Role of Subunit D in Ubiquinone-Binding Site of Vibrio cholerae NQR: Pocket Flexibility and Inhibitor Resistance

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ABSTRACT: The ion-pumping NADH: ubiquinone dehydrogenase (NQR) is a vital component of the respiratory chain of numerous species of marine and pathogenic bacteria, including Vibrio cholerae. This respiratory enzyme couples the transfer of electrons from NADH to ubiquinone (UQ) to the pumping of ions across the plasma membrane, producing a gradient that sustains multiple homeostatic processes. The binding site of UQ within the enzyme is an important functional and structural motif that could be used to design drugs against pathogenic bacteria. Our group recently located the UQ site in the interface between subunits B and D and identified the residues within subunit B that are important for UQ binding. In this study, we carried out alanine scanning mutagenesis of amino acid residues located in subunit D of V. cholerae NQR to understand their role in UQ binding and enzymatic catalysis. Moreover, molecular docking calculations were performed to characterize the structure of the site at the atomic level. The results show that mutations in these positions, in particular, in residues P185, L190, and F193, decrease the turnover rate and increase the Km for UQ. These mutants also showed an increase in the resistance against the inhibitor HQNO. The data indicate that residues in subunit D fulfill important structural roles, restricting and orienting UQ in a catalytically favorable position. In addition, mutations of these residues open the site and allow the simultaneous binding of substrate and inhibitors, producing partial inhibition, which appears to be a strategy used by Pseudomonas aeruginosa to avoid autopoisoning.

INTRODUCTION

The facultative aerobic, Gram-negative bacterium Vibrio cholerae most often grows in saltwater marine ecosystems, but certain strains can also colonize the human intestine, causing cholera, the life-threatening diarrheal disease. Cholera is closely associated with natural disasters and war-torn regions where clean water supplies are unavailable or tainted.1,2 In high salinity environments and in the human small intestine, this microorganism is able to thrive due to its ability to pump sodium ions across the cell membrane, creating a sodium motive force (SMF).3 The SMF can be utilized to power essential cellular processes, such as flagellar rotation, substrate transport, pH regulation, and ATP synthesis.3−8 In V. cholerae, the SMF is generated by the NADH: quinone oxidoreductase enzyme complex (NQR),3 which couples the transfer of electrons from NADH to ubiquinone (UQ) to the pumping of sodium ions.3−14 In V. cholerae, it has been shown that NQR regulates the transcription of the ToxT gene, which positively regulates the production of the cholera toxin, toxin coregulated pili, and accessory colonization factor.15,16 Mutations to the nqr operon, as well as NQR inhibitors, decrease the transcription of the toxT gene resulting in decreased production of the cholera toxin and other virulence factors.

NQR is composed of six subunits (A–F) and five confirmed redox cofactors that facilitate electron transfer: flavin adenine dinucleotide (FAD), 2Fe–2S center, two covalently bound flavin mononucleotide (FMN) cofactors, and riboflavin.5,9,17,18 These cofactors shuttle electrons from NADH, the initial electron donor, to UQ, the final electron acceptor.17,19−21 Interestingly, riboflavin’s use as a cofactor has been solely

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reported in NQR. Additionally, the covalently linked FMN cofactors are attached through a unique covalent bond found in a few other respiratory enzymes. Electrons from NADH are initially donated from NADH to the FAD cofactor located in subunit F and proceed to the other cofactors. From FAD, electrons are transferred to the $2\text{Fe}−2\text{S}$ center, also located within subunit F, to an FMN cofactor in subunit C. The electrons subsequently move to FMN in subunit B to riboflavin and finally to UQ. Crystallographic data suggests that an additional nonheme Fe cofactor could participate in the shuttling of electrons, but its presence has not been confirmed experimentally. The location of the final electron acceptor UQ has been a controversial topic with two main hypotheses. One model indicates that a binding site could be located in a hydrophilic pocket within the cytosolic subunit A, which would be able to accommodate the benzoquinone head, with the isoprenoid tail probably interacting with subunit B. However, our group has followed the original hypothesis by Unemoto’s group and the earlier work by Barquera’s group, and we have identified that the catalytic UQ binding site is located in the interface of subunits B and D, deeply embedded in the membrane core. In our previous report, we showed that most of the residues in subunit B that form part of the UQ binding pocket are highly conserved. Alanine was used as a replacement amino acid to remove the bulky functional groups of the residues.

