Purification and Characterization of the Recombinant Na^+-Translocating NADH:Quinone Oxidoreductase from *Vibrio cholerae*†

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ABSTRACT: The *nqr* operon from *Vibrio cholerae*, encoding the entire six-subunit, membrane-associated, Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR), was cloned under the regulation of the P_{BAD} promoter. The enzyme was successfully expressed in *V. cholerae*. To facilitate molecular genetics studies of this sodium-pumping enzyme, a host strain of *V. cholerae* was constructed in which the genomic copy of the *nqr* operon was deleted. By using a vector containing a six-histidine tag on the carboxy terminus of the NqrF subunit, the last subunit in the operon, the recombinant enzyme was readily purified by affinity chromatography in a highly active form from detergent-solubilized membranes of *V. cholerae*. The recombinant enzyme has a high specific activity in the presence of sodium. NADH consumption was assessed at a turnover number of 720 electrons per second. When purified using dodecyl maltoside (DM), the isolated enzyme contains approximately one bound ubiquinone, whereas if the detergent LDAO is used instead, the quinone content of the isolated enzyme is negligible. Furthermore, the recombinant enzyme, purified with DM, has a relatively low rate of reaction with O_2 ($10-20\ s^{-1}$). In steady state turnover, the isolated, recombinant enzyme exhibits up to 5-fold stimulation by sodium and functions as a primary sodium pump, as reported previously for Na^+-NQR from other bacterial sources. When reconstituted into liposomes, the recombinant Na^+-NQR generates a sodium gradient and a $\Delta\Psi$ across the membrane. SDS−PAGE resolves all six subunits, two of which, NqrB and NqrC, contain covalently bound flavin. A redox titration of the enzyme, monitored by UV−visible spectroscopy, reveals three $n = 2$ redox centers and one $n = 1$ redox center, for which the presence of three flavins and a 2Fe−2S center can account. The *V. cholerae* Na^+-NQR is well-suited for structural studies and for the use of molecular genetics techniques in addressing the mechanism by which NADH oxidation is coupled to the pumping of Na^+ across the membrane.

The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) is the main gateway for electrons into the aerobic respiratory chain of many marine and pathogenic bacteria (*1*−*4*). Na^+-NQR operates as a primary sodium pump, coupling an electron transfer reaction (from NADH[1] to ubiquinone) to Na^+ translocation across the membrane. It is likely that the enzyme pumps one sodium per electron across the membrane (*5*). The activity of Na^+-NQR generates a sodium motive force (smf) that can be used to do metabolic work, such as driving the flagellum or transporting substrates (*6*, *7*).

The enzyme has been isolated from the marine bacteria *Vibrio alginolyticus* and *Vibrio harveyi* and studied extensively (*2*, *4*). Na^+-NQR is an assembly of six different subunits (NqrA−F) which range in size from ∼21 to 50 kDa. The subunits of Na^+-NQR have no amino acid sequence

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\Psi$, membrane potential; DM, *n*-dodecyl β-maltoside; EDTA, ethylenedinitrilotetraacetic acid; E_m, midpoint potential (in millivolts); EPR, electron paramagnetic resonance; ETH-157, *N*,*N*′-dibenzyl-*N*,*N*′-diphenyl-1,2-phenylene diacetamide; FAD, flavin adenine dinucleotide; FMN, flavin adenine mononucleotide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; LDAO, *n*-dodecyl-*N*,*N*-dimethylamine *N*-oxide; NADH, reduced nicotinamide adenine dinucleotide; NTA, nitrilotriacetic acid; oxonol VI, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; PAGE, polyacrylamide gel electrophoresis; Q-1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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homologies to any of the subunits of Complex I, the H^+-pumping NADH:quinone oxidoreductase present in many bacteria as well as in the mitochondrion. However, given the functional similarity between Complex I and Na^+-NQR, it is probable that these enzymes also share some mechanistic features coupling redox chemistry to ion transport (*8*).

If one copy of each subunit is assumed, the aggregate molecular mass of Na^+-NQR is 210 kDa. The genes for all six subunits (*nqrA*−*F*) reside in a single operon. The enzyme contains at least four different cofactors: a noncovalently bound FAD and a 2Fe−2S center, both in subunit F, and two covalently bound FMNs, one in subunit B and the other in subunit C (*2*, *9*, *10*).

NqrF also includes a motif typical of NADH binding sites, suggesting that the substrate donates electrons to this subunit. Neither the order of electron flow through the different prosthetic groups nor the pathway for sodium translocation is yet defined.

The *nqr* operon is not confined to bacteria from marine environments, but has a much broader distribution and can be found in a number of bacterial genomes (*11*). Among these are many pathogens, including *Vibrio cholerae*. This also suggests that the use sodium as a chemiosmotic ion is widespread. In the case of *V. cholerae*, the ability of the organism to maintain a sodium motive force (smf) appears to be linked to pathogenesis. Häse and Mekalanos (*12*) have reported that when the *nqr* operon is interrupted, virulence factors are expressed at higher levels.

The ability to use genetic tools to study Na^+-NQR would clearly be advantageous in addressing specific questions about structure−function relationships in the enzyme and in studying the physiological role of the sodium motive force. For this reason, we have chosen to examine the Na^+-NQR from *V. cholerae*, an organism which has been shown to be congenial to genetic manipulation and for which the genome sequence has been determined. The six-gene *V. cholerae nqr* operon was cloned and expressed in its parent organism under the control of the P_{BAD} promoter. The expressed enzyme was purified using a six-histidine affinity tag, engineered at the carboxy terminus of NqrF. The isolated enzyme has high turnover. In the presence of sodium, the NADH:Q-1 oxidoreductase activity is ~100 μmol min^{-1} mg^{-1}. When the enzyme is reconstituted into proteoliposomes, catalytic turnover leads to generation of a sodium motive force.

This is the first report of a recombinant Na^+-NQR and demonstrates that *V. cholerae* can be used to produce this membrane protein in sufficient amounts for biochemical, biophysical, and structural studies.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. V. cholerae O395N1 was used as a source of genomic DNA. *V. cholerae* O395N1-toxT:lac::ΔnqrA-F (see below) was used as the host for expression of the *nqr* operon in the pBAD-TOPO vector. *Escherichia coli* TOP10 (Invitrogen) was used for cloning. *V. cholerae* and *E. coli* were grown in Luria-Bertani (LB) medium at 37 °C; when appropriate, ampicillin (100 μg/mL) and streptomycin (50 μg/mL) were added.

