Sequencing and Preliminary Characterization of the Na^+-Translocating NADH:Ubiquinone Oxidoreductase from *Vibrio harveyi*†

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ABSTRACT: The Na^+-translocating NADH: ubiquinone oxidoreductase (Na^+-NQR) generates an electrochemical Na^+ potential driven by aerobic respiration. Previous studies on the enzyme from *Vibrio alginolyticus* have shown that the Na^+-NQR has six subunits, and it is known to contain FAD and an FeS center as redox cofactors. In the current work, the enzyme from the marine bacterium *Vibrio harveyi* has been purified and characterized. In addition to FAD, a second flavin, tentatively identified as FMN, was discovered to be covalently attached to the NqrC subunit. The purified *V. harveyi* Na^+-NQR was reconstituted into proteoliposomes. The generation of a transmembrane electric potential by the enzyme upon NADH:Q_1 oxidoreduction was strictly dependent on Na^+, resistant to the protonophore CCCP, and sensitive to the sodium ionophore ETH-157, showing that the enzyme operates as a primary electrogenic sodium pump. Interior alkalinization of the inside-out proteoliposomes due to the operation of the Na^+-NQR was accelerated by CCCP, inhibited by valinomycin, and completely arrested by ETH-157. Hence, the protons required for ubiquinol formation must be taken up from the outside of the liposomes, which corresponds to the bacterial cytoplasm. The Na^+-NQR operon from this bacterium was sequenced, and the sequence shows strong homology to the previously reported Na^+-NQR operons from *V. alginolyticus* and *Haemophilus influenzae*. Homology studies show that a number of other bacteria, including a number of pathogenic species, also have an Na^+-NQR operon.

Unemoto and collaborators (*1*) first found that the marine bacterium *Vibrio alginolyticus* is able to generate a respiration-dependent electric potential and expel sodium against its gradient in the presence of uncouplers. Later they showed that this was due to the activity of a unique sodium-translocating NADH-ubiquinone oxidoreductase (Na^+-NQR), which is induced at alkaline pH and can generate an electrochemical Na^+ gradient during aerobic respiration (*1*−*3*). This Na^+ gradient can be used for flagellar rotation, amino acid transport, and possibly ATP synthesis (*4*−*7*). The enzyme works as an energy-conserving primary Na^+ pump, with a proposed Na^+/e^- stoichiometry of 1 (*8*). Biochemical studies of the Na^+-NQR have all used the enzyme from the marine bacterium *V. alginolyticus*. However, in addition to its function in marine bacteria (*9*), the Na^+-NQR has recently been identified in the pathogenic bacterium *Haemophilus influenzae* (*10*) and has been implicated in the virulence of *Vibrio cholerae* (*11*).

Purified Na^+-NQR from *V. alginolyticus* is known to contain FAD[1] and an FeS center as redox cofactors, and there are conflicting reports concerning the presence of an additional FMN cofactor (*12*− *16*). The enzyme can use either NADH or dNADH as an electron donor and ubiquinone, menadione, or ferricyanide as electron acceptor. The quinone reductase activity is dependent on Na^+ and is sensitive to HQNO (*12*, *17*−*20*). Na^+-NQR does not pump H^+ and shows no sequence homology to either the H^+-translocating NADH-ubiquinone oxidoreductases or the nonenergy-conserving NADH-ubiquinone oxidoreductases (*13*, *21*, *22*).

The Na^+-NQR operon in *V. alginolyticus* is composed of six structural genes that correspond to the six subunits present in the Na^+-NQR complex (*14*, *22*, *23*). Three subunits, NqrA, C, and F [previously designated as the α, γ, and β subunits, respectively (*12*)] are relatively hydrophilic, while the other three subunits, NqrB, D, and E are very hydrophobic and were not detected in early studies of the enzyme (*14*). NqrF

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; ETH-157, *N*,*N*′-dibenzyl-*N*,*N*′-diphenyl-1,2-phenylene diacetamide; FAD, flavin adenine dinucleotide; FMN, flavin adenine mononucleotide; Hepes, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]; HPLC, high-performance liquid chromatography; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; LDAO, lauryldimethylamine oxide; MALDI, matrix-assisted laser desorption ionization, Mes, 2-[*N*-morpholino]ethanosulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; dNADH, reduced nicotinamide hypoxanthine dinucleotide (deamino NADH); NADPH, reduced nicotinamide adenine dinucleotide phosphate; ORF, open reading frame; oxonol VI, bis-(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; Q1, 2,3-dimethoxy-5-methyl-6-[3-methyl-2-butenyl]-1,4benzoquinone; pyranin, 8-hydroxy-1,3,6-pyrenetrisulfonic acid; TCA, trichloroacetic acid; TOF, time-of-flight; $\Delta\Psi$, membrane potential.

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contains binding motifs for NADH, FAD, and the iron−sulfur center, and is involved in the NADH dehydrogenase activity (*13*, *20*−*22*). Little is known about the function of the other subunits. Sequence alignments show no homologies to known sodium transporting proteins.

