NADH:Ubiquinone Oxidoreductase of *Vibrio alginolyticus*: Purification, Properties, and Reconstitution of the Na^+ Pump†

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ABSTRACT: The Na^+-activated NADH:ubiquinone oxidoreductase of *Vibrio alginolyticus* was extracted from the membranes with lauryldimethylamine-*N*-oxide and purified by two successive anion exchange columns. This preparation, yielding four major and several minor stained bands after SDS−PAGE, retained the NADH-dehydrogenase activity (with menadione as an artificial electron acceptor) and ubiquinone-1 (Q) reductase activity. On further fractionation of the enzyme, the Q-reductase activity essentially disappeared. Chemical analyses revealed the presence of FAD but not FMN, of non-heme iron and of acid-labile sulfur and tightly-bound ubiquinone-8 in the purified Q-reductase preparation. The participation of an iron−sulfur cluster of the [2Fe-2S] type in the electron translocation was demonstrated by the appearance of a typical EPR signal for this prosthetic group after the reduction of Q-reductase with NADH. A strong EPR signal typical for a radical observed upon reduction of the enzyme might arise from the formation of quinone radicals. In the absence of Na^+, the path of the electrons apparently ends with the reduction of ubiquinone-1 to the semiquinone derivative which in the presence of O_2 becomes reoxidized with concomitant formation of superoxide radicals. In the presence of Na^+, these oxygen radicals are not formed and the semiquinone is further reduced to the quinol derivative. These results indicate that the Na^+-dependent step in the electron transfer catalyzed by NADH:ubiquinone oxidoreductase is the reduction of ubisemiquinone to ubiquinol. After reconstitution of the purified Q-reductase into proteoliposomes, NADH oxidation by ubiquinone-1 was coupled to Na^+ transport with an apparent stoichiometry of 0.5 Na^+ per NADH oxidized. The transport was stimulated by valinomycin ($+K^+$) or by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The transport of Na^+ is therefore a primary event and does not involve the intermediate formation of a proton gradient.

The first step in the respiratory chain of mitochondria and many aerobic bacteria is the transfer of electrons from NADH to ubiquinone. Several types of membrane-bound enzymes (NQR)[1] are known to catalyze this reaction. Members of the NQR-1 family couple the electron transfer to proton translocation across the membrane. The most complicated enzyme of this group is complex I of mammalian mitochondria which is composed of at least 41 different subunits (Walker, 1992). The counterparts of this enzyme in the bacteria *Paracoccus denitrificans* (Yagi, 1986; Yagi et al., 1991), *Escherichia coli* (Weidner et al., 1993; Leif et al., 1995), or *Thermus thermophilus* (Yagi et al., 1988) are composed of 14 different subunits that are homologous to the mammalian enzyme forming a more minimal catalytic entity. The prosthetic groups involved in the electron transfer within these complexes include FMN and several iron−sulfur clusters (Beinert & Albracht, 1982; De Jong et al., 1994; Yagi, 1993). Enzymes of the second group of NADH: ubiquinone oxidoreductases (NQR-2) do not couple electron transfer to proton translocation. These enzymes usually consist of a single polypepetide and have FAD as the only redox cofactor (Yagi, 1991).

Two different NADH:ubiquinone oxidoreductases have also been found in the membrane of the marine bacterium *Vibrio alginolyticus* (Hayashi & Unemoto, 1987; Hayashi et al., 1992). One of these, termed NQR-2 is of the non-energy-conserving type, whereas the other one, termed NQR-1, is believed to be coupled to Na^+ ion pumping. This enzyme is induced during aerobic growth at alkaline pH and may have the physiological advantage to keep the cytoplasmic pH near neutrality while pumping cations (Na^+) into the alkaline environment (Tokuda & Unemoto, 1981). Evidence for the existence of similar respiratory Na^+ pumps has been provided for other marine bacteria (Tokuda & Kogure, 1989). A similar enzyme has also been found in *Klebsiella pneumoniae* growing anaerobically on citrate (Dimroth & Thomer, 1989). In this organism, the Na^+-NQR-1 has an anabolic role in NADH synthesis via a reversed electron transfer from ubiquinol to NAD^+ which is driven by the electrochemical Na^+ gradient generated by the

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[1] Abbrevations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, 1,4-dithio-DL-threitol; DCCD, dicyclohexylcarbodiimide; LDAO, lauryldimethylamine-*N*-oxide; NQR-1, energy-conserving NADH:ubiquinone oxidoreductase; NQR-2, non-energy-conserving NADH: ubiquinone oxidoreductase; Q, ubiquinone-1.

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oxalacetate decarboxylase Na^+ pump (Pfenninger-Li & Dimroth, 1992).

Previous biochemical evidence on the Na^+-NQR-1 from *V. alginolyticus* indicated that the enzyme consists of only three subunits, with apparent molecular masses of 52 000, 46 000, and 32 000 Da (Hayashi & Unemoto, 1984, 1987). It was also reported that the enzyme contains FMN in the 52 kDa subunit and FAD in the 46 kDa subunit and that no other redox cofactors were present (Hayashi & Unemoto, 1986). We have shown recently that the enzyme is probably more complex than anticipated, consisting of more than three subunits, and that FAD is the only flavin-containing prosthetic group (Pfenninger-Li & Dimroth, 1995). The genes encoding the 52 and 32 kDa subunits of the *V. alginolyticus* NQR-1 were shown by two different groups to be physically connected to a cluster of together six open reading frames that was tentatively designated the *nqr* operon (Beattie et al., 1994; Hayashi et al., 1995). Further biochemical characterization of the protein is required to assess the participation of all of the gene products in the enzyme complex and to gain more insight into the catalytic mechanism. We report here a new purification of the enzyme, provide evidence that the complex consists probably of at least four different subunits and that an iron−sulfur cluster of the [2Fe-2S] type functions as an additional prosthetic group. We also show for the first time with proteoliposomes containing the purified enzyme that Na^+ transport by the NQR-1 is a primary event and not catalyzed by a combination of a H^+-translocating NQR-1 with a Na^+/H^+ antiporter.

EXPERIMENTAL PROCEDURES

Materials. Lauryldimethylamine *N*-oxide (LDAO) was purchased from Calbiochem (San Diego, CA, U.S.A.). ^{22}NaCl was obtained from Amersham International (Buckinghamshire, U.K.). Cyclohexylammonium salt of NADH (Na^+-content < 0.1%) was from Sigma (Buchs, Switzerland). Ubiquinone-1 and ubiquinone-8 were the generous gifts of Dr. Keller (F. Hoffman-La Roche and Co., Basel, Switzerland). All other reagents were of analytical grade excluding those used in cultivation media.