Cloning and Expression of the nqr Operon. The *V. cholerae nqr* operon was obtained from genomic DNA by a PCR. Genomic DNA was prepared using the "Wizard DNA genomic purification kit" from Promega. The following primers were used: forward, GAG GAA TAA TAA **ATG** ATT ACA ATA AAA AAG GGA TTG G; reverse, ACC ACC GAA GTC ATC CAG TAG. These primers correspond to the beginning of *nqrA* to the end of *nqrF*. The forward primer was designed to achieve expression of a native enzyme without including the N-terminal leader sequence usually introduced by this vector. The primer contains an in-frame stop codon and a translation reinitiation sequence, which consists of a ribosome-binding site and the first ATG of the protein (bold above). In the reverse primer, the native stop codon was removed to maintain the continuity of the reading frame through the C-terminal six-histidine tag.

PCR Conditions. "Herculase" DNA polymerase (Stratagene) was used to amplify the 5.8 kb fragment corresponding to the *nqr* operon. The PCR fragment was isolated by agarose gel electrophoresis and purified using the "Wizard PCR prep DNA purification system" (Promega). After purification, a "hanging adenine" was added by incubating the DNA in the presence of 1 unit of Taq DNA polymerase (BRL). The DNA was then introduced into a pBAD-TOPO vector. The ligation mix was then used to transform TOP-10 competent cells which were plated on LB agar−ampicillin plates and incubated at 37 °C. Transformants containing the *nqr* operon in the pBAD-TOPO vector were grown, and plasmid DNA was extracted using the "Wizard Plus miniprep DNA system" (Promega). The correct insertion and orientation of the *nqr* operon in the plasmid were checked by restriction analysis, and the fidelity of the PCR was confirmed by DNA sequencing. The complete operon was sequenced in both directions using internal primers designed according to the reported sequence (GenBank accession number AF117331). Sequencing was performed by the Biotechnology Center of the University of Illinois at Urbana-Champaign. The *nqr*-pBAD-TOPO construct was then introduced into *V. cholerae* O395N1-toxT:lac::ΔnqrA-F by electroporation using the conditions reported previously (*13*). Expression of the recombinant Na^+-NQR in *V. cholerae* was checked by Western blotting using antibodies against the six-histidine tag as described previously (*13*).

Deletion of the nqr Operon. The deletion of the *nqr* operon was achieved by homologous recombination. The *nqr* operon and surrounding sequences were amplified from genomic DNA by a PCR using the forward primer (GCC GGC CTG CGT CCT GTC GCT CGT) and the reverse primer (GGA ACA CCA TCA CGG TTC AGT) and cloned into pCR2.1 (Invitrogen). Internal deletion of the *nqrA*−*F* genes was achieved using internal *Nru*I sites. This results in an out-of-frame deletion of amino acids 114−447 of the *nqrA* gene, complete deletion of the *nqrB*−*D* genes, and deletion of amino acids 1−267 of the *nqrF* gene. The out-of-frame fusion of the *nqrA* and *nqF* genes terminates 24 amino acids downstream of the fusion site. The DNA was then subcloned into the pWM91 suicide vector (*14*) and introduced into the chromosome of the 0395N1-*toxT*::*lacZ* strain following sucrose selection as previously described (*15*).

Expression and Protein Purification. Expression in *V. cholerae* of the *nqr* operon in the pBAD vector was achieved by induction with 0.2% L-arabinose, added to the culture during the log phase of growth. Cells were grown until the end of the log phase and then harvested, washed with 10

mM Tris-HCl (pH 7.5), 200 mM NaCl, and 5 mM $MgSO_4$, and frozen at −80 °C until they were used.

The recombinant, His-tagged Na^+-NQR complex was purified by affinity chromatography using a Ni−NTA resin. Because sodium plays an essential role in the function of Na^+-NQR, the enzyme was prepared both with and without sodium present using buffers containing either sodium or potassium cation. As a first step, the bacterial membranes were isolated as described previously (*13*). The membrane fraction was washed with buffer containing either sodium or potassium salts [i.e., 50 mM sodium (potassium) phosphate (pH 8), 300 mM NaCl (KCl), 5% glycerol, and 5 mM imidazole] and then solubilized using 1% dodecyl maltoside (DM) or in some cases LDAO (see below) for 1 h at 4 °C. The solubilization mixture was centrifuged at 130000*g* for 30 min and then combined as a batch with Ni−NTA resin, which had been equilibrated with the washing buffer containing 0.1% DM (or LDAO). This mixture was gently agitated at 4 °C for 30 min and then poured into an empty column. The filled column was washed with 4 volumes of buffer containing 5 mM imidazole, followed by 5 volumes of buffer containing 10 mM imidazole, and the protein was then eluted from the column using buffer containing 50 mM imidazole. Imidazole and Ni^{2+} were removed from the protein sample by dialysis against Na^+ or K^+ phosphate buffer in the presence of 10 mM EDTA and 0.1% DM (or LDAO). The enzyme was then frozen in aliquots and kept at −80 °C.

Analytical Gel Filtration. A Superose 6 column (2.4 mL) in a "SMART purification system" (Pharmacia) with a two-wavelength absorbance monitor was used. A sample of 240 μg of purified Na^+-NQR was injected and then eluted with a buffer of 50 mM sodium phosphate (pH 8), 100 mM NaCl, 10 mM EDTA, 5% glycerol, and 0.05% DM.

To study the cofactors that were covalently bound, the enzyme was denatured and precipitated with TCA. TCA was added to the purified enzyme to a final concentration of 5%. The denatured protein was centrifuged, and the supernatant and pellet were collected. The supernatant was neutralized by the addition of aliquots of 1 M potassium phosphate (pH 7). The pellet was resuspended in 100 mM sodium phosphate buffer (pH 7) containing 1% SDS. Visible absorption spectra of the supernatant and resuspended pellet were recorded as well as the fluorescence spectrum of the supernatant (data not shown).