Although many bacteria have been reported to possess respiration-dependent primary Na^+ pumps (*9*, *24*, *25*), the DNA sequences of only two Na^+-NQRs have been reported, one from *V. alginolyticus* and another from *H. influenzae*, a nonmarine bacterium (*10*). Previous studies have also indicated the presence of Na^+-NQR in *Vibrio harveyi* (*9*, *26*), a marine bacterium studied mainly because of its luminescent system. In the current work, the purification and characterization of Na^+-NQR from *Vibrio harveyi* are described, together with the complete sequence of the *nqr* operon. The enzyme, as expected from the previous studies with the Na^+-NQR from *V. alginolyticus* (*16*), is demonstrated to be a primary sodium pump in reconstituted proteoliposomes. A new finding is that NqrC subunit of the *V. harveyi* Na^+-NQR contains a covalently bound flavin, which appears to be FMN attached to the protein via histidine-219. The enzyme appears to contain 1 equiv of each of the following prosthetic groups: FMN (covalent), FAD (noncovalent), an FeS cluster, and a tightly bound ubiquinone.

Recently, many microbial genomes have been fully or partially sequenced. By searching these sequence data, operons closely homologous to the known Na^+-NQRs are shown to be present in a number of bacteria, including several pathogenic bacteria: *V. cholerae* (*11*), *H. influenzae* (*10*), *Neisseria gonorrheae*, *Neisseria meningitidis*, *Yersinia pestis*, *Shewanella putrefaciens*, *Pseudomonas aeruginosa*, and *Porphyromonas gingivalis*. It appears that sodium bioenergetics and the Na^+- NQR in particular may play significant roles in pathogenic bacteria.

MATERIALS AND METHODS

Chemicals. Q_1 and horse heart cytochrome *c* were obtained from Sigma, oxonol VI from Molecular Probes, pyranin from Kodak, Bio-Beads from Bio-Rad, and trypsin (sequencing grade) was from Boehringer-Mannheim. Other reagents used were of analytical grade. Restriction enzymes were from GIBCO-BRL; Vent polymerase was obtained from New England Biolabs. Wizard Genomic DNA purification kit was from Promega, and QIAquick PCR purification and QIAquick gel extraction kits were from Qiagen.

Genome Sequencing Data. Preliminary genome sequence data from *V. cholerae*, *Porphyromonas gingivalis*, and *S. putrefaciens* were obtained from The Institute for Genomic Research (website at http://www.tigr.org). The sequence data of *Y. pestis*, *N. meningitidis* were produced by the Sequencing Group at the Sanger Centre and can be obtained from website at http://www.sanger.ac.uk. *N. gonorrheae* genome sequencing data were produced by University of Oklahoma's Advanced Center for Genome Technology website at http://www.genome.ou.edu. The sequence data of *Ps. aeruginosa* were produced by the Cystic Fibrosis Foundation, the University of Washington Genome Center, and PathoGenesis Corporation, website at http://www.pseudomonas.com.

General Methods. V. harveyi cells were grown at 30 °C in medium containing 250 mM NaCl, 10 mM KCl, 5 mM $MgSO_4$, 0.5% tryptone, 0.25% yeast extract, 0.4% (vol/vol) glycerol, 0.5 mM KH_2PO_4, and 50 mM Tris-HCl, pH 8.3. NADH dehydrogenase and Q_1 reductase activities of Na^+-NQR were measured at 30 °C using a Hitachi 557 spectrophotometer as described by Pfenninger-Li (*16*). Protein concentration was estimated by means of the bicinchoninic acid method using bovine serum albumin as a standard. Covalently bound heme was detected using the heme staining method described previously (*27*). Primer synthesis, DNA sequencing and the protein N-terminal sequencing were performed at the University of Illinois Biotechnology Center. Mass spectrometry was performed at the School of Chemical Sciences Mass Spectrometry Laboratory at the University of Illinois.

Purification of Na^+-NQR. V. harveyi cells were broken by using a French Press. Unbroken cells and cells debris were removed at 22500*g* (10 min). Membranes were collected by centrifugation (160000*g*, 90 min), washed once with buffer 1 (100 mM NaCl, 0.05 mM EDTA, 5% glycerol, and 10 mM Tris-HCl; pH 7.5), and resuspended in the same buffer. Membranes were solubilized with 1% LDAO as described before (*28*). The extract was applied onto a Fractogel TSK DEAE−650(S) column (26 × 60 mm) equilibrated with buffer 2 (buffer 1 containing 0.1% LDAO). The column was washed with buffer 2 containing 140 mM NaCl, and then the Na^+-NQR was eluted by linear NaCl gradient from 140 to 300 mM. Active fractions were applied directly onto a Q-Sepharose column (16 × 80 mm) equilibrated with buffer 2 containing 300 mM NaCl. The column was washed with three bed volumes of this buffer and the Na^+-NQR was eluted by 425 mM NaCl in buffer 2. The purified enzyme was then subject to SDS−PAGE and mass spectrometry.

Determination of the Na^+-NQR Cofactors. Noncovalently bound flavins were extracted from Na^+-NQR using the method described previously (*29*). Extracted flavins were separated by reversed-phase HPLC using an Altex Ultrasphere-ODS column (dimensions: 250 × 4.6 mm). The solvent was 20% methanol and 10 mM KH_2PO_4-KOH (pH 6.0), and the flow rate was 1 mL/min. Flavins were detected at 450 nm. The retention times for FAD and FMN standards were 6.1 and 9.4 min respectively. Covalently bound flavin was detected as described previously (*29*). Quinone was extracted and quantified as described by Shestopalov (*30*). The quinone extract from *Azotobacter vinelandii* membranes was used as the standard for Q_8.