### RESULTS

**Alanine Scanning Mutagenesis.** Alanine scanning mutagenesis was conducted on amino acid residues of subunit D adjacent to the identified UQ binding pocket to understand their function. Figure 1 shows the location of these residues within the binding site (Figure 1A,B), and their interactions with UQ and HQNO (2-n-heptyl-4-hydroxyquinoline N-oxide) (Figure 1C,D, respectively), an inhibitor that also occupies this site. In our experiments, we used alanine scanning mutagenesis of conserved residues in subunit B to locate residues involved in UQ binding and catalysis. Moreover, the functional data were corroborated by molecular docking. Our data helped us unambiguously locate the catalytic UQ binding site in the protein and to identify residues F185 and F211 in subunit B as critical parts of this motif. While the role of subunit B residues is now well understood, the other part of the UQ binding site has not been studied. The goal of this project is to characterize the role of residues of subunit D lining the UQ binding site, through the characterization of alanine mutants and the use of computational analysis methods. Our results indicate that although the residues of subunit D are not conserved, they fulfill important roles, delimiting the UQ binding site. Moreover, mutations of these residues, which have been reported in *Pseudomonas aeruginosa*, confer resistance against antibiotics targeting this site. The identification and characterization of the UQ binding site are important to understand the catalytic mechanism of NQR, and it has become evident that it can be used to treat infectious diseases. The work by Dibrov et al. is now showing that NQR inhibitors that target the UQ binding site clear the infection by the intracellular pathogen *Chlamydia trachomatis*, which, as recently shown by our group, depends on NQR to maintain the infection.

**Figure 1.** (A) Overall structure of NQR and location of the UQ binding pocket (orange circle) in the interface of subunits B (blue) and D (green). (B) Arrangement of subunits B and D residues in the UQ binding site (upper panel: front view; lower panel: top view). Subunit B residues 185 and 211 are highlighted in cyan. Subunit D residues are highlighted in orange. Best-docked pose of UQ (C) and HQNO (D) to NQR. Inset shows the top view of the binding site. (E) Sequence alignment of sections of subunit D involved in the UQ binding site.
could interact with UQ while maintaining the hydrophobic environment of the pocket. In the case of P185, the proline-to-glycine substitution is preferred, since glycine has “helix-breaking” properties similar to proline, while alanine has helix-forming properties.

Kinetic Characterization of the Mutants. To determine the role of each residue on enzyme activity and UQ binding, a kinetic characterization was conducted on the mutants. Enzyme activity was tested at varying concentrations of UQ at near-saturating concentrations of NADH (250 μM) and NaCl (50 mM), which allowed us to determine the turnover rate (kcat) and apparent Km for UQ (KmUQ) for the mutants and compare it to that of wild-type NQR. For all mutants, the observed kcat was significantly lower compared to wild-type NQR (Table 1 and Figure 2A). It should be noted that wild-type data was obtained from ref 27.

Table 1. Kinetic Properties of NQR Mutants

| Mutant | kcat (s⁻¹) | KmUQ (μM) | Kmapp (μM) | HQNO resistance (s⁻¹) |
|--------|------------|-----------|------------|-----------------------|
| F151A  | 278 ± 30   | 3.7 ± 1.5 | 0.5 ± 0.15 | 24 ± 1.3              |
| L155A  | 244 ± 4    | 4 ± 0.5   | 0.1 ± 0.04 | 38 ± 3                |
| L180A  | 312 ± 50   | 4.9 ± 2.3 | 0.3 ± 0.15 | 33 ± 3                |
| P185G  | 88 ± 5     | 0.5 ± 0.2 | 0.18 ± 0.1 | 49 ± 3                |
| F189A  | 219 ± 30   | 4.6 ± 2   | 0.2 ± 0.01 | 39 ± 0.6              |
| L190A  | 184 ± 20   | 8.7 ± 2   | 0.5 ± 0.1  | 41 ± 0.3              |
| F193A  | 123 ± 33   | 5.2 ± 2   | 0.8 ± 0.1  | 47 ± 1                |
| WT     | 490        | 2.7       | 0.2        | <5                    |

“Activity measurements were conducted in the presence of 250 μM NADH and 50 mM NaCl with different concentrations of HQNO. Apparent kinetic parameters were obtained by fitting the data from Figure 2 to eq 1, assuming saturating concentrations of all substrates. Wild-type data was obtained from ref 27.

Figure 2. Titration of NQR mutant activity with UQ (A) and HQNO (B). O, P185G; , L190A; ▲, F193A; △, L190A; □, L155A; ◆, F151A; ▲, F189A. Data points are shown as standard deviations of the mean (SDM) (n = 8).

Although all mutants had lower UQ reduction rates, the nonphysiologic NADH oxidation rates (>550 s⁻¹, not shown) were not affected by the mutations, indicating that the mutation solely influences the electron transfer following an initial donation by NADH (likely the transfer of electrons to UQ). The kinetic data obtained show that mutants P185G, L190A, and F193A have the lowest kcat. Moreover, the mutants L190A and F193A show an increase in the KmUQ of 2–3 times, compared to the wild-type enzyme. Interestingly, the P185G mutant had the lowest KmUQ values of all mutants (Table 1).