Enzymatic Activity. The activity of the enzyme was measured spectrophotometrically. Assays were carried out at room temperature (approximately 20 °C) in the same buffer that was used for dialysis. Activity was measured either as the level of oxidation of NADH (340 nm; $\epsilon = 6.22$ mM^{-1} cm^{-1}) (*3*, *16*, *17*) or as the level of reduction of Q-1. Since previous preparations of the enzyme have exhibited significant reaction rates with O_2, assays were performed both anaerobically and aerobically. One way to monitor Q-1 reductase activity is to use a wavelength corresponding to an isosbestic point for NADH oxidation (281.6 nm). The extinction coefficient for the difference spectrum between reduced and oxidized Q-1 at this wavelength was estimated to be 8.1 mM^{-1} cm^{-1}. The extinction coefficient was determined with reference to a literature value for Q-1 in ethanol (*18*), by making parallel dilutions from a concentrated stock solution into ethanol and in the assay buffer. However, when the enzyme was assayed under anaerobic conditions so that the only available electron acceptor was Q-1, the estimated rate of Q-1 reduction was ∼20% lower than expected from the NADH oxidation rate. The same activity values were obtained under aerobic assay conditions when freshly prepared enzyme in DM (but not LDAO) was used, indicating a negligible reaction with O_2 for this enzyme preparation. Hence, it is likely that the estimated extinction coefficient for Q-1 reduction at 281.6 nm (8.1 mM^{-1} cm^{-1}) is ∼20% too high.

The turnover number was calculated using a molecular mass for the recombinant Na^+-NQR complex of 215 kDa, which is ∼5 kDa higher than that of the native enzyme due to the additional tag on NqrF.

Spectrophotometric measurements were taken using an SLM-Aminco DW2000 apparatus, which was equipped with a stirred semi-micro quartz cuvette (Hellma) so that injections could be made while recording. The system was constructed in such a way that the headspace above the liquid in the cuvette could be flushed with a continuous flow of water-saturated nitrogen gas. In this way, oxygen could be removed from the samples before the reaction started. This was particularly important for the enzyme prepared with LDAO.

Quinone Analysis. Hydrophobic cofactors were extracted from the enzyme with a 6:4 mixture of methanol and petroleum ether, dried under flowing nitrogen gas, and then resuspended in ethanol. The resulting sample was analyzed for quinone content by HPLC using a Microsorb-MV 100 Å HPLC column (Varian) in a Perseptive BIOCAD Sprint system. The column was equilibrated and run with ethanol at a flow rate of 0.1 mL/min. The effluent was monitored at 278 nm. Retention times for the quinone components were calibrated using Q-8 (*19*) obtained by extraction of membranes from *V. cholerae* and *V. harveyi*, as well as commercially obtained Q-10, as standards.

Spectroelectrochemistry. For electrochemistry, purified Na^+-NQR in 0.05% DM, 50 mM sodium phosphate buffer (pH 8.0), and 100 mM NaCl was concentrated to approximately 0.2 mM. Redox titrations, monitored by UV−visible absorbance, were carried out by the method described in refs *20* and *21*. Spectra were recorded at a succession of redox potentials during both oxidative and reductive phases of the titration at 4 °C. The gold grid working electrode was chemically modified with a 1:1 mix of 2 mM cysteamine and a 2 mM mercaptopropionic acid solution. To accelerate the redox reaction, the following mediators were used at a final concentration of 45 μM each (midpoint potential vs Ag/AgCl/3 M KCl in parentheses): ferrocenyltrimethylammonium iodide (437 mV), 1,1′-dicarboxylferrocene (436 mV), diethyl-3-methylparaphenylenediamine (159 mV), ferricyanide (216 mV), dimethylparaphenylenediamine (163 mV), 1,1′-dimethylferrocene (133 mV), tetramethylparaphenylenediamine (62 mV), tetrachlorobenzoquinone (72 mV), 2,6-dichlorophenolindophenol (9 mV), ruthenium hexamine chloride (−8 mV), 1,2-naphthoquinone (−63 mV), trimethylhydroquinone (−108 mV), menadione (−220 mV), 2-hydroxy-1,4-naphthoquinone (−333 mV), anthraquinone 2-sulfonate (−433 mV), neutral red (−515 mV), and methyl viologen (−654 mV). At the given concentrations, and with a path length of 10−15 μm, no spectral contributions from the mediators in the visible range that was used could be detected in control experiments with samples lacking the

protein. Approximately 8 μL of the protein solution was sufficient to fill the spectroelectrochemical cell. Measurements were taken using the Ag/AgCl/3 M KCl reference electrode.

Electron Paramagnetic Resonance Conditions. Oxidized Na^+-NQR samples in 50 mM sodium phosphate buffer (pH 8), 100 mM NaCl, and 0.05% DM were transferred into quartz EPR tubes (4 mm outside diameter). The enzyme was frozen in liquid nitrogen. Samples were stored at −80 °C. No changes in the EPR signal were detected after incubation at this temperature for several weeks.

EPR experiments were performed on a Varian E-122 X-band (9.079 GHz) spectrometer. A microwave power of 0.2 mW was used with a modulation amplitude of 2.0 G at 100 kHz. Samples were run at 50 K using an Air Products Helitran cyrostat. The magnetic field was calibrated with a Varian NMR gaussmeter, and the microwave frequency was determined with an EIP frequency meter.

Other Methods. Reconstitution of the enzyme into liposomes and measurement of $\Delta\Psi$ using oxonol VI absorbance have been described previously (*2*). The protein concentration was measured using the bicichonic acid (BCA) method. SDS−PAGE in the presence of 6 M urea was performed using a Tris-Tricine discontinuous gel system (4% stacking gel, 16% resolving gel).

RESULTS

Cloning and Expression. The *nqr* operon was obtained by a single PCR and cloned into a pBAD-TOPO expression vector. This construct was then introduced into a *V. cholerae* strain from which the genomic *nqr* operon had been deleted. By using this plasmid, a six-histidine tag was introduced at the C-terminus of subunit F. Gene expression in the pBAD-TOPO vector is under control of an arabinose promoter (P_{BAD}). Expression of the recombinant Na^+-NQR was induced by the addition of 0.2% L-arabinose to the growth medium during the middle of log phase growth. This did not have any toxic effect on the cells. Western blotting following SDS−PAGE of cell fractions using anti-histidine tag antibodies confirmed that the recombinant enzyme was expressed and was localized to the bacterial membrane. The antibodies labeled a band with a molecular mass of approximately 46 kDa, consistent with the expected location of the His-tagged subunit F.