Bacterial Growth, Preparation of Plasma Membranes, and Isolation of NADH:Ubiquinone Oxidoreductase. V. alginolyticus (DSM 2171^T, Braunschweig, Germany) was grown aerobically at 37 °C in a complex medium containing 5.0 g of polypeptone/L, 5.0 g of yeast extract/L, 4.0 g of K_2HPO_4/L, 2.0 g of glucose/L, and 30 g of NaCl/L. The pH of the medium was adjusted to 8.5 with Tris base. Cells were selected that grew on agar plates with the complex medium in the presence of 5 μM CCCP (Tokuda & Unemoto, 1983). A single colony was picked and used to inoculate 5 mL of liquid medium. After transfer to 200 mL and then to 2.0 L, the dense culture was used to inoculate 100 L of medium in a fermenter (300 L volume). The cells were harvested in the exponential phase after 12−16 h, yielding about 0.5 kg of wet packed cells. The cells were frozen at −70 °C until use without loss of both NADH-dehydrogenase and Q-reductase activities. The enzyme was extracted from the membranes with 1% LDAO and purified by chromatography on DEAE-Sephacel and Q-Sepharose columns as described (Pfenninger-Li & Dimroth, 1995). The enzyme (protein concentration > 1 mg/mL) dissolved in 10 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM EDTA, 10% glycerol, 0.1 M NaCl and 0.1% LDAO can be stored in liquid N_2 without significant loss of activity. Repeated freezing and thawing, however, leads to the loss of Q-reductase activity.

In some experiments, the Q-Sepharose fraction was further purified by Superdex 200 column chromatography. Therefore, the concentrated Q-Sepharose fraction was applied to a Superdex 200 column equilibrated with 10 mM Tris/HCl buffer, pH 8.0, containing 10% (v/v) glycerol, 0.1% (w/v) LDAO, and 0.1 M NaCl. Hydroxyapatite chromatography was carried out with a column from Merck (Hydroxyapatite 75/5), equilibrated with 10 mM sodium phosphate buffer, pH 6.8, containing 10% (v/v) glycerol and 0.1% (w/v) LDAO. The active fractions (NADH-dehydrogenase) were eluted with a linear gradient of phosphate buffer (10−120 mM sodium phosphate, pH 6.8). In another experiment, 0.6 mL of concentrated Q-Sepharose fraction was loaded carefully onto a 12 mL gradient of 5%−20% (w/v) sucrose in 50 mM Tris/HCl buffer, pH 8.0, containing 50 mM NaCl and 0.1% (w/v) LDAO and centrifuged for 16 h at 100 000*g*. 0.5 mL fractions were collected and assayed for NADH-dehydrogenase and Q-reductase activities.

Determination of Enzyme Activities and Protein. Both NADH-dehydrogenase and Q-reductase activities were assayed at 25 °C in 1.0 mL quarz cuvettes ($d = 1$ cm). The standard assay for NADH-dehydrogenase activity contained the following in 1.0 mL: 0.1 mM NADH, 0.1 mM menadione (sometimes 15 μM ubiquinone-1 instead of menadione), 0.2 M NaCl, and 20 mM Tris/HCl, pH 8.0. The reaction was started by addition of the enzyme, and the initial velocity of the absorbance decrease at 340 nm was monitored ($\epsilon_{340} = 6.22$ mM^{-1} cm^{-1}). If membrane vesicles were used for measuring NADH-dehydrogenase activity, then 5 mM KCN was included in the assay solution to inhibit NADH oxidation by the respiratory chain. The Q-reductase activity was measured by following the formation of ubiquinol-1 from ubiquinone-1. The standard assay mixture contained the following in 1.0 mL: 0.1 mM NADH, 15 μM ubiquinone-1, 0.2 M NaCl, 0.01% (w/v) LDAO, 20 mM Tris/HCl, pH 8.0, and the enzyme. Changes in absorbance difference at the wavelength pair 248−268 nm were recorded with a Shimadzu UV-3000 dual wavelength/double-beam recording spectrophotometer, and the rate of ubiquinol-1 formation was calculated based on the extinction coefficient $\Delta\epsilon_{248-268} = 7.8$ mM^{-1} cm^{-1}.

Protein was determined by the bicinchoninic acid method (Smith et al., 1985) using the reagent obtained from Pierce and bovine serum albumin as standard.

N-Terminal Sequencing. NADH:ubiquinone oxidoreductase subunits were separated on SDS−PAGE according to Schägger and von Jagow (1987) and electroblotted onto a hydrophobic poly(vinylidene difluoride) (PVDF) membrane (Millipore). The blot was stained with 0.1% Coomassie Blue in 50% methanol and destained in 50% methanol. Membrane pieces with the respective subunits were directly used for N-terminal sequence analysis using a protein sequencer (model 470/A; Applied Biosystems) with HPLC-on-line PTH (phenylthiohydantoin)-amino acid detection (HPLC model 120, Applied Biosystems).

Characterization of Enzyme. To examine the effects of Na^+, K^+, and Li^+ salts on the NADH-dehydrogenase and Q-reductase activities, "Na^+-free" buffers were prepared by using chemicals containing the lowest Na^+ contaminations

available. The buffers were checked for Na^+ concentration with an atomic absorption spectrophotometer.

The pH optimum of NADH-dehydrogenase and Q-reductase was assayed in a polybuffer system containing the following in a total volume of 1.0 mL: 0.1 mM NADH, 15 μM ubiquinone-1, 0.01% LDAO, 0.2 M NaCl, and 20 mM each of MES, MOPS, and TRICINE (for pH 6.0) or 20 mM each of MOPS, TRICINE, and glycine (for pH 7−9), both adjusted to the desired pH with KOH.

Extraction and Determination of Quinones. The quinones were extracted from *V. alginolyticus* membranes with petroleum ether (Redfearn, 1967) and then analyzed by HPLC (Takada et al., 1984). Therefore, aliquots of 0.1 mL of *V. alginolyticus* membrane vesicles (20 mg of protein/mL, washed two times with buffer) or 0.1 mL of isolated enzyme (1.5−2.5 mg of protein/mL) were transferred into glass tubes with glass stoppers. After addition of 0.4 mL of methanol (−20 °C, HPLC grade) and 0.5 mL of petroleum ether (bp 40−60 °C), the mixture was covered and vortexed for 1−2 min and then centrifuged (15 000*g*, 2 min) to separate the two layers. The upper layer was removed and combined with that from a second extraction with 0.3 mL of petroleum ether. After addition of 0.2 mL of 95% ethanol, the solvent was evaporated in a Speed Vac (6 h). The yellow pellet was dissolved in 0.05 mL of 100% ethanol and analyzed by difference spectroscopy, oxidized−reduced (by a trace of sodium borohydride) between 230 and 320 nm as described by Kröger (1978). The type of quinone was analyzed by injecting the solution onto a ODS Hypersil C_{18} reversed-phase column (5 μm, 250 × 4 mm Hewlett Packed). The mobile phase was prepared by dissolving 7.0 g of $NaClO_4 \cdot H_2O$ in 1000 mL of a mixture of ethanol (puriss.pa), methanol (HPLC grade), and 70% $HClO_4$ (puriss.pa) (700:300:1). The flow rate was 1.0 mL/min. The signal was detected with a UV-detector at 275 nm. The quinone standards eluted as follows: Q-1, 3.7 min; Q-6, 5.2 min; Q-8, 6.4 min; and Q-10, 8.6 min.

Determination of Non-Heme Iron, Acid-Labile Sulfur, and Flavins. For the complexation of extraneous iron ions 200 μL samples were incubated with 10 mg of Chelex 100 resin for 1 h at 4 °C with shaking. The resin was subsequently removed by centrifugation.