Identification of the Flavin Ligand. A total of 3.4 μg of purified Na^+-NQR was electrophoresed on an SDS−PAGE gel, and the fluorescent NqrC subunit (apparent molecular mass of 32 kDa), was excised from the gel and washed with 20 mM NH_4HCO_3 and 50% acetonitrile. The washed gel band was dried and rehydrated in 20 mM NH_4HCO_3, and 0.5 μg of trypsin (in 1 mM HCl) was added. The mixture was incubated at 30 °C for 24 h. After incubation, the gel was washed with 60% acetonitrile, 0.1% trifluoroacetic acid, and then dried. The trypsin digest was separeted by HPLC. The effluent was monitored at 220 nm and by fluorescence (excitation 444 nm, emission 525 nm). The material from the fluorescent peak from the HPLC separation was used for N-terminal sequence analysis. The sample was placed into a sequencing cartridge and subjected to a total of 10

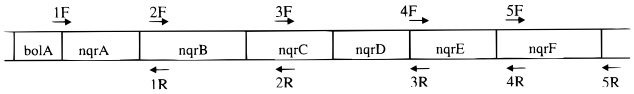

FIGURE 1: Corresponding positions of the designed primers on the Na^+-NQR operon from *V. alginolyticus*. Arrows indicates the direction of the primers, forward (F) or reverse (R). Five pairs of primers were designed based on published Na^+-NQR gene sequence from *V. alginolyticus* (GenBank accesion number AB008030).

cycles of Edman degradation to determine the amino acid sequence.

Mass Spectrometry. Molecular mass determination was determined using the Voyager DESTR (Perseptive Biosystems) MALDI/TOF spectrometer. The matrix was prepared by dissolving 1.5 mg of [2-(4-hydroxyphenylazo(benzoic acid)] (HABA) in 1 mL of 66% acetonitrile (ACN), 33% water, 0.1% trifluoroacetic acid (TFA) mix. A total of 400 pmol of Na^+-NQR was precipitated by adding 100 μL of 10% TCA solution, and the pellet was washed twice with water and redissolved in 99% formic acid. The sample was diluted to a final concentration of 45 pmol/μL with the formic acid. One microliter of a 1:20 dilution of protein to matrix was spotted on to the target for analysis. All mass spectra were externally calibrated.

Reconstitution of Proteoliposomes with Na^+-NQR. Proteoliposomes for $\Delta\Psi$ generation experiments were prepared mixing 1.74 mL of buffer A (100 mM Hepes-Tris and 0.05 mM EDTA; pH 6.5), with 0.26 mL 0.5 M *n*-octylglucoside and 20 mg of soybean phosphatidylcholine (Type-2S). The mixture was sonicated to homogeneity and Na^+-NQR (0.7 nmol) was added. The mixture was stirred for 30 min at room temperature and then Bio-Beads (540 mg) were added and incubated 3 more hours. After that, the solvent was separated from the Bio-Beads and centrifuged at 127000*g* for 90 min. Proteoliposomes with entrapped pyranin were prepared as above, but buffer B was used instead (100 mM Hepes, 25 mM NaOH, 25 mM KOH, 0.7 mM pyranin, and 0.05 mM EDTA titrated by Mes to pH 6.5). The proteoliposomes were washed once with buffer B without pyranin.

Generation of $\Delta\Psi$ by Na^+-NQR was monitored by oxonol VI. The sample contained 100 mM Hepes-Tris (sodium free medium) or 100 mM Hepes-NaOH, pH 7.5; 5 μg/mL oxanol VI, 100 μM Q_1, and proteoliposomes (28 μg protein/mL). The mixture was preincubated for 5 min prior to addition of the substrate. $\Delta\Psi$ was estimated spectrophotometrically (dual-wave measurements of the optical density difference at 625−587 nm) using a Shimadzu UV-3000 spectrophotometer. The reaction was initiated by adding NADH (0.4 mM). Generation of ΔpH by Na^+-NQR was monitored by fluorescence from entrapped pyranin, using a Hitachi F-2000 fluorimeter at 510 nm (excitation, 458 nm). The sample contained buffer B, 100 μM Q_1 and proteoliposomes (5 μg protein/mL). The mixture was preincubated for 5 min and the reaction was initiated by the additon of NADH (0.4 mM).

Sequencing of Na^+-NQR Operon. Genomic DNA was purified from *V. harveyi* using the Wizard Genomic DNA purification kit. Five pairs of primers were designed based on published homologous Na^+-NQR gene sequence from *V. alginolyticus*. PCR was performed using Vent Polymerase and genomic DNA from *V. harveyi* as template. The obtained PCR products were then sequenced. The corresponding position of the primers is shown in Figure 1. The primers were designed based on the sequence of *V. alginolyticus*; therefore, some deviations could be expected in the primers. Following PCR, the entire operon was cloned from genomic DNA into a pUC19 plasmid and was sequenced again to correct the possible errors.

Table 1: Purification of Na^+-NQR from *V. harveyi*

purification step	total activity (units)[a]	specific activity (units/mg)	yield (%)	purification (*n*-fold)
membrane vesicles	1079	1.5	100	
LDAO extract	1007	3.9	93.3	2.5
Fractogel TSK DEAE-650(S)	481	16.9	47.8	11
Q-sepharose	365	42.5	36.2	27.7

[a] Using NADH: menadione oxidoreductase activity. One unit is defined as 1 μmol of NADH consumed/min.