Purification and Activity. After detergent solubilization of the bacterial membranes, Na^+-NQR was purified in a single chromatographic step using a Ni−NTA resin. The best results were obtained using the detergent dodecyl maltoside (DM). Buffers used in the enzyme preparation also contained sodium, which helped to stabilize the enzyme. Preparations made in potassium-containing buffers were considerably less stable. Enzyme activity can be measured by following either NADH oxidation at 340 nm (ϵ = 6.22 mM^{-1} cm^{-1}) or quinone reduction at 281.6 nm (ϵ = 8.1 mM^{-1} cm^{-1}). When the level of oxidation of NADH was measured, preparations of the recombinant enzyme routinely gave specific activities of ∼100 μmol min^{-1} mg^{-1}, or ∼700 electrons per second. Somewhat smaller values (∼77 μmol min^{-1} mg^{-1}) were obtained when the level of quinone reduction was measured using the estimated extinction coefficient at 281.6 nm (ϵ = 8.1 mM^{-1} cm^{-1}).

In the past, menadione has often been used as an electron acceptor when the level of NADH oxidation was measured. With the *V. cholerae* enzyme, use of menadione gave an activity of ∼3 μmol of NADH min^{-1} mg^{-1}, which is considerably lower than previously reported values. This activity is not sensitive to HQNO, and it is not increased at high concentrations of sodium, indicating that the menadione reaction short-circuits the coupled redox reaction of the enzyme. The low NADH:menadione activity of the current preparation probably indicates that a nonphysiological menadione interaction site is less accessible in this enzyme preparation.

The nearly 1:1 stoichiometry of NADH oxidation and quinol reduction contrasts with some previously reports of preparations of the enzyme from other sources (*17*) in which the rate of NADH oxidation was substantially greater than the rate of quinol reduction. For example, the enzyme from *V. alginolyticus* consumed 120 μmol of NADH min^{-1} mg^{-1} (NADH:menadione activity), but when Q-1 was used as the acceptor, only 1 μmol of quinone min^{-1} mg^{-1} was reduced (*17*). The *V. harveyi* enzyme consumed 100−110 μmol of NADH min^{-1} mg^{-1} (NADH:menadione activity), but Q-1 was reduced at a rate of 38−42 μmol min^{-1} mg^{-1} (*22*).

Thus, in most previous preparations, the rate of NADH oxidation was considerably higher than the rate of reduction of ubiquinone. Apparently, these preparations are able to donate electrons to alternate electron acceptors. In agreement with Bogachev et al. (*22*), we have found that preparations made with LDAO show significant O_2 consumption, but when DM is used instead of LDAO, the extent of O_2 consumption is dramatically reduced. There are some indications, here and in earlier reports (*22*), that LDAO itself may also be able to act as an electron acceptor. In the current work, when DM was used as a detergent, O_2 consumption was negligible (1−2 μmol min^{-1} mg^{-1}) compared to Q-1 reduction (∼77 μmol min^{-1} mg^{-1}). Unless otherwise indicated, all preparations used in this work were made in DM and contained no LDAO.

The fact that the DM preparation consumes very little oxygen during turnover suggests that the rate of quinone reduction is actually equal to the rate of NADH oxidation. Indeed, these activities were not affected when the reaction was performed under anaerobic conditions. Taken together, these results suggest that the discrepancy between NADH oxidase and Q-1 reductase activities, reported above, is likely due to a systematic error in the spectrophotometric measurement of Q-1 activity (see Materials and Methods). If a 1:1 stoichiometry is assumed, the extinction coefficient that should be used to monitor quinone reduction under the reported assay conditions is 6.2 mM^{-1} cm^{-1} at 281.6 nm.

Sodium Stimulation and HQNO Inhibition of NADH: Ubiquinone Oxidoreductase Activity. As previously reported for Na^+-NQR enzymes, the steady state activity is sensitive to the presence of sodium in the assay buffer. At 200 mM Na^+, the NADH:quinone-1 oxidoreductase activity is 5 times higher than in the absence of sodium (Figure 1). K^+ or Li^+ is not nearly as effective at activating Na^+-NQR, even at high concentrations. This specific requirement for sodium is similar to that reported for the enzymes from *V. alginolyticus* and *V. harveyi* (*2*, *3*).

Another characteristic of all previously studied Na^+-NQRs is their high sensitivity to HQNO (*23*). The quinone reductase

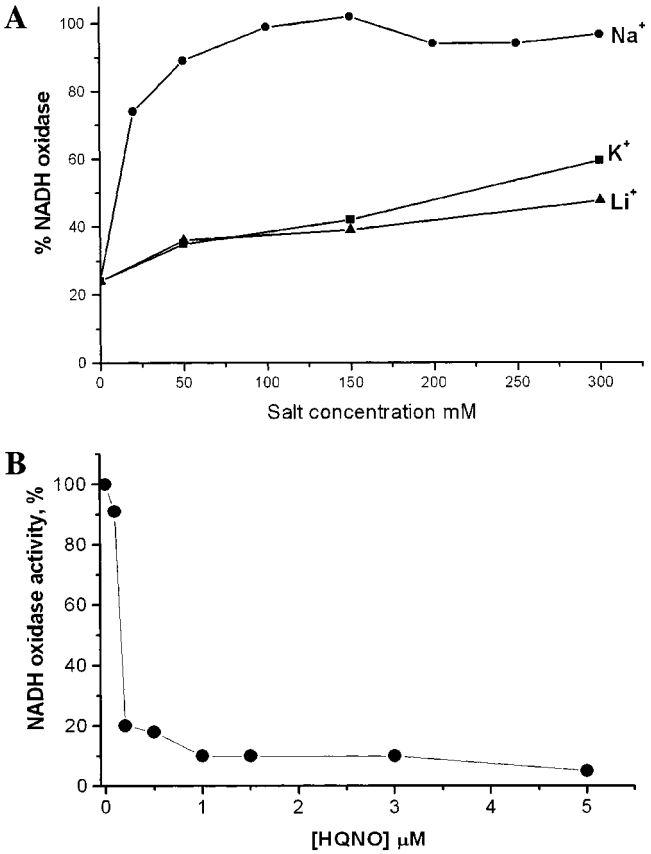

FIGURE 1: Steady state turnover of the recombinant Na^+-NQR from *V. cholerae*. Coupled oxidation of NADH and reduction of Q-1 was assessed by following the disappearance of NADH at 340 nm: (A) effect of various alkali cations and (B) effect of HQNO. Samples contained 200 μg of purified enzyme (prepared in K^+ buffer), 20 mM Tris-HCl (pH 8), 0.1% DM, and NaCl, KCl, or LiCl as indicated; 100 μM NADH and 10 μM Q-1 were added as substrates.

activity of the recombinant *V. cholerae* enzyme is strongly inhibited by HQNO, with activity being nearly abolished at 2 μM HQNO (Figure 1).