In addition to activity characterizations, HQNO titrations were performed with the mutants. HQNO is a compound naturally produced by different types of bacteria, including P. aeruginosa. This molecule is an inhibitor of many respiratory enzymes, but it is particularly effective against V. cholerae NQR, with sub-μM Kiapp values (Table 1). HQNO was proposed to interact with the UQ binding site, which would make it a competitive inhibitor vs UQ. However, the data show that HQNO is a mixed-type inhibitor, complicating the interpretation of the data. Our group showed that HQNO indeed interacts with the UQ binding site, competing with UQ (giving rise to the competitive component) and with ubiquinol, the product of the reaction, explaining the uncompetitive component of the mixed inhibition. Titrations of the activity using HQNO show that most mutants have a similar Kiapp compared to the wild-type enzyme. However, in all cases tested, the activity could not be completely inhibited with HQNO, and a resistant component was evident (Figure 2B and Table 1). The mutants P185G, L190A, and F193A showed the highest HQNO-resistant components. Due to the significant changes observed, these three mutants were selected for further characterization.

HQNO Inhibitory Mechanism. The inhibition mechanism of HQNO was studied in the mutants by performing UQ titrations at varying concentrations of HQNO (Figure 3). The data obtained was globally fitted to the equation for different types of inhibitors, including competitive, uncompetitive, mixed (not shown), and mixed partial (eq 1). The data was best fitted to the function describing mixed-partial-type inhibition, as clearly shown in the double reciprocal plot with intersecting lines in the second or third quadrant, although all mutants had lower UQ reduction rates, the nonphysiologic NADH oxidation rates (>550 s⁻¹, not shown) were not affected by the mutations, indicating that the mutation solely influences the electron transfer following an initial donation by NADH (likely the transfer of electrons to UQ). The kinetic data obtained show that mutants P185G, L190A, and F193A have the lowest kcat. Moreover, the mutants L190A and F193A show an increase in the KmUQ of 2–3 times, compared to the wild-type enzyme. Interestingly, the P185G mutant had the lowest KmUQ values of all mutants (Table 1).

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characteristic of this type of inhibition (Figure 3). Two inhibition constants were obtained, corresponding to the competitive and uncompetitive components of the mixed inhibition ($K_{ic}$ and $K_{iu}$), respectively (Table 2). Moreover, the $k_{catR}$ was also obtained, corresponding to the kinetic component that is resistant to saturating concentrations of the inhibitor. This component indicates that the enzyme can bind the substrate and the inhibitor at the same time and still be active. As shown in Table 2, the $K_{ic}$ and $K_{iu}$ values for the mutants are similar ($0.1$ to $0.3 \mu M$), consistent with the intercepting pattern near the abscissa. The inhibitor-resistant component was also calculated for the three mutants, with values ranging from $30$ to $50\%$, which are comparable or higher to the values reported for <i>P. aeruginosa</i> NQR, which is naturally resistant to this inhibitor.

Taken together, the data indicate that these residues, except P185, play important-but-not-essential roles in the UQ binding site. P185, the only conserved residue, appears to have a critical structural role that cannot be fulfilled by glycine or other residues. Interestingly, the mutation of these residues confers significant resistance to HQNO.

**Molecular Docking.** To understand the role of these residues on the structure of the UQ binding pocket and their influence on the substrate and inhibitor binding, molecular docking was performed using the crystal structure of wild-type <i>V. cholerae</i> NQR (PDB ID: 4P6V) and the predicted structure of the mutants. The residues were mutated in silico using UCSF Chimera. Figures 4 and 5 show the lowest energy poses of UQ and HQNO in the binding pocket, respectively. In wild-type NQR, UQ is deeply embedded in the

### Table 2. Kinetic Properties of NQR Mutants

| mutant  | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $K_{ic}$ (μM) | $K_{iu}$ (μM) | $k_{catR}$ ($s^{-1}$) |
|---------|----------------------|------------|---------------|---------------|----------------------|
| WT      | 490                  | 2.7        | 0.2           | 0.2           | <5                   |
| P185G   | $95 \pm 4$           | $2.7 \pm 0.5$ | $0.11 \pm 0.04$ | $0.15 \pm 0.04$ | $30 \pm 1$          |
| L190A   | $172 \pm 11$         | $5.7 \pm 1.1$ | $0.37 \pm 0.20$ | $0.21 \pm 0.06$ | $45 \pm 3$           |
| F193A   | $133 \pm 8$          | $5.4 \pm 1.0$ | $0.26 \pm 0.13$ | $0.17 \pm 0.06$ | $49 \pm 3$           |

Activity measurements were conducted in the presence of 250 μM NADH and 50 mM NaCl with different concentrations of UQ-1 or HQNO. Kinetic parameters were obtained by globally fitting the data from Figure 3 to eq 1. Wild-type data was obtained from ref 27.
The docking data suggest that HQNO is bound directly to the UQ binding site in the wild-type enzyme (Figures 1D and 5A). In the L190 and F193 mutants, HQNO is not bound as deeply, and it appears relatively rotated (Figure 5B,C), consistent with the slight increase in the $K_{UQ}$ for this inhibitor. Due to this rotation, the distance between HQNO and subunit B increases, and it could be possible to accommodate both UQ and HQNO in this site, a likely explanation for the partial inhibition. As shown in Figure 4D, the binding of HQNO to mutant P185G is nearly identical to wild type (Figure 5D).