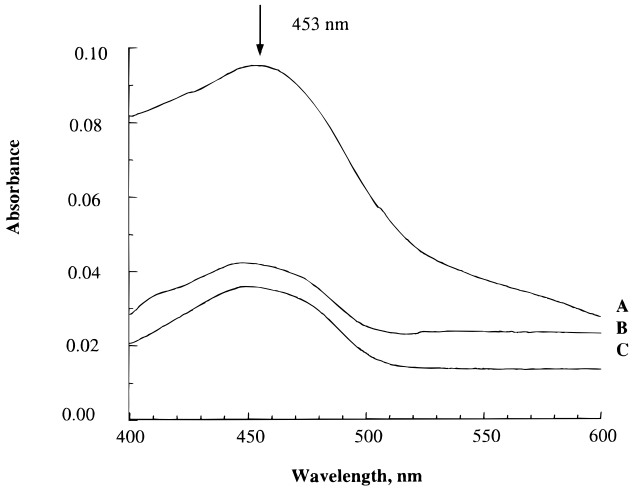

FIGURE 2: Visible difference spectra (oxidized minus dithionite reduced) of Na^+-NQR. The characteristic peak for flavins at 450 nm is indicated with an arrow. (A) Purified Na^+-NQR, the spectrum was measured in 50 mM Hepes, 150 mM NaCl, and 0.1% LDAO, pH 7.5; protein concentration was 0.78 mg/mL. (B) Difference spectrum of the supernatant after TCA precipitation of the protein. A total of 0.65 mg of the Na^+-NQR was precipitated with 5% TCA solution. After centrifugation the pellet was washed with 5% TCA. The combined supernatant fractions were neutralized by adding 1 M K_2HPO_4 (up to pH 7.0) and used for determination of the noncovalently bound flavin. The pellet was resuspended in 100 mM NaH_2PO_4 (pH 7.0) containing 1% SDS and used for determination of the covalently bound flavin. (C) Difference spectrum of the pellet resuspended in SDS solution.

RESULTS

Catalytic Properties. As shown in Table 1, the purified Na^+-NQR from *V. harveyi* has a specific NADH dehydrogenase activity of 42 units/mg (a 27.7-fold purification compared to cell membranes). SDS−PAGE (not shown) shows bands corresponding to each of the six gene products, similar to the result obtained by Nakayama and collaborators with the enzyme from *V. alginolyticus* (*14*). However, there are additional bands which are likely to be contaminants.

The oxidized minus reduced spectrum of the purified enzyme (Figure 2A) has a peak at 453 nm, typical of flavoproteins. The enzyme is able to use menadione as an electron acceptor and can utilize NADH or dNADH (but not NADPH) as donor. The apparent K_m for NADH is 30 μM and for dNADH is 80 μM. The purified Na^+-NQR reduced Q_1 with a specific activity of 11.2 inits/mg. This reaction is

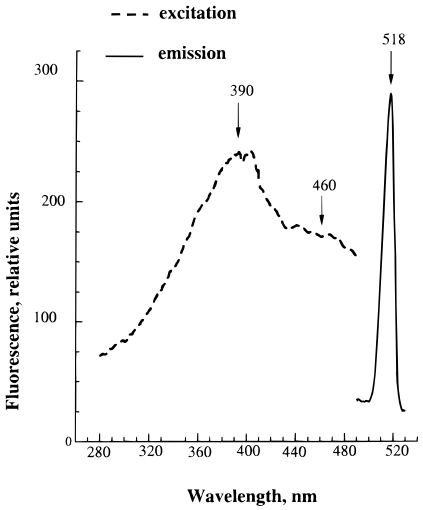

FIGURE 3: Fluorescence spectra of NqrC subunit. Purified Na^+-NQR was electrophoresed isolated following SDS−PAGE. The fluorescent band (NqrC subunit) was taken from the gel and its fluorescence spectra were recorded in 10 mM NaH_2PO_4 pH 7 (emission at 518 nm and excitation at 360 nm).

strongly dependent on sodium concentration. The apparent K_m for sodium in the absence of potassium and magnesium is about 100 mM. The Q_1-reductase activity of the purified enzyme is strongly inhibited by HQNO with $I_{0.5}$ of about 0.15 μM.

Cofactor Determination. Flavin. The soluble flavin in Na^+-NQR was determined in the partially purified preparation (after the Fractogel TSK DEAE column) as well as in the final isolated enzyme, by solvent extraction followed by HPLC. In all cases, FAD was the only flavin found.

The total flavin content of the purified Na^+-NQR was also determined after precipitation of the protein by trichloroacetic acid (TCA). The protein was denatured by TCA and the oxidized minus reduced spectra of the supernatant and the pellet (resuspended in SDS) were recorded (Figure 2, panels B and C). The spectra show that flavins are present in both fractions: 3.1 nmol/mg protein in the supernatant and 2.9 nmol/mg in the pellet. Since the Na^+-NQR molecular weight is 211 kDa (1 mg of protein corresponds to 4.76 nmol), this suggests that the enzyme probably contains one noncovalently bound FAD and one covalently bound flavin.