SDS−PAGE and Covalent Flavins. A number of different preparations of *V. cholerae* Na^+-NQR were subjected to SDS−PAGE in the presence of 6 M urea (see Materials and Methods). Figure 2 shows the results obtained with two preparations, one prepared with sodium salts and the second with potassium salts. The preparations appear to be identical. Figure 2A shows a gel of these two enzyme preparations after Coomassie staining. The six structural subunits of the Na^+-NQR complex (A−F) can be seen, including all three hydrophobic subunits (B, D, and E). There are no major contaminants.

Previous studies on Na^+-NQR from *V. harveyi* (*2*) and *V. alginolyticus* (*9*) have found that subunits B and C of the enzyme fluoresce because each contains a covalently bound FMN. When an SDS gel of the recombinant *V. cholerae* Na^+-NQR is exposed to UV illumination (prior to staining), two fluorescent bands corresponding in molecular mass to subunits B and C are observed (Figure 2B), confirming the presence of the covalently bound flavins in the *V. cholerae* enzyme.

Gel Filtration. To determine the homogeneity of the Na^+-NQR preparation, the purified enzyme was subjected to gel filtration chromatography. Elution was followed using a two-

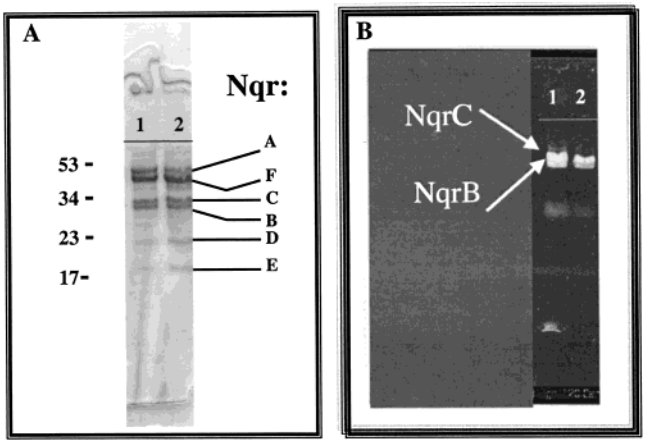

FIGURE 2: SDS−PAGE of the purified Na^+-NQR from *V. cholerae*. (A) With Coomassie staining. (B) A similar gel under UV illumination. Lane 1: Na^+ preparation; lane 2: K^+ preparation. The letters A−F in panel A denote the six subunits of the Na^+-NQR complex. NqrB and -C are denoted in panel B. A discontinuous gel system was used with 4 to 16% acrylamide in the presence of SDS and 6 M urea; 30 μg of protein was loaded in each lane.

wavelength absorbance monitor set at 280 and 450 nm to show protein and flavin, respectively. The elution profiles (not shown) at each of the two wavelengths gave a single major peak, showing that the preparation is homogeneous. The apparent molecular mass from this column is 360 kDa, using soluble proteins for calibration. It is likely that the protein is a monomer (calculated molecular mass of 215 kDa) with bound detergent; a more complete hydrodynamic analysis will be necessary to make this determination.

UV−Visible and EPR Spectroscopies. UV−vis absorption spectra of the reduced and oxidized enzyme under a variety of conditions were recorded using enzyme prepared from several different batches of cell growth. Figure 3A shows the spectrum of the purified Na^+-NQR (as isolated, air-oxidized). There is a broad absorbance maximum at 450−460 nm and a peak at 378 nm, both characteristics of flavoproteins. Addition of potassium ferricyanide did not significantly change the absorbance spectrum, indicating that the enzyme as isolated is in the fully oxidized state. The enzyme can be reduced by its substrate (NADH) or by dithionite. The oxidized-minus-reduced difference spectrum exhibits peaks around 450−460 nm as expected of flavins (Figure 3B).

In addition to the FMN prosthetic groups, which are covalently attached to subunits B and C, Na^+-NQR has been shown to contain a noncovalently bound FAD, believed to be bound to subunit F. To confirm the presence of noncovalently bound flavin in the recombinant *V. cholerae* Na^+-NQR, the enzyme was denatured and the protein precipitated using TCA. The oxidized-minus-reduced spectra of the resuspended pellet and supernatant (Figure 3C) both show a peak near 450 nm with a shape indicative of flavin(s). The extracted flavin has a fluorescence emission spectrum with a peak at 525 nm (not shown), typical of flavins. Qualitatively, these data are consistent with previous studies on Na^+-NQR, which have concluded that the enzyme contains two molecules of covalently bound FMN and one molecule of noncovalently bound FAD (*2, 8, 24*).