### DISCUSSION

In this work, the role of amino acid residues in NQR subunit D lining the UQ binding pocket was analyzed. In a previous study, residues of subunit B within the pocket were shown to be highly conserved across bacterial lineages. Sequence analyses show that subunit D residues within the binding pocket are not conserved, except P185, which is conserved across all bacterial species. Although the residue variability in these positions could indicate that they are not important for UQ binding, the data indicate that these residues fulfill important roles, maintaining the pocket structure and confining UQ to the catalytically relevant orientation in the binding site.

**Role of Subunit D Residues in UQ Binding.** Although most of these residues are not conserved in the NQR family, their mutants show significant changes in the catalytic parameters, as shown in Table 1 and Figure 2. In all of these cases, a decrease in activity was found, as well as a moderate increase in the $K_{UQ}$ and a significant increase in the resistance to the inhibitor HQNO. To understand the effects of these changes, mutants P185G, L190A, and F193A (which produced the more pronounced effects) were characterized. The most drastic decline in activity occurred for mutant P185G, with a 5-fold decrease in $k_{cat}$ as to that of the wild-type enzyme. Interestingly, the $K_{UQ}$ of this mutant has also the lowest of all, and it required a small concentration of UQ to be fully active. The decrease in $k_{cat}$ could be attributed to the disruption of the structure of the enzyme, since proline is a helix breaker. Alteration of the helix structure could cause a larger change in the overall structure of the binding pocket or even the entire complex, decreasing the activity. Alternatively, this decrease in activity could be due to the binding of UQ in a location that is close, but not in the UQ binding site. Molecular docking revealed sharp differences between the mutants and wild type in how UQ binds within the pocket (Figure 4). In the wild type, UQ binds deeply within the center of subunits B and D with its ring in the direction of subunit D. For P185G, UQ is located above the binding pocket and off-center, more closely associating with subunit D. The decrease in the turnover rate would also produce the observed decrease in $K_{m}$, since this parameter contains $k_{cat}$ in the denominator ($k_{-1} + k_{cat}/k_{1}$).

The mutants L190A and F193A showed a 2-fold decrease in the $k_{cat}$ compared to the wild-type enzyme and also had the two highest $K_{UQ}$ values, 2−3 times greater than that of wild type. The results suggest that these residues interact directly with the substrate in the binding pocket and upon modification, higher concentrations of the substrate are required. The results obtained from molecular docking help to understand this behavior. The lowest energy pose for UQ in the F193A mutant is located above the UQ pocket in a vertical orientation, with the isoprenoid tail pointing downwards into the pocket, instead of lying planar with respect to F185 and F211 residues in subunit B, as is the case for the wild-type pose. The lowest energy pose of the L190A mutant shows UQ above the binding site, which is not close to that of the wild type at all; instead, it is inverted and rotated to face subunit D. Even though L190 and L193 residues are not conserved, in all cases found, these positions contain bulky aromatic and aliphatic residues. These residues serve as the lid of the UQ binding site and stabilize the substrate in the proper position that allow high electron transfer rates. Once the lid is removed, by adding small residues such as alanine, UQ is not properly oriented, and the small changes observed in the distance between riboflavin and UQ could have a deep effect on electron transfer. This difference could explain the lowered $k_{cat}$ and higher $K_{m}$ values obtained for these mutants.

**Role of Subunit D Residues in Inhibitor Resistance.** The mutants showed an increase in the resistance to HQNO, in addition to the changes in the kinetic parameters. In mutants P185G, L190A, and F193A, HQNO behaved as a partial-mixed inhibitor vs UQ. These results differed greatly from the wild-type enzyme, which has been shown to be inhibited by sub-micromolar concentrations of HQNO with a mixed-type inhibition. Remarkably, under the partial-mixed mechanism of inhibition, the enzyme is functional at saturating concentrations of HQNO, and it is able to bind the substrate and inhibitor at the same time. Molecular docking shows that HQNO is bound deeply in the wild-type pocket. Analogous to what was observed for UQ, in mutants L190A and F193A, HQNO is found in a different orientation and appears rotated, explaining the increase in the $K_{UQ}$ for the inhibitor. Moreover, in these mutants, we observed that HQNO interacts more closely with subunit D, opening a space that could accommodate the substrate and inhibitor. For P185G, HQNO appears to bind in the same position as in wild type. As previously mentioned, proline serves as a helix breaker and the disruption of this residue could cause larger structural effects to the helix and probably in the entire UQ binding pocket, which cannot be replicated by our docking analysis even when the MD relaxation step is incorporated. It is likely that major structural alterations caused by this mutation could cause a decrease in catalytic activity and probably stabilize the interaction with HQNO. In previous reports, it was proposed that the ion-pumping mechanism of NQR is kinetically controlled, which supports a mechanism in which the enzyme would undergo significant conformational changes. The crystallographic structure of NQR shows that several of the cofactors are separated by distances that would not allow electron transfer at physiologically relevant rates, further suggesting a conformationally driven mechanism. Our group
The linear pathway, is >30 Å. Thus, significant inhibition of integrated electron carriers in ribosomes is required to transfer at physiologically relevant rates. For instance, the distance between many cofactors, including riboflavin and the ubiquinone-binding site, is too large to support electron transfer at physiologically relevant rates. Another important aspect that supports the role of significant inhibition in integrated electron carriers is that the mutations could produce long-range effects that could interfere with other steps, which, in a highly dynamic system as NQR, might produce changes in a distant UQ site or other structures. Another important aspect that supports the role of large conformational changes in the mechanism of NQR is that the distance between many cofactors, including riboflavin and the ubiquinone-binding site, is too large to support electron transfer at physiologically relevant rates. For instance, the distance between FMN in subunit B, the crystallographic riboflavin, and the catalytic UQ binding site characterized in this study, which comprises the last three electron carriers in the linear pathway, is >30 Å. Thus, significant subunit reorganization must occur during the catalytic process to bring cofactors together and allow the redox reactions to proceed.