When the purified Na^+-NQR protein was analyzed by SDS−PAGE, one protein band exhibited strong yellow/green fluorescence under UV illumination, suggesting a covalently bound group attached to the protein. The most likely candidates are flavins and cytochrome *c*. To determine if this fluorescence was due to a covalently bound heme on a contaminating polypeptide, the gel was stained for heme-induced peroxidase activity using tetramethylbenzidine, as described by Thomas (*27*). No evidence of covalent heme was found. The fluorescent protein was excised from the gel and extracted. Fluorescence excitation and emission spectra of extracted band were recorded (Figure 3). The emission spectrum has a peak at 518 nm, which is indicative of a flavin group. The fluorescent protein band was transferred to a poly(vinyliden difluoride) (PVDF) membrane for N-terminal sequencing. The result shows that the protein has ASNNDSIKKT as its N-terminal sequence, confirming that this is the NqrC subunit of Na^+-NQR. Further evidence for the presence of a covalently bound flavin in Na^+-NQR was provided by a mass spectrometric analysis of the enzyme. On the basis of the translated sequence, the molecular mass of NqrC should be 27 674 Da (without the first methionine). However, a molecular mass of 28 120 Da was determined experimentally for this subunit by MALDI mass spectrometry. The difference, 446 Da, corresponds within error to the molecular mass of FMN (454 Da), indicating that the covalently bound flavin is probably FMN.

Identification of the Flavin Ligand. The fluorescent NqrC subunit was digested with trypsin, and the resulting peptides were separated by HPLC. The HPLC chromatogram shows one major fluorescent peak (35.14 min), which has a corresponding peak in the absorbance profile recorded at 220 nm (not shown). The material from the fluorescent peak from the HPLC separation was subject to a total of 10 cycles of Edman degradation. The sample gave a clear sequence: **GGAPEGSEXG**. This corresponds to one of the expected NqrC tryptic peptides, from G211 to K253. The "X" corresponds to the place where histidine-219 is expected, but did not appear. This result strongly suggests that the flavin is linked to histidine in position 219 in the NqrC subunit.

Ubiquinone. Quinone analysis of the partially purified Na^+-NQR by solvent extraction followed by HPLC showed that the content of ubiquinone-8 is approximately stoichiometric with the amount of FAD extracted in the enzyme. This result is in agreement with the observations reported by Pfenninger-Li and co-workers (*16*) for the enzyme from *V. alginolyticus*. This shows that, like the *V. alginolyticus* enzyme, Na^+-NQR from *V. harveyi* contains a quinone-binding site, which binds quinone tightly enough to retain a significant fraction through the enzyme purification procedure.

Reconstituition. The Na^+-NQR was successfully reconstituted into liposomes using the detergent *n*-octylglucoside and Bio-Beads. The resulting proteoliposomes were used to study the formation of $\Delta\Psi$ and ΔpH by Na^+-NQR. Figure 4A shows that NADH:Q_1 oxidoreduction by the reconstituted enzyme in sodium-containing medium results in a change in oxonol VI fluorescence, indicating the formation of $\Delta\Psi$ (interior positive) by the proteoliposomes. The signal decay observed approximately 30 s after NADH addition is due to exhaustion of the substrate. The formation of $\Delta\Psi$ was stimulated by monensin (Figure 4C), slightly inhibited by the protonophore CCCP (Figure 4B), and arrested entirely by the sodium ionophore ETH-157 (Figure 4E) or by a combination of CCCP and nigericin (Figure 4D). Formation of $\Delta\Psi$ was not observed in sodium-free medium (Figure 4F). These data indicate that Na^+-NQR from *V. harveyi* operates as an electrogenic sodium pump, and that the enzyme is not able to use protons instead of sodium.

pH changes in the interior of the proteoliposomes during NADH:Q_1 oxidoreduction were followed by measuring the fluorescence of entrapped pyranin. Under these conditions, proteoliposomes generated a ΔpH (interior alkaline) (Figure 5). This process was stimulated by CCCP (Figure 5B); it was slightly decreased by valinomycin (Figure 5C) and could be completely abolished by the sodium ionophore ETH-157 (Figure 5D), nigericin plus CCCP (Figure 5E), alamethicin (Figure 5F), or gramicidin D (Figure 5, panels A−F).

The observed interior alkalinization can be explained by two different mechanisms: (i) electrophoretic efflux of

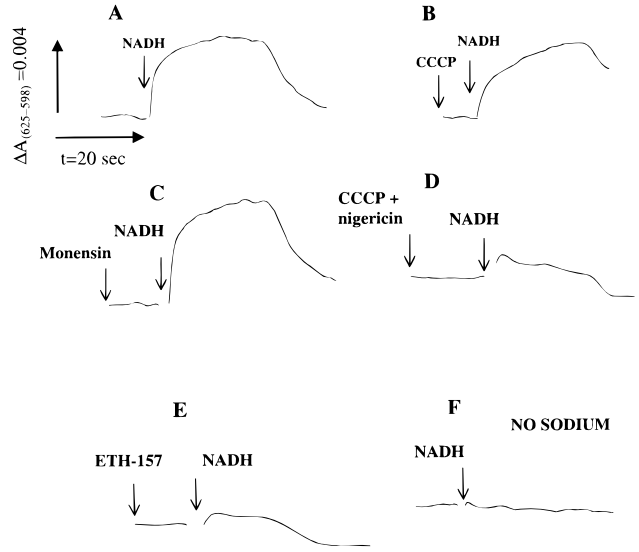

FIGURE 4: $\Delta\Psi$ generation by reconstituted Na^+-NQR. $\Delta\Psi$ was measured following the changes in absorbance of oxonol VI (625−587 nm) in the presence of 50 mM NaCl. Additions were made where indicated by the arrows. In all cases the reaction was started with the additon of NADH (0.4 mM). (A) Only NADH; (B) 1 μM CCCP; (C) 2 μM monensin; (D) 1 μM CCCP, 0.5 μM nigericin; (E) 5 μM ETH-157; (F) sodium-free medium.