Na^+-NQR isolated from *V. alginolyticus* and *V. harveyi* contains a radical signal that can be observed by EPR

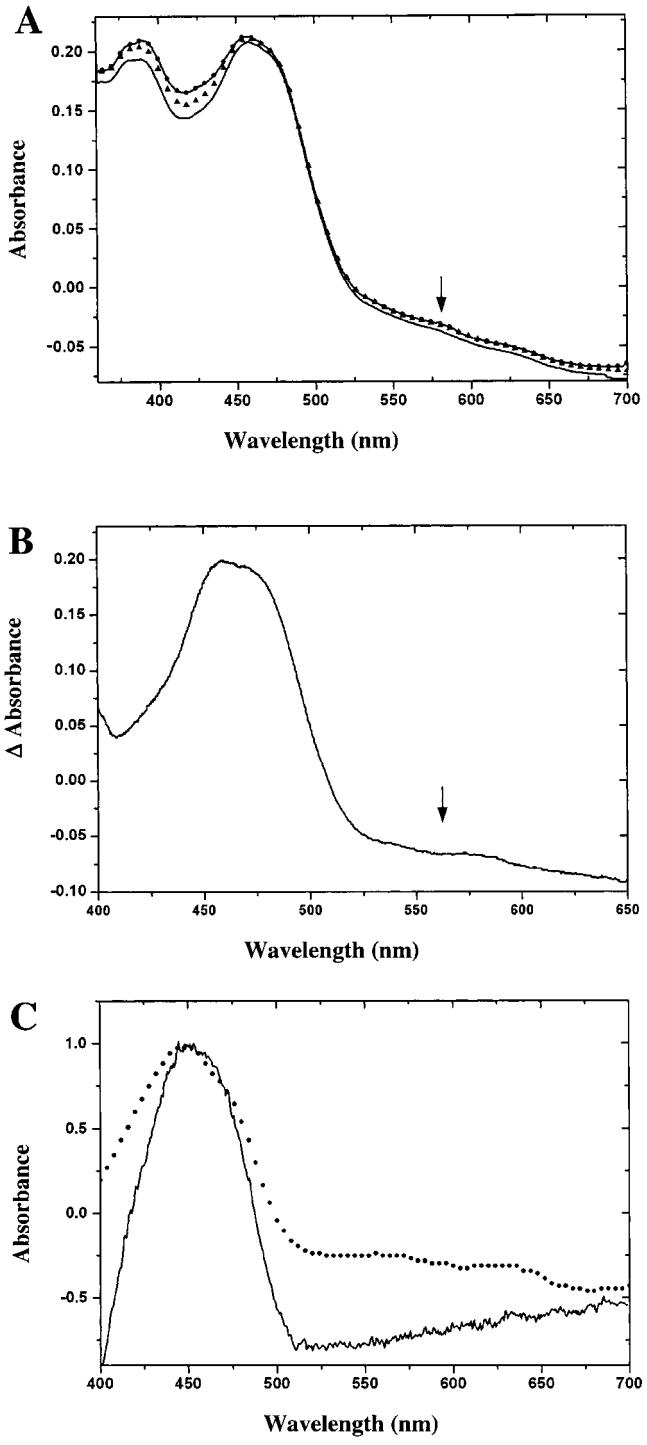

FIGURE 3: UV−visible spectra of the purified Na^+-NQR from *V. cholerae*. (A) Oxidized spectra: (—) air-oxidized enzyme, (•) enzyme in the presence of 50 μM $K_3Fe(CN)_6$ in air, and (▲) anaerobic enzyme in the presence of 50 μM $K_3Fe(CN)_6$. One milligram of protein per milliliter was used in each sample. (B) Oxidized-minus-reduced spectrum. Spectrum B was normalized to the amount of protein used in trace A. Enzyme was added to 1 mL of sodium phosphate buffer (pH 8), 100 mM NaCl, 10 mM EDTA, and 0.1% DM, and the air-oxidized spectrum was recorded. Then dithionite was added (a few grains) and the spectrum recorded again. (C) Oxidized-minus-reduced spectra of the Na^+-NQR from *V. cholerae* after trichloroacetic acid denaturation showing bound and free flavins: (•••) resuspended pellet and (—) supernatant. Enzyme (650 μg) was denatured with 5% TCA. The pellet and supernatant were recovered, and their spectra were recorded under air-oxidized conditions. Samples were then reduced by dithionite and the spectra recorded again.

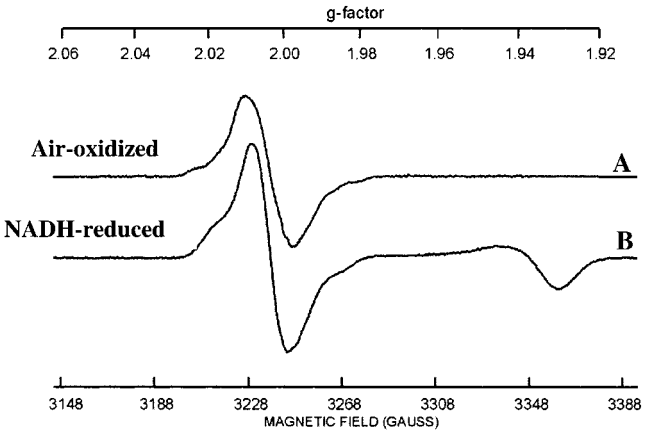

FIGURE 4: X-Band EPR spectra of *V. cholerae* Na^+-NQR, (A) air-oxidized and (B) reduced by NADH (10 mM). The protein concentration was 100 μM (21.7 mg/mL). The spectra were recorded at 50 K with a microwave power of 0.2 mW, a microwave frequency of 9.08211 GHz, and a modulation amplitude of 0.2 mT.

spectroscopy (*22*). To investigate the properties of this radical in the recombinant enzyme from *V. cholerae*, EPR spectra were acquired and compared with those previously reported. Figure 4 shows the spectra of the NADH-reduced and air-oxidized forms of the enzyme. A signal arising from the radical can be seen near $g = 2$ in both the spectra of the oxidized and reduced enzyme. An additional signal, which has been assigned to a 2Fe−2S center, can be observed in the spectrum of the reduced enzyme (see below). Remarkably, the intensity of the radical signal is independent of the redox state of the sample. On the basis of the line width and g value (2.0034), we tentatively assign the radical observed in the oxidized form of the enzyme as a neutral flavin semiquinone (*25*). This assignment is supported by the visible spectrum in Figure 3B. Note the intensity at 560 nm, a wavelength characteristic of neutral flavin radicals (*26*) (see the arrow in Figure 3B). Since this radical appears to be present in both the oxidized and reduced forms of the enzyme, it is possible that the flavin from which it arises is not a redox active cofactor in the enzyme.