Non-heme iron was determined colorimetrically with neocuproin (2,9-dimethyl-1,10-phenantroline) and ferrozine-[3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)-1,2,4-triazine] as described (Fish, 1988). Acid-labile sulfur was analyzed using methylene blue (Cline, 1969). Flavins were identified by HPLC analysis and quantified spectrofluorimetrically (Pfenninger-Li & Dimroth, 1995). Enzyme-bound flavins were released by treating samples with ice-cold trichloroacetic acid. The neutralized supernatant was subjected to HPLC at room temperature on an ODS Hypersil C_{18} reversed-phase (5 μm, 4 mm × 250 mm) column with 5 mM ammonium acetate buffer, pH 6.0, containing 0%−80% methanol as eluent (1.0 mL/min). FAD eluted after 13 min, FMN after 15 min, and riboflavin after 19 min.

Detection of Iron−Sulfur Clusters and Radicals by EPR Spectroscopy. Samples of *V. alginolyticus* membranes (65−70 mg/mL) or purified NADH:ubiquinone oxidoreductase (3−8 mg/mL) were inspected with EPR spectroscopy on a Bruker ECS-106 X-band EPR spectrometer. The field modulation frequency was 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter. The X-band frequency was measured with a HP 5350B microwave frequency counter. The microwave power incident to the cavity was measured with a HP 432 B power meter. Simulation was carried out as described earlier (Beinert & Albracht, 1982). Quantification of EPR signals was carried out by direct double integration of the experimental spectra (Albracht, 1984; Aasa & Vänngård, 1975) or by comparison with a well-fitting simulation (Albracht et al., 1979).

Detection of Superoxide Radicals. The formation of superoxide radicals was detected by chemiluminescence elicited by their reaction with luminol (Michelson, 1978). The experiments were carried out with Berthold Biolumat LB95000C. The assay mixtures contained the following in 0.27 mL at 25 °C: 48 μmol of Tris/HCl (pH 8.0), 10 nmol of luminol, 7 nmol of ubiquinone-1, and 2 μg of purified NADH:ubiquinone oxidoreductase (Q-Sepharose fraction). The chemiluminescence was initiated by automatically injecting 100 μL (0.1 μmol) of NADH into the cuvette, which was placed in the measuring position. The effect of different salts on chemiluminescence signals was studied by adding 10 μL solutions of proper concentrations into the reaction mixtures.

Reconstitution of Proteoliposomes Containing NADH:Ubiquinone Oxidoreductase. To form liposomes, 40 mg of phosphatidylcholine from soybean or 40 mg of *E. coli* phospholipids was suspended in 1.0 mL of 10 mM Tris/HCl buffer, pH 8.0, containing 0.1 M K_2SO_4 and 1 mM DTT (reconstitution buffer) and vortexed under N_2 until a translucent solution had formed. This was cooled with ice and sonicated 3 × 1 min in a water-type sonicator. The incubation mixtures contained in a total volume of 0.7 mL 10 mM Tris/HCl buffer, pH 8.0, 0.1 M K_2SO_4, 20 mg of sonicated phosphatidylcholine, 2.7% octyl glucoside, and NADH:ubiquinone oxidoreductase (0.1 mg of protein). The order of additions was always detergent first followed by enzyme. After 1−2 min incubation at room temperature, the incubation mixture was diluted into 25 mL of the reconstitution buffer (25 °C) and the solution was centrifuged at 150 000*g* for 45 min. The pellet was resuspended in 0.6 mL of reconstitution buffer, and the freshly prepared proteoliposomes were used immediately for the Na^+ transport assays.

Determination of Na^+ Transport into Proteoliposomes. The reconstituted proteoliposomes (0.15 mL) were incubated at 25 °C in a total volume of 0.4 mL of 10 mM Tris/HCl buffer, pH 8.0, containing 0.1 M K_2SO_4, 1 mM DTT, and 2.5 mM $^{22}NaCl$ (200−300 cpm/nmol). The uptake of $^{22}Na^+$ was initiated by adding 0.1 μmol of ubiquinone-1 and 0.1 μmol of NADH (cyclohexylammonium salt). Samples (0.09 mL) were removed after appropriate incubation periods and filtered over a small column (0.4−0.5 mL) of Dowex 50-K^+ to separate free $^{22}Na^+$ ions from those entrapped within the proteoliposomes. The entrapped $^{22}Na^+$ that was eluted from the column with 0.6 mL of 10 mM Tris/HCl buffer, pH 8.0, containing 0.1 M K_2SO_4 and 1 mM DTT was determined by γ-scintillation counting.

RESULTS

Purification of NADH:Ubiquinone Oxidoreductase from V. alginolyticus and N-Terminal Amino Acid Sequences.

Table 1: Survey of the Purification of the NADH:Ubiquinone Oxidoreductase (QR) from *V. alginolyticus*[a]

	total activity (units)		specific activity (units/mg)		yield (%)		purification (*n*-fold)	
purification step	NDH	QR	NDH	QR	NDH	QR	NDH	QR
membrane vesicles	240		1.2					
LDAO extract	340	56.7	4.2	0.7	100	100		
DEAE-Sephacel	276	46	13.8	2.3	81.1	81.1	3.3	3.3
Q-Sepharose	79.2	13	88	14.5	23.3	22.9	21	20.7
Superdex 200 (A)	64.41	10.6	92	15.1	18.9	18.7	21.9	21.6
Superdex 200 (B)	36	0.3	120	1.0	10.6	5.3	28.6	1.42

[a] Also shown are the NADH-dehydrogenase activities determined with menadione as electron acceptor (NDH). The starting material was 10 g of wet packed cells.

Table 2: Comparison of Polypeptides Predicted from the DNA Sequence of the *nqr* Operon with Those Identified by SDS−PAGE[a]

subunit	calcd. molecular mass (from DNA sequence; Da)	apparent molecular mass (SDS−PAGE; Da)	apparent subunit	comments
NqrA	48 622	50 700	α	N-terminal sequence
NqrB	46 809	33 300 (?)	a	very hydrophobic
NqrC	27 703	31 700	γ	N-terminal sequence
NqrD	22 602		b	very hydrophobic
NqrE	21 540		c	very hydrophobic
NqrF	45 274	45 900	β	binding motifs for [2Fe-2S], FAD, and NADH

[a] The hydrophilic subunits are designated by Greek letters, and the (tentative) hydrophobic subunits are designated by Latin letters.

Previously the purification of a three-subunit complex of NQR-1 was described with the detergent Liponox (Hayashi & Unemoto, 1984). As this detergent was not available to us we investigated the suitability of a series of ionic, zwitterionic, and non-ionic detergents for solubilizing and purifying the NADH:ubiquinone oxidoreductase from *V. alginolyticus*. The tendency to form aggregates with other proteins was dependent on the detergent and turned out to be one of the major problems. To find a suitable detergent, membrane extract prepared with a certain detergent was applied to a gel filtration column (Superose 6 or Superdex 200) equilibrated with buffer containing $^1/_{10}-^1/_5$ of the detergent concentration of the membrane extract. An elution profile with symmetrical peaks that could be well separated was taken as criterion for the presence of NQR-1 in defined micelles with the detergent. It was found that extraction of the membranes with 1% LDAO or 1% dodecyl maltoside was suitable for further purification of the enzyme.

The enzyme was partially purified from the 1% LDAO extract by anion exchange chromatography on DEAE-Sephacel and Q-Sepharose columns as described under Experimental Procedures. The specific activities of NADH-dehydrogenase and Q-reductase both increased 21-fold over that of the LDAO extract to 88 and 14.5 units/mg, respectively (Table 1). Both activities were obtained in a yield of 23%.