In a previous report by our group, *P. aeruginosa* NQR (Pa-NQR) was characterized. The results showed that Pa-NQR possesses inhibition resistance to HQNO with a partial-mixed-type inhibition. Our results showed that *P. aeruginosa* NQR contains mutations in residues 151 and 155, which confer significant resistance against HQNO (naturally produced by *P. aeruginosa*) (Table 1), and in mutants P185G, L190A, and F193A. According to the data found in this work (Table 1), these two positions are optimal to confer HQNO resistance, since the mutants show partial inhibition with a significant HQNO-resistant component, while maintaining some of the highest enzymatic activity.

### CONCLUSIONS

The results indicate that residues of the UQ binding site in subunit D play major roles in catalytic UQ binding site, allowing the proper location and orientation of UQ in the site. Moreover, these residues also play roles in pocket structure and flexibility, and subtle changes in this site can confer resistance against HQNO, which is naturally produced by several types of bacteria, allowing the binding of the substrate and inhibitor at the same time, which can be beneficial to avoid autopoisoning.

### MATERIALS AND METHODS

#### Plasmid Constructs

The mutations were obtained with a site-directed mutagenesis kit (Agilent Technologies) using primers designed to mutate residues of interest to alanine (or glycine in the case of the P185 residue), using the primers found in Table 3. Mutations were subcloned in-frame into *S*-KpnI and *S*-EcoRI restriction enzyme sites in an *nqr*-pBAD/HisB construct. Included in the construct were a triplicate Gly repeat spacer and a six-histidine sequence. Mutant *nqr*-pBAD/HisB plasmids were transformed into *V. cholerae* O395 strain with a deleted genomic *nqr* operon (*Δnqr*) for subsequent protein expression. All mutations were confirmed through DNA sequencing (Operon MWG).

#### Protein Expression and Purification

*V. cholerae* Δnqr cells carrying the pBAD plasmid containing wild-type or mutant NQR operon were grown in Luria Broth media, as described previously by Tuz et al. The expression of NQR was induced using arabinose. Induced cells were harvested, washed, and lysed via sonication (60 s pulsed sonication, 50% duty cycle). Cell membranes were collected by differential centrifugation and solubilized in buffer containing 0.3% n-dodecyl-β-D-maltoside (DDM), 5 mM imidazole, 50 mM Na2HPO4, 300 mM NaCl, 5% glycerol, pH 8.0. Protein purification was done using Ni-NTA affinity chromatography and DEAE-sepharose ion-exchange chromatography.

#### Activity Measurements

UQ reductase activity was measured spectrophotometrically at 282 nm, using a molar extinction coefficient of 10.2 mM−1 cm−1. Enzyme activity assays were performed in buffer containing 250 μM K2-NADH, 50 mM NaCl, 50 mM Tris—HCl, 1 mM EDTA, 5% glycerol, 0.05% DDM, pH 8.0. Saturation kinetics were measured at varying concentrations of UQ-1 (0–50 μM) and HQNO (0–10 μM).

To characterize the inhibition mechanism of HQNO, UQ-1 titration experiments were performed at several fixed-variable concentrations of HQNO. The data were fitted to the functions of competitive, uncompetitive, and mixed-type inhibitions. However, the function that best described the behavior is that of partial-mixed-type inhibition (eq 1),

\[
v = \frac{k_{cat} \left[ \frac{[S]}{K_m} + \frac{[I]}{K_{in}} \right]}{1 + \frac{[I]}{K_{in}} + \frac{[S]}{K_m} + \frac{[I]}{K_{in}} \left[ \frac{[S]}{K_m} \right]} \tag{1}\]

where *v* is the turnover rate, *k*_{cat} is the turnover rate at saturating concentrations of the substrate in the absence of the inhibitor, *k*_{cat} is the turnover rate obtained at saturating concentrations of the substrate and inhibitor, *K*_{in} is the concentration of UQ [I] is the concentration of HQNO, Km is the Michaelis–Menten constant, and *K*_{cat} and *K*_{in} are the inhibition constants of the competitive and uncompetitive components.