In the spectrum of the reduced enzyme (Figure 4B), there is also an EPR signal arising from the 2Fe−2S center. The g values (2.0183, 1.9378, and 1.9325) are similar to those reported previously for this center in Na^+-NQR (*17*, *22*). Double integration shows that the ratio of spins arising from the radical and the 2Fe−2S center is approximately 1. Because the flavin radical and 2Fe−2S signals overlap, an accurate determination of this ratio was obtained by simulation of the first integral (absorption) spectrum (not shown). This yields a radical to 2Fe−2S ratio of 1.05 + 10%.

No EPR signals, other than those of the flavin radical and the 2Fe−2S center, are observed at temperatures down to 10 K.

Redox Titrations. A complete spectroelectrochemical redox titration of the recombinant Na^+-NQR was carried out. This is the first redox titration that accounts for all the possible cofactors participating in electron transfer in Na^+-NQR. The points in Figure 5 indicate the absorbance at 460 nm during the oxidative and reductive phases of the titration. The flavins and the 2Fe−2S center absorb at this wavelength (*27*). The solid line in Figure 5 is the theoretical prediction, which gives the best fit to the data. The model includes four Nernstian

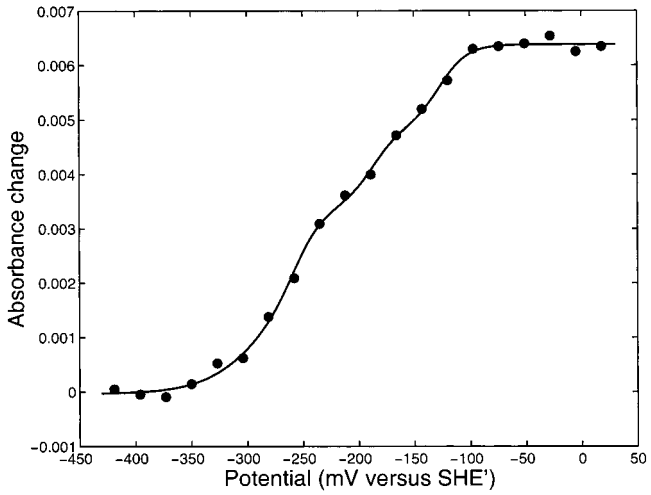

FIGURE 5: Redox titration taken in the oxidative direction of the *V. cholerae* Na^+-NQR monitored by UV−visible absorbance at 460 nm. Solid circles represent the measured data. The continuous line shows the best fit for a sum of Nernstian potentials (vs SHE′), consisting of three $n = 2$ midpoint potentials (−257, −187, and −127 mV) and one $n = 1$ midpoint potential (−292 mV).

components: three $n = 2$ components (−257, −187, and −127 mV, vs SHE′, pH 7) and one $n = 1$ component (−292 mV). Presumably, the three $n = 2$ components are flavins and the $n = 1$ component is the 2Fe−2S center.

In a previously reported redox titration (*28*), where a one-component fit was used to analyze the data, a midpoint potential of −295 mV was reported, and assigned to FAD. It is unlikely that this corresponds to any single component in the current four-component fit, even though one of these components does have similar parameters ($E_m = -257$ mV, $n = 2$). The midpoint potential assigned to the $n = 1$ component (−292 mV) is in rough agreement with the value obtained for the 2Fe−2S center in the *V. harveyi* enzyme (−267 mV) in an EPR-monitored redox titration (*22*).

Quinone Content of the Recombinant Na^+-NQR. It has been reported that Na^+-NQR from *V. alginolyticus* contains a tightly bound quinone (*17*). This is also the case for the *V. cholerae* enzyme, although the results are dependent on the detergent used in the preparation. Enzyme prepared using DM contains approximately 1 mol of ubiquinone-8 per mole of protein, whereas the enzyme prepared using LDAO is completely devoid of quinone.

Na^+ Translocation. The most striking characteristic of Na^+-NQR is its ability to selectively translocate sodium ions electrogenically across the membrane. The *V. cholerae* recombinant enzyme was reconstituted into liposomes using a method described in ref *2*. The reconstituted Na^+-NQR is fully active, demonstrating that the enzyme is stable during the procedure, and also showing that the enzyme is incorporated with the NADH binding domain facing the outside of the liposome. Hence, the enzyme is expected to pump Na^+ to the inside of the liposomes, generating a potential which is positive inside. The sodium gradient has the effect of slowing enzyme turnover. This can be seen most clearly when the $\Delta\Psi$ is collapsed by addition of a sodium ionophore. Figure 6 shows the change in absorbance at 340 nm, which reflects the oxidation of NADH during turnover of the reconstituted enzyme. The addition of the sodium ionophore