Inspection of the thus purified enzyme by SDS−PAGE revealed four dominant polypeptides with apparent molecular masses of 50.7, 45.9, 33.3, and 31.7 kDa, respectively, besides various less densely stained polypeptides (Figure 1). The polypeptides with apparent molecular masses of 50.7, 45.9, and 31.7 kDa correspond to the three (hydrophilic) subunits α, β, and γ noted in a previous study of the enzyme (Hayashi & Unemoto, 1987). According to the DNA sequence (Beattie et al., 1994; Hayashi et al., 1995) the enzyme presumably contains three additional hydrophobic subunits which will be termed a, b, and c according to decreasing molecular mass in analogy to the terminology of the F_1F_o ATPase subunits (see also Table 2). The polypeptide with the apparent molecular mass of 33.3 kDa (Figure 1) was tentatively assigned as the a subunit. Attempts to further purify the enzyme failed probably because the complex tends to dissociate. Chromatography of the purified NQR-1 on Superdex 200 yielded two fractions, termed A and B (Figure 2). Only fraction A, which contained the major portion of the protein, retained substantial amounts of Q-reductase activity and consisted of the four polypeptides mentioned above. In fraction B the polypeptide designated as the a subunit was missing leading to a drop of the Q-reductase activity to about 1 unit/mg of protein. The NADH-dehydrogenase activity of this preparation, however, increased to about 120 units/mg of protein (Table 1). A similar loss of this polypeptide occurred during hydroxyapatite chromatography, again being accompanied by a significant decrease of the quinone reductase activity and an increase of the NADH-dehydrogenase activity.

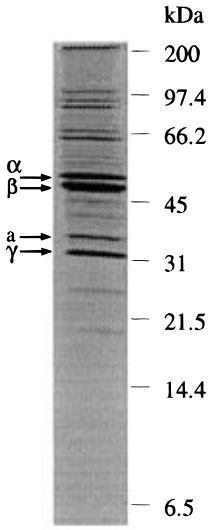

FIGURE 1: Coomassie-stained SDS−PAGE of the purified Na^+-translocating NADH:ubiquinone oxidoreductase from *V. alginolyticus* (Q-Sepharose fraction). 10 μg of purified enzyme was applied to a 16.5% SDS polyacrylamide gel (Schägger & von Jagow, 1987), electrophoresed, and stained with Coomassie. Molecular mass markers are also shown.

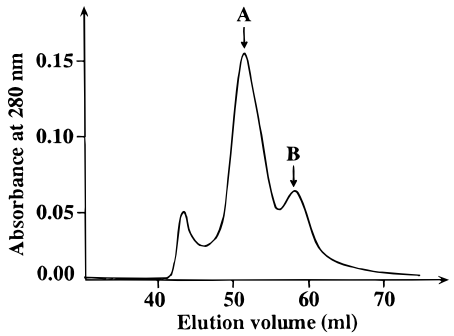

FIGURE 2: Elution profile of NQR-1 from Superdex 200. A 2.1 mg Q-Sepharose fraction was loaded on a Superdex 200 column. For details see Experimental Procedures.

Following sucrose density gradient centrifugation of NQR-1, the NADH-dehydrogenase activity was found in a yellow fraction near the top of the gradient. Analysis of this fraction by SDS−PAGE revealed the presence of only one polypeptide with apparent molecular mass of 45.9 kDa. This fraction was completely devoid of Q-reductase activity. These results show that the enzyme tends to dissociate and further indicate that the 45.9 kDa polypeptide catalyzes the NADH-dehydrogenase reaction without the participation of other polypeptides. These additional subunits are required, however, for the Q-reductase activity.

N-terminal sequences have been obtained from the subunits with 50.7 kDa (α) and 31.7 kDa (γ). These were identical to the N-terminal sequences described previously and matched the N-termini of NqrA and NqrC as deduced from the DNA sequence (Hayashi et al., 1994; Beattie et al., 1994). From the other proteins encoded by the *nqr* gene cluster NqrF (45 274 Da) contains binding motifs for NADH, FAD, and an iron−sulfur cluster (Rich et al., 1995). As the isolated subunit of 45 900 Da (β) was a catalytically active NADH-dehydrogenase (Hayashi & Unemoto, 1984, 1986; see also above), this subunit is defined as NqrF. NqrF and the fourth of the major stained polypeptides on SDS−PAGE (apparent molecular mass 33.3 kDa, designated as subunit a) have blocked N-termini. The a subunit easily dissociates from the complex during purification, leading to the disappearence of the Q-reductase activity (e.g., in fraction B from the Superdex column, Figure 2). It is most likely identical with NqrB, a very hydrophobic protein with a predicted molecular mass of 46 809 Da that due to this hydrophobicity may move unusually fast on SDS−PAGE. We have no evidence yet whether NqrD and NqrE with the predicted molecular masses of 22 602 and 21 540 Da, respectively, are subunits of the NQR. Polypeptides with the expected mobilities were faintly stained on SDS−PAGE, but no N-terminal sequences could be obtained.

Catalytic Properties. Purified NQR-1 was specific for NADH or deamino-NADH but could not oxidize NADPH. Menadione, ferricyanide, or ubiquinone-1 served as electron acceptors for NADH oxidation. NADH oxidation by ubiquinone-1 followed typical saturation kinetics, yielding an apparent K_m for NADH of 29 μM (with 15 μM ubiquinone-1) and an apparent K_m for ubiquinone-1 of 3.3 μM (with 100 μM NADH) and a V_{max} of 26 units/mg of protein. Please note that the rate of NADH oxidation by ubiquinone-1 was about 4 times less than that by menadione that was used for the determination of the NADH-dehydrogenase in Table 1. The pH optimum for the NADH-dehydrogenase or the Q-reductase activity was 8.0. The NADH-dehydrogenase activity was stimulated by various salts with no difference between KCl and NaCl. Maximal activation occurred at a concentration of 0.2 M of either of these salts. Sodium ions were specifically required, however, for the Q-reductase activity. Under the standard assay conditions, the enzyme was half-saturated at 60 mM NaCl. The affinity of the enzyme decreased to an apparent K_m for NaCl of 10 mM in the additional presence of either 0.2 M KCl or 10 mM $MgCl_2$. The NADH-dehydrogenase activity was not affected by adding phospholipids, but the Q-reductase activity increased 2−3-fold by adding 0.25 mg of sonicated L-α-phospatidylcholine from soybean to 1.0 mL of the assay mixture.

The Q-reductase activity was inhibited by HQNO with K_i of 0.4 μM and by $AgNO_3$ with K_i of 0.2 μM. The NADH-dehydrogenase activity was inhibited by Ag^+ ions at similar concentrations whereas HQNO was not inhibiting. Incubation of the enzyme with 1 mM DCCD at pH 6.5 or 8.0 at 25 °C with or without 0.1 M NaCl caused 20% or 80% inhibition of the Q-reductase activity after 10 or 60 min, respectively. The presence of 25 μM rotenone which had no effect on the NADH-dehydrogenase activity reduced the quinone reductase by 25%. Many of the results reported here for our purified enzyme are in accord with those reported previously for other preparations of this enzyme (Hayashi & Unemoto, 1984; Hayashi et al., 1992).