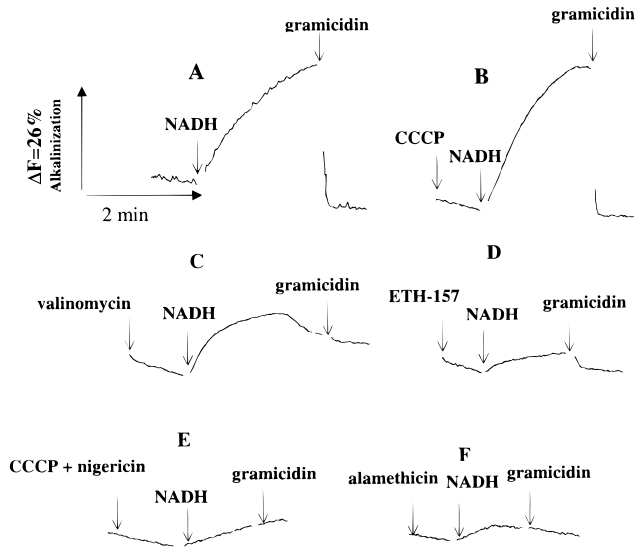

FIGURE 5: ΔpH generation by reconstituted Na^+-NQR. pH changes in the interior of the proteoliposomes were measured by the change in fluorescence of entrapped pyranin (excitation, 458 nm). Additions were made where indicated by the arrows. (A) 1 μg/mL gramicidin; (B) 1 μM CCCP + 1 μg/mL gramicidin; (C) 1 μM valinomycin + 1 μg/mL gramicidin; (D) 5 μM ETH-157 + 1 μg/mL gramicidin; (E) (1 μM CCCP + 0.5 μM nigericin) + 1 μg/mL gramicidin; (F) 5 μg/mL alamethicin + 1 μg/mL gramicidin.

protons to neutralize sodium influx and/or (ii) by proton consumption for the formation of ubiquinol. The latter will be seen only if the protons are taken up from the inside of the liposomes, which corresponds to the bacterial periplasm. If the interior alkalinization of the proteoliposomes arises from electrophoretic proton flow, it should be stimulated by protonophores and strongly inhibited by sodium ionophores. If this alkalinization is caused by the formation of quinol, this process should be partially sensitive to protonophores and resistant to sodium ionophores. Experimentallly, the formation of ΔpH was slightly increased by CCCP (Figure 5B) and strongly decreased by the sodium ionophore ETH-157 (Figure 5D). This indicates that the interior alkalinization results from proton efflux to discharge the $\Delta\Psi$ created by sodium pumping and not from net proton consumption for the formation of quinol. It also means that the protons for ubiquinol production are taken up from outside of the liposomes, which corresponds to the bacterial cytoplasm. The limited effect of valinomycin on ΔpH formation can be probably attributed to a low initial concentration of potassium inside the vesicles.

Sequencing of Na^+-NQR. The Na^+-NQR operon from *V. harveyi* was sequenced as described in the Materials and Methods. The sequence is highly homologous to the Na^+-NQR in *V. alginolyticus* and encodes six polypeptides. Reading frame 1 contains the genes for NqrA, B, and D, and reading frame 2 contains NqrC, E, and F. The final gene sequence of Na^+-NQR from *V. harveyi* ($\sim$ 6 kb) has been deposited in GenBank (accession number AF165980).

The program TMPRED (Prediction of Transmembrane Regions and Orientation program; http://www.isrec.isb-sib.ch) was used to predict the transmembrane helices and membrane sidedness. The NqrA subunit has no predicted transmembrane helices. NqrB, D, and E are predicted to be very hydrophobic and to contain 8, 5, and 6 transmembrane helices, respectively. NqrC has one predicted transmembrane helix. Direct amino acid sequencing shows that the N-terminal methionine is missing from NqrC, which is also the case for the enzyme from *V. alginolyticus* (*23*). NqrF is predicted to have two transmembrane helices near the N-terminus, and in addition, NqrF includes sequence motifs suggesting the presence of an iron−sulfur center, FAD, and NADH binding sites, in agreement with the predictions of Rich and collaborators (*13*).