#### Molecular Docking

Molecular docking of HQNO and UQ-1 was performed with the wild-type structure of *V. cholerae* NQR (1), and in mutants P185G, L190A, and F193A. Mutations of these residues were generated in UCSF Chimera.

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**Table 3. NQR Subunit D Mutation Primers**

| mutant | sense primer sequence (5′→3′) |
|--------|-----------------------------|
| F151A  | GTAAGCAGGTATTCTTGCGGCCGTTTGAAGC |
| L155A  | TTTGTTTCTTCCGCTGAGCTTGGCCGGCTACGTTAAACCTTGG |
| L180A  | TGGATTACACCCGACAGGCGGATGCTACTGCGACCCTTC |
| P185G  | CGGACCTGATGCTACCGAGGTTTACAGATTCTTCCTGATC |
| F189A  | CGGACCTTCTAGCATTCTCGGGATCGGCTATGATTGGG |
| L190A  | GCAATCTTCTCTGATGCCGCGCACTATGTGTTGGGCAATTCG |

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The interface of the BDE subunits, where the UQ binding site is located, of these models was prepared for UCSF DOCK 6.6 (3) by removing all hydrogen atoms with UCSF Chimera 1.9 (2). All models’ molecular surfaces were generated using DMS from DOCK 6.6. Sphgen, another Dock 6.6 tool, was utilized to generate vacancy spheres with a radius between 1.0 Å and 5.0 Å (3). The spheres were generated within a 36−36−36 Å box surrounding the BDE interface. Grid scores were used to rank docked poses, with the lowest grid score corresponding to the highest-ranked pose. The highest-ranked pose for both HQNO and UQ-I from the wild-type docking was then transposed into the mutant structures, and a grid score was calculated.

**ASSOCIATED CONTENT**

**Accession Codes**

PD accession number for *V. cholerae* NQR is 4P6V.

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**

(1) Jutla, A.; Khan, R.; Colwell, R. Natural Disasters and Cholera Outbreaks: Current Understanding and Future Outlook. *Curr. Environ. Health Rep.* 2017, 4, 99−107.

(2) Watson, J. T.; Gayter, M.; Connolly, M. A. Epidemics after Natural Disasters. *Emerging Infect. Dis.* 2007, 13, 1−5.

(3) Hase, C. C.; Barquera, B. Role of Sodium Bioenergetics in *Vibrio cholerae*. *Biochim. Biophys. Acta, Bioenerg.* 2001, 1505, 169−178.

(4) Kojima, S.; Yamamoto, K.; Kagawashi, I.; Homma, M. The Polar Flagellar Motor of *Vibrio cholerae* Is Driven by an Na+ Motive Force. *J. Bacteriol.* 1999, 181, 1927−1930.

(5) Hase, C. C.; Fedorova, N. D.; Galperin, M. Y.; Dibrov, P. A. Sodium Ion Cycle in Bacterial Pathogens: Evidence from Cross-Genome Comparisons. *Microbiol. Mol. Biol. Rev.* 2001, 65, 353−370.

(6) Skulachev, V. P. Sodium Bioenergetics. *Trends Biochem. Sci.* 1984, 9, 483−485.

(7) Reyes-Prieto, A.; Barquera, B.; Juárez, O. Origin and Evolution of the Sodium-Pumping NADH: Ubiquinone Oxidoreductase. *PLoS One* 2014, 9, No. e96696.

(8) Dibrov, P.; Dibrov, E.; Pierce, G. N. Na+-NQR (Na+ +Translocating NADH:Ubiquinone Oxidoreductase) as a Novel Target for Antibiotics. *FEBS Microbiol. Res.* 2017, 41, 653−671.

(9) Juárez, O.; Barquera, B. Insights into the Mechanism of Electron Transfer and Sodium Translocation of the Na+-Pumping NADH:Quinone Oxidoreductase. *Biochim. Biophys. Acta, Bioenerg.* 2012, 1817, 1823−1832.

(10) Hayashi, M.; Nakayama, Y.; Unemoto, T. Recent Progress in the Na+-Translocating NADH-Quinone Reductase from the Marine Vibrio alginolyticus. *Biochim. Biophys. Acta, Bioenerg.* 2001, 1505, 37−44.

(11) Steuber, J.; Vohl, G.; Muras, V.; Toulouse, C.; Claüßen, B.; Vorburger, T.; Fritz, G. The Structure of Na+-Translocating of NADH:Ubiquinone Oxidoreductase of *Vibrio cholerae*: Implications on Coupling between Electron Transfer and Na+ Transport. *Biol. Chem.* 2015, 396, 1015−1030.

(12) Bogachér, A. V.; Bertecchi, Y. V.; Barquera, B.; Verkhovsky, M. I. Sodium-Dependent Steps in the Redox Reactions of the Na+-Motive NADH:Quinone Oxidoreductase from *Vibrio harveyi*. *Biochemistry* 2001, 40, 7318−7323.