Cofactors of NQR-1. The catalytically active NQR-1 (fraction A from the Superdex 200 column) that contained the four (main) subunits mentioned in the previous chapter exhibited after concentration a yellowish-brown color characteristic of iron−sulfur proteins. Analysis revealed the presence of 8.4 nmol of non-heme iron/mg and 8.0 nmol of acid-labile sulfur/mg. Additionally, 2.0 nmol of FAD/mg was found in the purified NQR-1. Other flavins were not present in the enzyme, and the previously reported FMN content (Hayashi & Unemoto, 1986) could not be confirmed. The preparation also contained ubiquinone-8 (2.0 nmol/mg), which was extracted with petroleum ether and identified by HPLC and difference spectroscopy as described by Kröger (1978) and probably phospholipids (0.7 nmol/mg) as revealed from the organic phosphorous content. Traces of menaquinone were found in the *V. alginolyticus* membranes, but this quinone was not present in the purified enzyme. Please note that ubiquinone-8 and FAD were determined after extraction with organic solvent or dissociation from the protein by trichloroacetic acid treatment. Since both procedures are likely to not quantitatively extract the compounds to be determined, the ubiquinone-8 and FAD contents should be regarded as the minimum values. For determining non-heme iron and acid-labile sulfur more direct methods were applied. The figures obtained in these analyses are therefore more likely to represent the actual amounts within the protein (see also the Discussion Section). In summary, these data could indicate that FAD, iron−sulfur cluster(s), and tightly-bound ubiquinone-8 are electron-carrying cofactors of the Na^+-NQR-1 of *V. alginolyticus.*

Participation of the FAD in the electron transfer has recently been shown spectroscopically by the disappearance of a band around 450 nm that is typical for an oxidized flavin, following NADH addition to the purified enzyme (Pfenninger-Li & Dimroth, 1995). The properties of the enzyme have also been studied by EPR. When an aerobic suspension

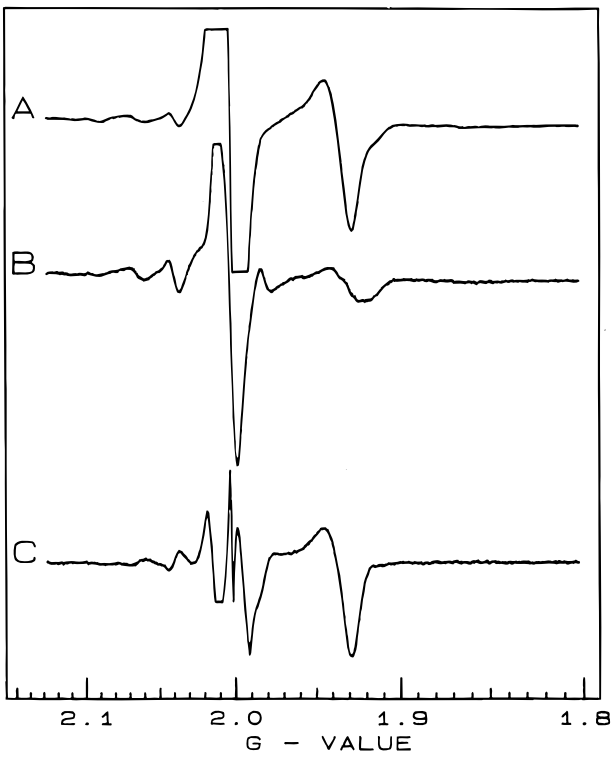

FIGURE 3: EPR spectra of plasma membranes of *V. alginolyticus* reduced with NADH or succinate. (A). Membranes (65 mg/mL) were mixed with NADH (8 mM final concentration) and frozen in liquid nitrogen after 2 min at 0 °C. (B) Membranes were incubated with 40 mM succinate for 3 min at 0 °C and then frozen in liquid nitrogen. The induced signals in the $g = 2.1-1.8$ region were exactly the same as those induced by succinate after 10 min at 30 °C. (C) Difference spectrum A minus B. EPR conditions: microwave frequency, 9422.6 MHz; microwave power incident to the cavity, 2.6 mW; modulation amplitude, 1.27 mT; temperature, 45 K.

of membranes of *V. alginolyticus* was inspected by EPR between 5 and 80 K, the following signals were observed (not shown): (i) an axial $g = 6$ line of an axial high-spin Fe(III) ion (probably high-spin heme); (ii) a signal at $g = 4.3$ due to a rhombic high-spin d^5 system, in proteins usually due to extraneous Fe(III); (iii) lines at $g_z = 2.97$ and $g_y = 2.22$ like those of low-spin heme a; (iv) a small signal due to contaminating Mn(II) and a small radical signal. No signals due to Cu-A could be observed.

In a preparation incubated with 40 mM succinate for 10 min at 30 °C, the signal of the low-spin heme was completely abolished, while that of the high-spin heme decreased by 70%. The radical signal remained essentially unchanged, and a weak signal in the $g = 1.93$ region was induced (Figure 3, trace B). The g values and temperature dependence of the latter signal indicated that it was due to the reduced $[2Fe\text{-}2S]^+$ cluster of succinate dehydrogenase. No signal due to a possible Rieske Fe-S protein could be detected.

When membranes were reduced with 8 mM NADH for 2 min at 0 °C, both the low-spin and high-spin hemes responded as with succinate. There were major changes in the $g = 1.9-2$ region, however (Figure 3, trace A). A large radical signal was induced, and a strong line around $g = 1.94$ appeared. A difference spectrum (Figure 3, trace C) shows the extra reduction by NADH (except for the $g = 2$ region, which produced errors due to overflow). We have also measured the radical signal at 80 K and 2.6 μW. It represented a spin concentration of about 20 μM. The $g = 1.94$ line is the g_{xy} region of a nearly axial signal from a $[2Fe\text{-}2S]^+$ cluster. The optimal temperature for the detection was 45 K. By comparison with a simulation (obtained as described below) the spin concentration was calculated to about 4 μM. No additional signal could be observed at temperatures down to 5 K, so no [4Fe-4S] clusters could be detected. Addition of dithionite did not change this situation.

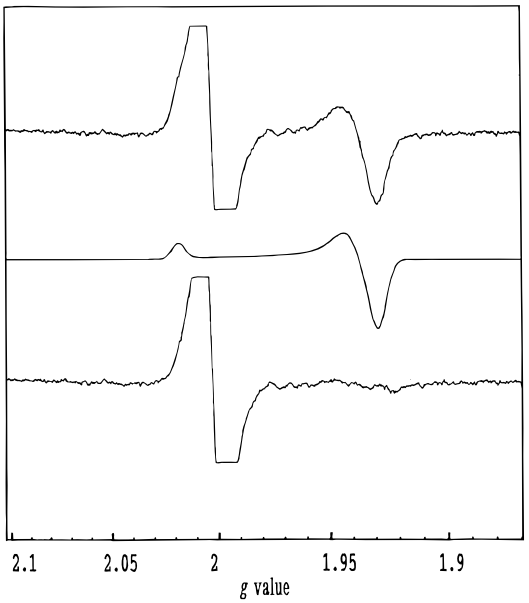

FIGURE 4: EPR spectra of reduced purified NADH:ubiquinone oxidoreductase. In the top trace enzyme obtained after the DEAE-Sephacel column was mixed with succinate and NADH (final concentrations 20 and 8 mM, respectively) and frozen in liquid nitrogen after 2 min at 0 °C. Middle trace: computer simulation with parameters $g_{xyz} = 1.929, 1.937, 2.017$ and widths $(x, y, z) = 1.4, 2.0, 1.2$ mT. Lower trace: difference spectrum upper minus middle trace. EPR conditions: microwave frequency, 9414.3 MHz; microwave power, 1 mW; modulation amplitude, 0.638 mT; temperature, 45 K.