Homology Study. Although it has been shown that Na^+-NQR exists in marine and moderately halophilic bacteria (*9*), the DNA sequences of only two Na^+-NQR's have been previously reported in the literature, one from *V. alginolyticus* (*13*, *31*), and another from *H. influenzae* (*10*), a nonmarine bacterium. By searching unfinished genomes with Blast, the entire Na^+-NQR operon was found in several other bacteria, including *V. cholerae* (see also ref *34*), *N. gonorrheae*, *N. meningitidis*, *Yersinia pestis*, *S. putrefaciens*, *Ps. aeruginosa*, *P. gingivalis*. The deduced Na^+-NQR protein sequences from these bacteria suggest that all six structural subunits share strong homology. NqrC, whose length varies considerably among the different bacteria, is the least conserved subunit with identity scores ranging 28.9−80.8% with respect to that of *V. harveyi* (Figure 6). In contrast, the length of the other five subunits is conserved, and the amino acid sequence identity ranges 33.2−99.5%. *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Actinobacillus actinomycetemcommitans* also have genes homologous to all six subunits of Na^+-NQR, but in these organisms, the genes are not organized into a single operon. The Blast search also revealed several more distant homologies. Four of the Na^+-NQR subunits, NqrB, C, D, and E showed homology to nitrogen fixation proteins in *Rhodobacter capsulatus* (*32*) as well as to predicted gene products from *Escerichia coli*. NqrB, D and E were found to be homologous to three gene products in *Thermotoga maritima*, possibly involved in electron-transfer reactions (*33*).

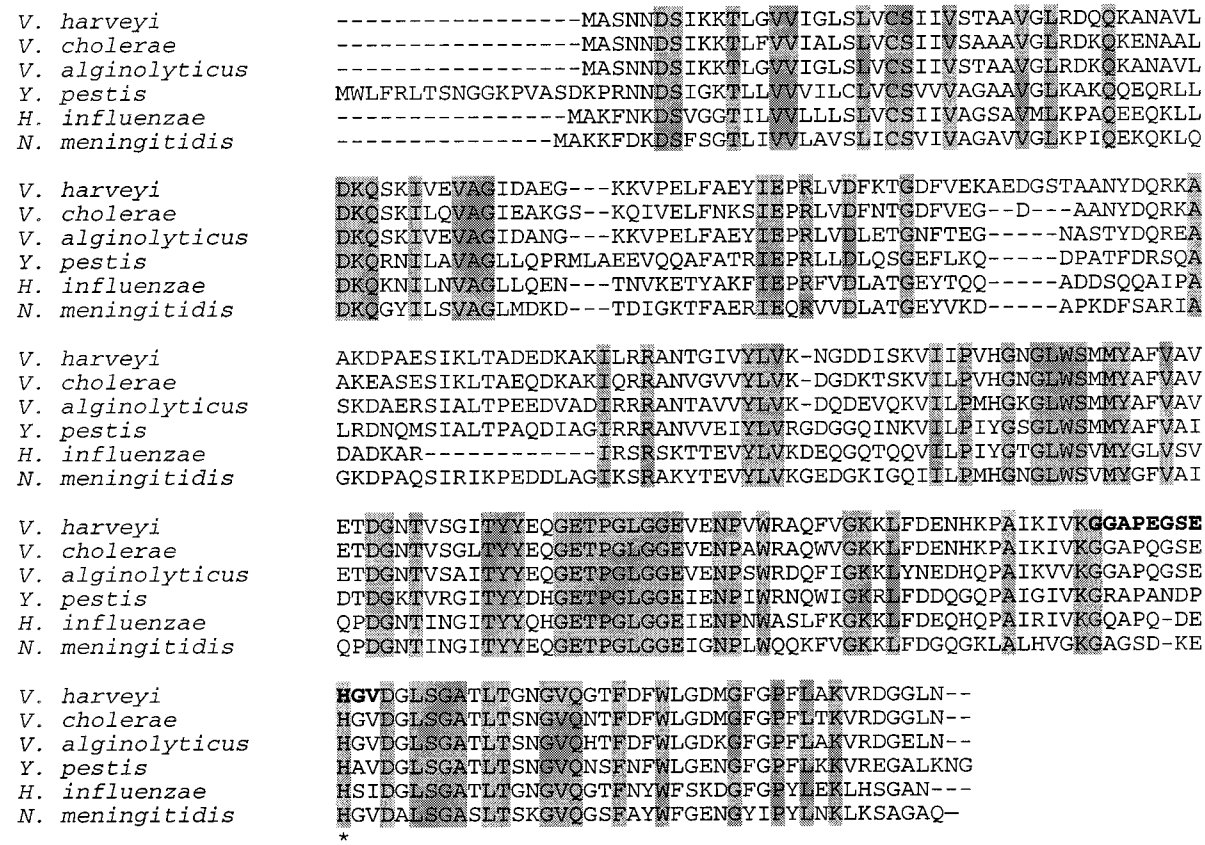

FIGURE 6: Sequence alignment of NqrC subunit. Conserved residues are shaded. Part of the sequence of the fluorescent peptide which was isolated is shown in bold leters, and H219 is indicated (*). The sequence of *V. harveyi* has been submitted to GenBank and is available under accesion number AF165980. The GenBank accession numbers for the other sequences are *V. alginolyticus*, AB008030; *V. cholerae*, AAD29962-67; *H. influenzae*, U32702.

DISCUSSION

Enzyme Activity. The Na^+-NQR operon from *V. harveyi* has been cloned and sequenced, and the properties of the purified enzyme have been studied. The catalytic properties of the enzyme (i.e., K_m for NADH, dNADH, sodium; K_i for HQNO) are very similar to those of the enzyme from *V. alginolyticus*. Clearly, the *V. harveyi* Na^+-NQR is closely related to the enzyme in *V. alginolyticus*.

