R. B. (2004) Mutagenesis study of the 2Fe-2S center and the FAD-binding site of the Na\(^+\)-translocating NADH:ubiquinone oxidoreductase from *Vibrio cholerae*. *Biochemistry* **43**, 12322–12330

15. Roitel, O., Sergienko, E., and Branlant, G. (1999) Dimers generated from tetrameric phosphorylating glycerolaldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* are inactive but exhibit cooperativity in NAD binding. *Biochemistry* **38**, 16084–16091

16. Júarez, 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

17. Neehaul, Y., Júarez, O., Barquera, B., and Hellwig, P. (2013) Infrared spectroscopic evidence of a redox-dependent conformational change involving ion binding residue NqrB-D397 in the Na\(^+\)-pumping NADH:quinone oxidoreductase from *Vibrio cholerae*. *Biochemistry* **52**, 3085–3093

18. Schmidt, S., Biegel, E., and Müller, V. (2009) The ins and outs of Na\(^+\) bioenergetics in *Acetobacterium woodii*. *Biochim. Biophys. Acta* **1787**, 691–696

19. Gouaux, E., and Mackinnon, R. (2005) Principles of selective ion transport in channels and pumps. *Science* **310**, 1461–1465