Figure 4 (upper trace) shows a spectrum of enzyme purified up to the DEAE-Sephacel stage, to which succinate and NADH were added. The large radical and the line at $g = 1.94$ are clearly observable. There were no other signals detectable. The middle trace is a simulation [$g_{xyz} = 1.929$, 1.937, 2.017, and widths $(x, y, z) = 1.4, 2.0, 1.2$ mT]. The lower trace is a difference spectrum. The figure shows that the Fe-S cluster is a 2Fe type with slightly rhombic symmetry. Similar spectra were obtained from the enzyme obtained after the Q-Sepharose column. In all samples the spin concentration of the radical (recorded at 100 K) was much larger (6−14 times) than that of the Fe-S cluster (recorded at 45 K). We have also checked the possible effects of addition of NaCl, Q-1, HQNO, superoxide dismutase, or a combination of those, upon the NADH-induced EPR signals. No obvious effects could be detected. Highly purified NADH-dehydrogenase (but essentially devoid of Q-reductase activity, Superdex 200, fraction B) reduced with NADH only showed a very weak radical signal, whereas no $g = 1.94$ line could be detected.

Inactivation of NADH-Dehydrogenase by Incubation with NADH and Catalysis of Superoxide Radical Formation. On incubation of the aerobic enzyme (Q-Sepharose fraction) with NADH in the absence of an electron acceptor such as ubiquinone-1 or menadione, the NADH-dehydrogenase activity was destroyed with a half-inactivation time of about 2 min. The same incubation in the absence of O_2, however,

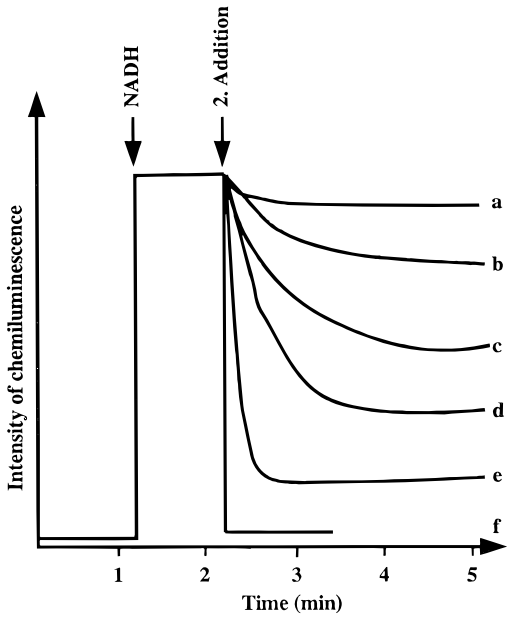

FIGURE 5: Formation of superoxide radicals with the purified NADH:ubiquinone oxidoreductase from *V. alginolyticus* (Q-Sepharose fraction) as determined by chemoluminescence. The assay mixtures contained the enzyme, ubiquinone-1, and luminol. After initiating superoxide radical formation by NADH addition, the effect of the following compounds (2. addition) on the chemoluminescence was followed: (a) 0.1 M NaCl and 2 μM HQNO, (b) 1.0 mM NaCl, (c) 10 mM NaCl, (d) 50 mM NaCl, (e) 0.1 M NaCl and (f) 200 units of superoxide dismutase. No chemoluminescence was observed by omitting either the enzyme or luminol.

did not inactivate the enzyme (data not shown). These results could indicate the formation of superoxide radicals from a radical intermediate in the electron transfer chain (e.g., flavin or quinone radicals) that would then inactivate the enzyme. This interpretation may hold, although superoxide dismutase did not prevent the inactivation. Apparently the superoxide radicals could destroy the enzyme at the site of their formation before they became accessible to the dismutase.

To follow more directly the formation of superoxide radicals, a chemoluminescence assay with luminol as light emitter was used. On the addition of NADH to a mixture of luminol and the purified NQR-1, a chemoluminescence signal was obtained that immediately disappeared after adding superoxide dismutase, indicating that the signal arises from the formation of superoxide radicals (not shown). About 3−4 times higher chemoluminescence signals were obtained in the additional presence of ubiquinone-1. This signal again was immediately abolished by superoxide dismutase (Figure 5). It was stable for about 5 min in the absence of Na^+ ions but, interestingly, decreased with increasing velocity after increasing amounts of Na^+ ions were added (from 1 to 100 mM). The signal declined only slowly with 0.1 M LiCl and was not affected at all by 0.1 M KCl (data not shown). With 0.1 M NaCl and the Q-reductase inhibitor HQNO (2 μM) no significant decrease of the signal was observed. A plausible explanation of these results would be the formation of enzyme-bound ubiquinone-1 radicals by electron transfer from NADH. In the absence of Na^+ ions or in the presence of HQNO these radicals could react with O_2 to yield the superoxide radicals and regenerating the oxidized ubiquinone moiety. In the presence of Na^+ ions, however, the ubiquinone radicals apparently take up a second

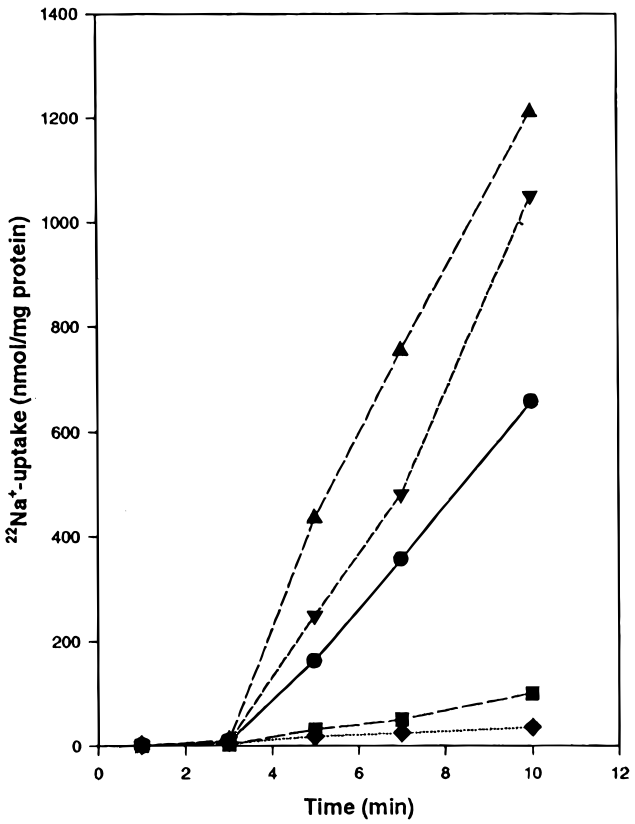

FIGURE 6: Kinetics of $^{22}Na^+$ uptake into reconstituted NADH:ubiquinone oxidoreductase-containing proteoliposomes. Enzyme purified through Q-Sepharose chromatography was applied in all experiments except those symbolized with ■, in which fraction B from the Superdex 200 column was used. Reconstitution of proteoliposomes and determination of $^{22}Na^+$ transport were performed as described under Experimental Procedures. The transport was initiated after 3 min by completing the incubation mixtures with ubiquinone-1 and NADH. Additions and omissions were as follows: (●) no addition; (▲) plus 1.0 μM valinomycin; (▼) plus 5 μM carbonyl cyanide *m*-chlorophenylhydrazone; (◆) plus 20 μM HQNO or without NADH.

electron to yield ubiquinol and thus reduce the formation of superoxide radicals.