Cofactors of Na^+-NQR. It is generally agreed that the *V. alginolyticus* Na^+-NQR contains FAD and an FeS cluster (*12*, *15*, *16*). Hayashi and Unemoto (*12*) also reported the presence of a noncovalently bound FMN in the NqrA subunit. The current work shows that the Na^+-NQR from *V. harveyi* also contains noncovalently bound FAD and, in addition, has a second flavin (probably FMN) covalently bound to the NqrC subunit. Furthermore, we observed that the NqrC subunit from the enzyme purifed from *V. alginolyticus* also exhibits fluorescence under UV illumination, suggesting that this is a common property of the enzyme.

Mewis and co-workers (*34*) have reported that there are more than 20 enzymes that contain covalently bound flavins. These covalent flavoproteins can be divided in two groups according to the type of linkage to the flavin; in one group, the flavin binds to the protein via the 8α-methylene group of the isoalloxazine ring, and in the second group, the flavin binds via the C6 atom of the isoalloxazine ring. The first group includes enzymes in which the flavin (FAD or FMN) is bound via tyrosine, histidine, or cysteine. In the second group, the flavin (FMN only) has only been found attached to cysteine (*34*). There is no recognized sequence motif around the covalent attachment site. But in several of the covalent flavoproteins, there is separate binding motif associated with the bound flavin, consisting of a Gly-X_m-Gly-X_n-Gly dinucleotide binding sequence close to the amino terminus (*34*). Figure 6 shows an alignment of the NqrC subunit sequences. There are several conserved residues that could potentially be the covalent site of flavin attachment, one conserved cysteine, one histidine, and three tyrosine residues. The fluorescent peptide isolated from NqrC contains the only conserved histidine (H219), suggesting that the covalent flavin is attached to histidine-219 in NqrC. The histidine moiety can be covalently bound to the flavin via the N(3) or N(1)-imidazole nitrogen and the current data do not distinguish between these options (*34*). The mass spectrometry data indicate, but do not definitively prove, that the covalent flavin is FMN. Possibly, NqrC binds to FMN via an 8α-N(3) histidyl linkage, similar to that recently reported in sarcosine oxidase (*35*). This will require further examination. In any event, these data indicate that the NqrC subunit from the *V. harveyi* enzyme is directly involved in electron transfer.

Ubiquinone. The *V. harveyi* Na^+-NQR contains a substoichiometric amount of ubiquinone-8 associated with the purified enzyme. This suggests that, like the *V. alginolyticus* enzyme (*16*), Na^+-NQR from *V. harveyi* contains a quinone-binding site, which binds tightly enough to retain a significant fraction (i.e., at least 0.1) of the bound molecules through the enzyme purification procedure. The presence of this

apparently tightly bound quinone has been interpreted to mean that the enzyme has two quinone-binding sites, one for a quinone cofactor, possibly involved in sodium translocation, and a second, catalytically exchangeable, site by which the enzyme comunicates with the Q-pool (*13*, *16*).

Reconstitution. The reconstituted *V. harveyi* Na^+-NQR functions as a primary sodium pump to generate a transmembrane $\Delta\Psi$. This agrees with earlier findings on the *V. alginolyticus* enzyme (*16*). In the current work, the movement of protons linked to NADH:Q oxidoreduction by Na^+-NQR has been examined for the first time in a reconstituted system. We show that a ΔpH is created by a secondary process, caused by electrophoretic H^+ flow to discharge the $\Delta\Psi$ generated by the primary Na^+ pump. The enzyme does not generate ΔpH directly, and the protons required for the formation of ubiquinol must be taken up from the cytoplasmic side of the membrane, the same side as protons produced by the oxidation of NADH.

Homology Studies. The predicted Na^+-NQR protein sequence from *V. harveyi* is closely homologous to those for *V. alginolyticus* (*23*, *31*) and *H. influenzae* (*10*) but shows very little similarity to H^+-translocating-NADH-ubiqinone oxidoreductases. In addition, Na^+-NRQ sequences are now available from a number of disease-causing bacteria including *V. cholerae* (*11*), *N. gonorrheae*, *N. meningitidis*, *Yersinia pestis*, *S. putrefaciens*, *Ps. aeruginosa*, and *P. ginginvalis*. The larger number of sequences has made it possible use sequence alignments to make predictions with more confidence, and these confirm all of the previously identified cofactor-binding motifs (NADH, FAD, and FeS) (*12*, *13*, *21*).

The presence of the genes encoding Na^+-NQR in a number of nonmarine bacteria, including several prominent human pathogens, suggests that this enzyme may be of some general importance. Some strains of *H. influenzae* contain an active Na^+-NQR and require up to 200 mM NaCl for optimal growth (*10*), and *Yersinia pestis* is also able to survive at Na^+ concentrations as high as 150 mM (*36*). Of particular interest is the recent demonstration that the expression of the virulence genes in *V. cholerae* is affected by changes in membrane Na^+ flux (*11*). Furthermore, inhibition of Na^+-NQR by HQNO (or deletion of the genes) causes elevated toxT activity, suggesting that Na^+-NQR may play a role in the virulence of the bacterium. Now that a number of pathogenic bacteria are known to have the genes for Na^+-NQR, the questions of whether Na^+-NQR is expressed in these bacteria and whether this enzyme has a role in virulence deserve to be investigated.

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