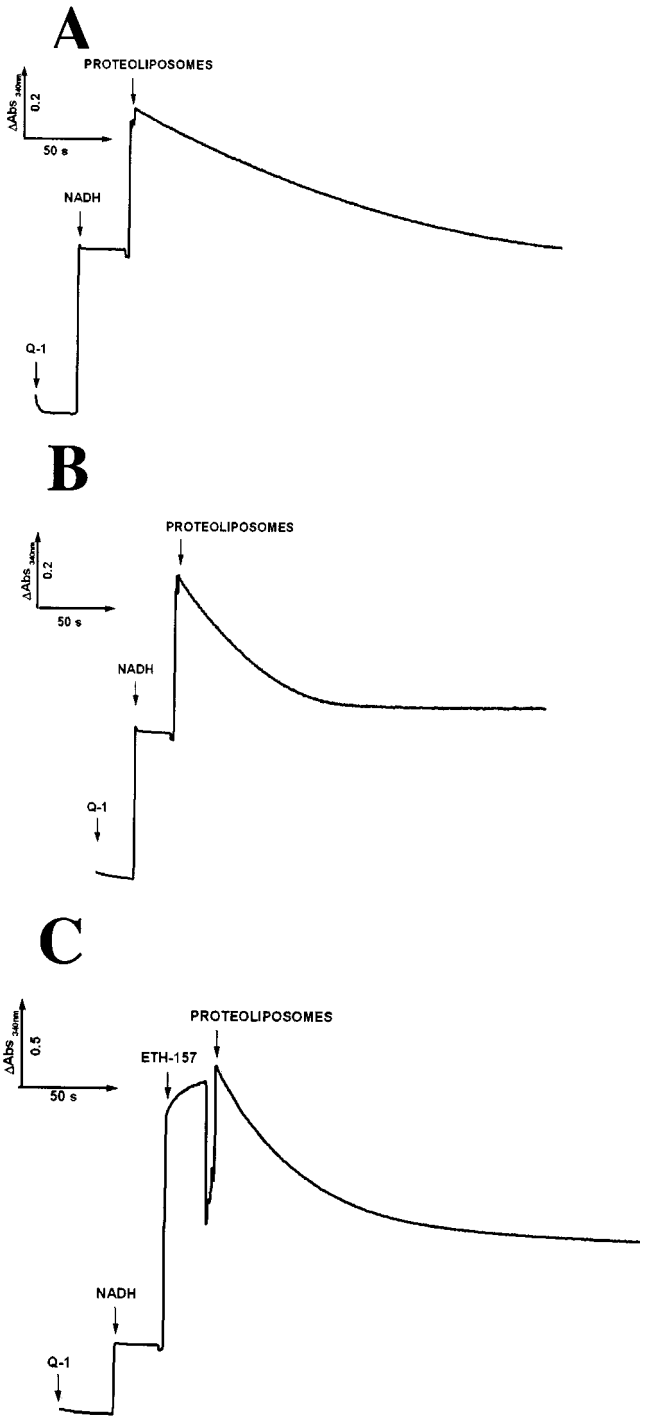

FIGURE 6: NADH oxidase activity of Na^+-NQR reconstituted in liposomes. Activity was followed by the change in absorbance at 340 nm: (A) no sodium added, (B) 200 mM NaCl added, and (C) 200 mM NaCl with 50 μM ETH-157 added. Sample conditions: 100 mM Hepes (adjusted to pH 6.5 using Tris base) and 50 μM EDTA. Additions of 10 μM Q-1, 100 μM NADH, and proteoliposomes were made at the indicated points.

ETH-157, in the presence of sodium, leads to an 8-fold increase in activity. The generation of $\Delta\Psi$ by the enzyme was observed directly by following changes in the absorbance of oxonol VI (*2*). As shown in Figure 7, enzyme turnover caused a dramatic change in the absorbance of this dye. The effect was reversed by the addition of the sodium ionophore ETH-157. These results demonstrate that the recombinant Na^+-NQR from *V. cholerae* is able to generate a $\Delta\Psi$, and

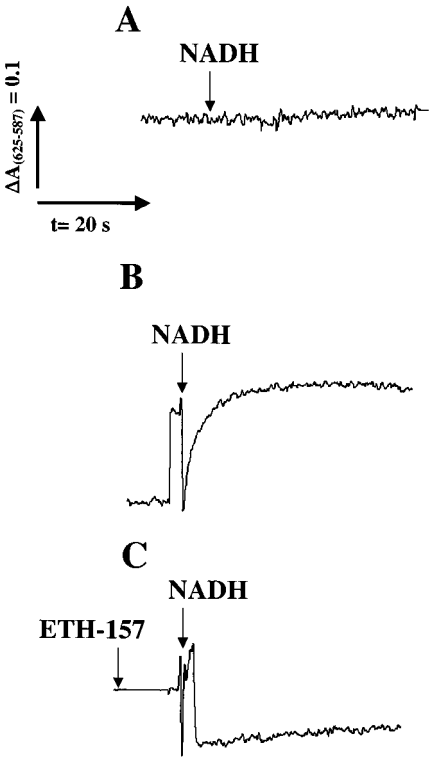

FIGURE 7: Generation of a membrane potential by Na^+-NQR from *V. cholerae* incorporated into liposomes. The membrane potential was measured by the change in absorbance of oxonol VI (625 minus 587 nm). The K^+ preparation of the enzyme was used. Activity was initiated by adding 10 μM Q-1 and 100 μM NADH. (A) No sodium added, with 500 μg/mL oxonol VI in Hepes buffer. (B) Same conditions as for panel A but in the presence of 200 mM NaCl. (C) Same conditions as for panel B but in the presence of 50 μM ETH-157.

that this membrane potential is the result of the sodium gradient created by the redox activity of the enzyme.

CONCLUSIONS

This work reports for the first time the successful recombinant expression of the *nqr* operon encoding Na^+-NQR from *V. cholerae.* A deletion of the genomic copy of the *nqr* operon was made in the *V. cholerae* host strain to facilitate molecular genetics studies using the plasmid-borne copy of *nqr*.

A six-histidine tag was engineered at the end of the NqrF subunit and resulted in a fully assembled enzyme that could be easily purified by affinity chromatography. The resulting preparation is stable, has a very high specific activity, and, very importantly, does not react with O_2 to any significant extent.

Na^+-NQR contains an intrinsic free radical that can be observed by EPR independent of the detergent used or whether sodium or potassium was used in the preparation. Our preliminary data suggest that the radical observed in the oxidized form of the enzyme is a neutral flavin semiquinone. Further characterization of the flavin radical is currently in progress.

Biochemical characterization shows that the *V. cholerae* enzyme is very similar to Na^+-NQR previously isolated from *V. alginolyticus* and *V. harveyi*. Hence, the presence of the histidine tag on NqrF does not interfere with the function of the enzyme. All six subunits and the previously reported redox cofactors [at least three flavins (two covalent FMNs and one noncovalent FAD), a 2Fe−2S center, and tightly bound ubiquinone-8] are present. Furthermore, when incorporated into proteoliposomes, the enzyme is able to translocate sodium across the membrane, producing a $\Delta\Psi$.

The high-quality biochemical preparation and the ability to generate site-directed mutants make this an excellent system for further study of the structure and mechanism of this fascinating enzyme. The experimental advantages of studying sodium as a translocated ion include the ability to localize the ion to either side of the membrane bilayer, or even sequestered within the protein. It is also feasible to experimentally use a larger range of concentrations than is typically possible with protons.

The *V. cholerae* Na^+-NQR enzyme is of particular interest because it appears to play a role in the virulence of the organism. Interruption of the *nqr* operon in *V. cholerae* results in an increased level of expression of virulence factors (*12*). This observation raises a number of interesting questions about the role of Na^+-NQR in the survival of *V. cholerae* as a free-living organism and how sodium bioenergetics influences the physiology of the organism. The ability to genetically modify Na^+-NQR within *V. cholerae* will provide a new and important tool with which to address these questions.

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