Reconstitution of the Na^+ Transport Activity of NADH: Ubiquinone Oxidoreductase. Pilot experiments with different reconstitution procedures revealed that the best results were obtained with the detergent dilution method with octyl glucoside as detergent. Kinetics of $^{22}Na^+$ uptake into proteoliposomes containing the purified NADH:ubiquinone oxidoreductase are shown in Figure 6. Almost no $^{22}Na^+$ was taken up in the absence of NADH and ubiquinone-1, but after these substrates were added the radioactive alkali ions were rapidly accumulated. After the complete inhibition of the quinone reductase activity by incubation of the proteoliposomes for 10 min with 20 μM HQNO, the Na^+ transport was also abolished. Also shown in Figure 6 is the effect of certain ionophores on the transport of $^{22}Na^+$ ions. The addition of the Na^+-carrying ionophore monensin completely abolished $^{22}Na^+$ uptake as expected. Valinomycin (in the presence of K^+) or the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone increased the Na^+ transport rate 2.1- and 1.4-fold, respectively. The probable action of these ion carriers is to dissipate the rate-limiting membrane potential generated by the Na^+ pump. Our results thus indicate an electrogenic Na^+ transport by the NADH:ubiquinone oxidoreductase. Stimulation rather than inhibition of Na^+

transport by the uncoupler further indicates that the transport of Na^+ is a primary event and not involving the intermediate formation of a proton gradient.

The initial rates of Na^+ transport and of quinone reduction by the protoliposomes in the absence of ionophores were 81 and 160 nmol min^{-1} (mg of protein)$^{-1}$, respectively. In the presence of valinomycin, the Na^+ transport rate increased to 170 nmol min^{-1} (mg of protein)$^{-1}$ and that of quinone reduction increased to 310 nmol min^{-1} (mg of protein)$^{-1}$. Thus, the apparent stoichiometry of the pump was about 0.5 Na^+ ions transported per reduction of 1 ubiquinone. This figure should be regarded as a minimum value because reconstituted proteoliposomes are usually less perfectly coupled than the ion pumps operating under in vivo conditions. The maximal rate of quinone reduction by the reconstituted proteoliposomes [310 nmol min^{-1} (mg of protein)$^{-1}$] was rather low. This low rate can be explained by the low Na^+ concentrations that had to be used in these experiments in order to detect $^{22}Na^+$ uptake. At the same Na^+ concentration the soluble Q-reductase has a specific activity of 1 μmol min^{-1} (mg of protein)$^{-1}$. It is expected that this activity decreases upon incorporation of the enzyme into proteoliposomes because enzyme specimens with the NADH binding site oriented to the internal surface are inactive with NADH added from the outside. The Na^+ transport activity was only observed with the enzyme complex retaining the quinone reductase activity but not with preparations which had (almost) lost this activity (e.g., fraction B of Superdex column). In summary, these results demonstrate for the first time that a NADH:ubiquinone oxidoreductase complex isolated from *V. alginolyticus* membranes acts as a primary Na^+ pump.

DISCUSSION

We show here that the NADH:ubiquinone oxidoreductase consists of more than the three subunits regarded previously to present the physiological form of the complex (Hayashi & Unemoto, 1987). In Table 2 the polypeptides predicted from the recently determined DNA sequence (Beattie et al., 1994; Hayashi et al., 1995) were compared with those present in the purified NQR-1. The three hydrophilic polypeptides of the complex (α, β, γ) correlate with NqrA, NqrF, and NqrC according to the N-terminal sequences (α, γ), and the presence of NADH and FAD binding motifs (β), respectively (see Results). The mobility of these polypeptides on SDS−PAGE was as expected from the molecular masses calculated from the DNA sequence. Subunit a which has a blocked N-terminus was readily lost during chromatographic procedures and simultaneously the Q-reductase activity was abolished. This protein is therefore clearly a subunit with an important function in the complex. It is probably identical to NqrB, a very hydrophobic protein of 46 809 Da. The greater mobility of this subunit on SDS−PAGE than expected from its molecular mass probably results from its binding of more SDS than usual; this is a common property of hydrophobic polypeptides (Pos et al., 1995; Di Berardino & Dimroth, 1995).

Previous biochemical studies had indicated that the isolated three subunit enzyme contained FMN in the α subunit and FAD in the β subunit and that these were the only redox cofactors. A possible mechanism of ion-coupling was proposed on this basis (Unemoto & Hayashi, 1989). However, the results presented here and elsewhere show that FMN is not a cofactor of the enzyme (Pfenninger-Li & Dimroth, 1995) and that FAD and an iron−sulfur center are redox cofactors. If we assume that one copy of each of the six subunits predicted from the DNA sequence is present in the enzyme complex, then its molecular mass (without the cofactors) would be 212 550 Da (Table 2). Hence, 1 mg of pure protein would correspond to 4.7 nmol. We found 8.4 nmol of non-heme iron and 8.0 nmol of acid-labile sulfur per mg of our purified NQR-1. These figures are in accordance with the presence of one [2Fe-2S] cluster per enzyme molecule. From EPR we observed a spin concentration from the $[2Fe\text{-}2S]^+$ cluster in the membrane (65 mg of protein/mL) of 4 μM (see Results). This is equivalent to 0.06 nmol/mg of membrane protein and after 76-fold purification of NQR-1 from the membrane vesicles to the Superdex 200 (A) fraction (Table 1) would correspond to 4.7 nmol/mg of protein. This figure again is in accord with the presence of one [2Fe-2S] cluster per enzyme molecule. The apparent ubiquinone-8 and FAD content of purified NQR-1 was 2 nmol per mg of protein each, which corresponds to only about 0.5 mol per mol of enzyme. The lower than expected amounts of these compounds analyzed are probably due to losses during their isolation from the protein.

FAD is the likely acceptor of the two electrons from NADH as in other NADH oxidizing flavoproteins. Redox titrations which revealed a single midpoint potential of a flavin prosthetic group of −295 mV are in accord with this supposition (Bourne & Rich, 1992). The reduced $FADH_2$ may then reduce the [2Fe-2S] cluster by a one-electron transfer, producing the flavin radical as the second product. After reoxidation of the iron−sulfur cluster, electron transfer from the flavin radical would reduce it again and would regenerate the oxidized flavin ready to take over two electrons from NADH again. It is interesting that Na^+ ions had no effect on the midpoint potential of the flavin. The first two anticipated reduction steps of the electron transfer chain in NQR-1 are therefore probably not directly involved in the transport of Na^+ ions. This sequence of reduction steps is in accord with the location of the binding sites for NADH, FAD, and the [2Fe-2S] cluster on the same (β) subunit.