(13) Juárez, O.; Shea, M. E.; Makhadze, G. I.; Barquera, B. The Role and Specificity of the Catalytic and Regulatory Cation-Binding Sites of the Na+-Pumping NADH:Ubiquinone Oxidoreductase from *Vibrio cholerae*. *J. Biol. Chem.* 2011, 286, 26383−26390.

(14) Juárez, O.; Athey, K.; Gillespie, P.; Barquera, B. Acid Residues in the Transmembrane Helices of the Na+-Pumping NADH:Quinone Oxidoreductase from *Vibrio cholerae* Involved in Sodium Translocation. *Biochemistry* 2009, 48, 9516−9524.

(15) Hase, C. C.; Mekalanos, J. J. TcpP Protein Is a Positive Regulator of Virulence Gene Expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A.* 1998, 95, 730−734.

(16) Minato, Y.; Fassio, S. R.; Reddekkopp, R. L.; Hase, C. C. Inhibition of the Sodium-Translocating NADH:Ubiquinone Oxidoreductase [Na+-NQR] Decreases Cholera Toxin Production in *Vibrio cholerae* O1 at the Late Exponential Growth Phase. *Microb. Pathog.* 2014, 66, 36−39.

(17) Juárez, O.; Nilges, M. J.; Gillespie, P.; Cotton, J.; Barquera, B. Riboflavin Is an Active Redox Cofactor in the Na+-Pumping NADH:Quinone Oxidoreductase (Na+-NQR) from *Vibrio cholerae*. *J. Biol. Chem.* 2008, 283, 33162−33167.

(18) Steuber, J.; Vohl, G.; Casutt, M. S.; Vorburger, T.; Diederichs, K.; Fritz, G. Structure of the *V. cholerae* Na+-Pumping NADH: Quinone Oxidoreductase. *Nature* 2014, 516, 62−67.

(19) Neehaul, Y.; Juárez, O.; Barquera, B.; Hellwig, P. Thermodynamic Contribution to the Regulation of Electron Transfer in the Na+ +Pumping NADH:Quinone Oxidoreductase from *Vibrio cholerae*. *Biochemistry* 2012, 51, 4072−4077.

(20) Strickland, M.; Juárez, O.; Neehaul, Y.; Cook, D. A.; Barquera, B.; Hellwig, P. The Conformational Changes Induced by Ubiquinone Binding in the Na+-Pumping NADH:Ubiquinone Oxidoreductase (Na+-NQR) Are Kinetically Controlled by Conserved Glycines 140 and 141 of the NqrB Subunit. *J. Biol. Chem.* 2014, 289, 23723−23733.

(21) Juárez, O.; Morgan, J. E.; Barquera, B. The Electron Transfer Pathway of the Na+-Pumping NADH:Quinone Oxidoreductase from *Vibrio cholerae*. *J. Biol. Chem.* 2009, 284, 8963−8972.

(22) Barquera, B.; Zhou, W.; Morgan, J. E.; Gennis, R. B. Riboflavin Is a Component of the Na+-Pumping NADH:Quinone Oxidoreductase from *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A.* 2002, 99, 10322−10324.

(23) Backiel, J.; Zagorevski, D. V.; Wang, Z.; Nilges, M. J.; Barquera, B. Covalent Binding of Flavins to RnfG and RnfD in the Rnf Complex from *Vibrio cholerae*. *Biochemistry* 2008, 47, 11273−11284.

(24) Barquera, B.; Hase, C. C.; Gennis, R. B. Expression and Mutagenesis of the NqrC Subunit of the NQR Respiratory Na+ Pump from *Vibrio cholerae* with Covalently Attached FMN. *FEBS Lett.* 2001, 492, 45−49.

(25) Hayashi, M.; Nakayama, Y.; Yasui, M.; Maeda, M.; Furushiki, K.; Unemoto, T. FMN Is Covalently Attached to a Threonine Residue in the NqrB and NqrC Subunits of Na+-Translocating NADH-Quinone Reductase from *Vibrio alginolyticus*. *FEBS Lett.* 2001, 488, 5−8.

(26) Fang, X.; Liang, P.; Raha, D. A.; Rosas-Lemus, M.; Chakravarty, S.; Tzu, K.; Juárez, O. Kinetic Characterization of *Vibrio cholerae* ApoE: Substrate Specificity and Regulatory Mechanisms. *PLoS One* 2017, 12, No. e0186805.

(27) Tzu, K.; Mezic, K. G.; Xu, T.; Barquera, B.; Juárez, O. The Kinetic Reaction Mechanism of the *Vibrio cholerae* Sodium-Dependent NADH Dehydrogenase. *J. Biol. Chem.* 2015, 290, 20009−20021.
(28) Casutt, M. S.; Nedièklov, R.; Wendelspiess, S.; Vossler, S.; Gerken, U.; Murai, M.; Miyoshi, H.; Möller, H. M.; Steuber, J. Localization of Ubiquinone-8 in the Na+-Pumping NADH:Quinone Oxidoreductase from Vibrio cholerae. J. Biol. Chem. 2011, 286, 40075–40082.
(29) Ito, T.; Murai, M.; Ninokura, S.; Kitazumi, Y.; Mezic, K. G.; Cress, B. F.; Koffas, M. A. G.; Morgan, J. E.; Barquera, B.; Miyoshi, H. Identification of the Binding Sites for Ubiquinone and Inhibitors in the Na+-Pumping NADH-Ubiquinone Oxidoreductase from Vibrio cholerae by Photoaffinity Labeling. J. Biol. Chem. 2017, 292, 7727–7742.
(30) Hayashi, M.; Shibata, N.; Nakayama, Y.; Yoshikawa, K.; Unemoto, T. Korormicin Insensitivity in Vibrio alginolyticus Is Correlated with a Single Point Mutation of Gly-140 in the NqB Subunit of the Na+-Translocating NADH-Quinone Reductase. Arch. Biochem. Biophys. 2002, 401, 173–177.
(31) Juárez, O.; Neehail, Y.; Turk, E.; Chahboun, N.; DeMicco, J. M.; Hellwig, P.; Barquera, B. The Role of Glycine Residues 140 and 141 of Subunit B in the Functional Ubiquinone Binding Site of the Na+-Pumping NADH-Quinone Oxidoreductase from Vibrio cholerae. J. Biol. Chem. 2012, 287, 25678–25685.
(32) Tuz, K.; Li, C.; Fang, X.; Raba, D. A.; Liang, P.; Minh, D. D. L.; Juárez, O. Identification of the Catalytic Ubiquinone-Binding Site of Vibrio cholerae Sodium-Dependent NADH Dehydrogenase: A Novel Ubiquinone-Binding Motif. J. Biol. Chem. 2017, 292, 3039–3048.
(33) Raba, D. A.; Rosas-Lemus, M.; Menzer, W. M.; Li, C.; Fang, X.; Liang, P.; Tuz, K.; Minh, D. D. L.; Juárez, O. Characterization of the Pseudomonas aeruginosa NQR Complex, a Bacterial Proton Pump with Roles in Autopoisoning Resistance. J. Biol. Chem. 2018, 293, 15664–15677.
(34) Dibrov, P.; Dibrov, E.; Maddaford, T. G.; Kenneth, M.; Nelson, J.; Resch, C.; Pierce, G. N. Development of a Novel Rationally Designed Antibiotic to Inhibit a Nontraditional Bacterial Target. Can. J. Physiol. Pharmacol. 2017, 95, 595–603.
(35) Liang, P.; Rosas-Lemus, M.; Patel, D.; Fang, X.; Tuz, K.; Oscar Juárez, X. Dynamic Energy Dependency of Chlamydia trachomatis on Host Cell Metabolism during Intracellular Growth: Role of Sodium-Based Energetics in Chlamydial ATP Generation. J. Biol. Chem. 2018, 293, 510–522.
(36) Nakayama, Y.; Hayashi, M.; Yoshikawa, K.; Mochida, K.; Unemoto, T. Inhibitor Studies of a New Antibiotic, Korormicin, 2-n-Heptyl-4-Hydroxyquinoline N-Oxide and Ag+ toward the Na+-Translocating NADH:Quinone Reductase from the Marine Vibrio alginolyticus. Biol. Pharm. Bull. 1999, 22, 1064–1067.
(37) Gunasekaran, K.; Nagarajaram, H.; Ramakrishnan, C.; Balaram, P. Stereochemical Punctuation Marks in Protein Structures: Glycine and Proline Containing Helix Stop Signals. J. Mol. Biol. 1998, 275, 917–932.
(38) Vila, J.; Williams, R. L.; Grant, J. A.; Wójcik, J.; Scheraga, H. A. The Intrinsic Helix-Forming Tendency of L-Alanine. Proc. Natl. Acad. Sci. U.S.A. 1992, 89, 7821–7825.
(39) Hazan, R.; Que, Y. A.; Maura, D.; Strobol, B.; Majcherczyk, P. A.; Hopper, L. R.; Wilbur, D. J.; Hreha, T. N.; Barquera, B.; Rahme, L. G. Auto Poisoning of the Respiratory Chain by a Quorum-Sensing-Regulated Molecule Favors Biofilm Formation and Antibiotic Tolerance. Curr. Biol. 2016, 26, 195–206.
(40) Zemke, A. C.; Bomberger, J. M. Microbiology: Social Suicide for a Good Cause. Curr. Biol. 2016, 26, R80–R82.
(41) Wagner, M. In Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems; Segel, I. H., Ed.; Wiley: New York, 2009; Vol. 53.
(42) Petterson, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera?A Visualization System for Exploratory Research and Analysis. J. Comput. Chem. 2004, 25, 1605–1612.
(43) Segel, I. H. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems; Wiley and Sons: New York, 1993.