It is interesting that the purified NQR-1 contained tightly-bound ubiquinone-8. As ubiquinone-8 was also the only ubiquinone species detectable in *V. alginolyticus* membranes, previous reports on the participation of ubiquinone-10 in the respiratory chain of *V. alginolyticus* could not be confirmed (Unemoto & Hayashi, 1979). We propose that the tightly-bound quinone (Q_A) takes part in the electron transfer chain and that it receives the electron from the iron−sulfur center and delivers it to a second quinone (Q_B), which is ubiquinone-1 in our in vitro system and ubiquinone-8 under physiological conditions in the membrane. The difference between the two quinones is that Q_A switches between the oxidized and semiquinone states whereas Q_B can be oxidized, half-reduced, or fully reduced. In addition, Q_B should be less tightly-bound because after reduction to the quinol state it has to dissociate from the enzyme and deliver the electrons to the quinol oxidase. The reduction of a mobile quinone (Q_B) to quinol by two one-electron steps via a firmly-bound quinone (Q_A) switching between oxidized and semiquinone forms would be analogous to the photosynthetic reaction center.

While the involvement of two quinones in NQR-1 is by no means proven, it assists in the explanation of pertinent observations. Hayashi and Unemoto (1984) have shown previously that the reduction of ubiquinone to ubiquinol proceeds by two one-electron transfer steps and that Na^+ ions are indispensable for ubiquinol formation. In the absence of Na^+ ions, superoxide radicals are formed apparently by one-electron transfer from the semiquinone state to oxygen which in the overall reaction leads to an oxidation of NADH by oxygen and not by the quinone. We used here a luminescence method to detect superoxide radical formation with which the previous results were confirmed. An important extension of these findings was the observation that superoxide radical formation was dependent on the absence of Na^+ ions or on the presence of the inhibitor HQNO. These results therefore indicate that the Na^+-dependent and HQNO-sensitive step in the electron transfer by NQR-1 is the one-electron reduction of ubiquinone-1 from the semiquinone to the quinol state. It is interesting that superoxide radical formation was not completely dissipated if ubiquinone-1 was omitted from the incubations with NQR-1 and NADH. These radicals could therefore derive from the semiquinone state of the tightly-bound ubiquinone-8 (Q_A).

The EPR experiments indicate that NADH:ubiquinone oxidoreductase is the major redox enzyme in the respiratory chain of *V. alginolyticus*. The amount of succinate dehydrogenase is estimated to be about 5-fold less. From EPR there is no evidence for a Rieske Fe-S protein or a Cu-A species. This might mean that the low-spin heme a-type EPR signal is due to a quinol oxidase. The NADH-reduced purified enzyme showed only EPR signals of a [2Fe-2S] cluster and a radical, presumably due to ubisemiquinone. Always the radical concentration was much greater than that of the Fe-S cluster (by a factor 5 in the membranes and 6−14 in the purified enzyme). This excludes FAD as the source. Hence we conclude that it is due to the semiquinone form of Q-8 (Q_A) stabilized by binding to the enzyme. The redox potential of the Fe-S cluster has to be much lower than that of the fumarate/succinate couple, since excess succinate could not reduce it in membrane preparations. Purification of the enzyme to the Superdex 200 (fraction B) stage resulted in a virtually complete loss of the NADH-induced EPR signals of the radicals and the [2Fe-2S] cluster. This indicates that the NqrB protein is essential for the stabilization of these two species.

Although NQR-1 of *V. alginolyticus* has been described more than ten years ago to function as a primary Na^+ pump, direct proof for this supposition has not been provided. A very important result of this paper, therefore, is the direct demonstration of Na^+ pumping with reconstituted proteoliposomes containing purified NQR-1. A direct coupling mechanism for the Na^+ transport could be envisaged in which Q_B bound to the integral membrane sector of the protein by its reduction to the semiquinone state would acquire a Na^+ specific site. This site would be occupied by Na^+ ions from an appropriate access channel compensating the unfavorable negative charge on the semiquinone in a medium of low dielectric (Rich et al., 1995). Only a semiquinone that has been electrically "neutralized" by Na^+ binding may be able to take over a second electron in the low-dielectric environment. This reduction to the quinol state is associated with a dramatic increase of the p*K* values of its two OH-groups. This promotes the protonation of the fully reduced quinol from the appropriate membrane side pushing the bound Na^+ ions into the release channel from which they are delivered to the other side of the membrane.

In summary, the results of this report present unequivocal proof that NQR-1 from *V. alginolyticus* functions as a primary Na^+ pump. The enzyme consists of three different hydrophilic and probably three different hydrophobic subunits and has a FAD and a [2Fe-2S] cluster as prosthetic groups. Electron transfer to ubiquinone-1 involves the semiquinone radical as an intermediate which may be (part) of the Na^+ binding site. The further reduction to ubiquinol is Na^+-dependent and may therefore be directly coupled to Na^+ ion pumping. The Na^+-translocating NQR-1 of *V. alginolyticus* provides a suitable model system to study the catalytic mechanism of NADH:ubiquinone oxidoreductase and its coupling to ion translocation. The Na^+-NQR-1 seems to be simpler than any of its H^+ translocating counterparts (six instead of 14 to >40 subunits) and is apparently easier to handle because it can be purified in a catalytically competent state allowing the reconstitution into proteoliposomes with retention of the Na^+ transport activity. To our knowledge, this has not been possible for any of the H^+-translocating NQR-1 enzymes despite considerable efforts.

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REFERENCES

Aasa, R., & Vänngård, T. (1975) *J. Magn. Reson. 19*, 308−315.
Albracht, S. P. J. (1984) in *Current Topics in Bioenergetics* (Lee, C. P., Ed.) Vol. 13, pp 79−106 Academic Press, New York.
Albracht, S. P. J., Leeuwerik, F. J., & van Swol, B. (1979) *FEBS Lett. 104*, 197−200.
Beattie, P., Tan, K., Bourne, R. M., Leach, D., Rich, P. R., & Ward, F. B. (1994) *FEBS Lett. 356*, 333−338.
Beinert, H., & Albracht, S. P. J. (1982) *Biochim. Biophys. Acta 683*, 245−277.
Boure, R. M., & Rich, P. (1992) *Biochem. Soc. Trans. 20*, 577−582.
Cline, J. D. (1969) *Limnol. Oceanogr. 14*, 454−458.
De Jong, A. M. P., Kotlyar, A. B., & Albracht, S. P. J. (1994) *Biochim. Biophys. Acta 1186*, 163−171.
Di Berardino, M., & Dimroth, P. (1995) *Eur. J. Biochem. 231*, 790−801.
Dimroth, P., & Thomer, A. (1989) *Arch. Microbiol. 151*, 439−444.
Fish, W. W. (1988) *Methods Enzymol. 158*, 357−364.
Hayashi, M., & Unemoto, T. (1984) *Biochim. Biophys. Acta 767*, 470−478.
Hayashi, M., & Unemoto, T. (1986) *FEBS Lett. 202*, 327−330.
Hayashi, M., & Unemoto, T. (1987) *Biochim. Biophys. Acta 890*, 47−54.
Hayashi, M., Hirai, K., & Unemoto, T. (1994) *FEBS Lett. 356*, 330−332.
Hayashi, M., Hirai, K., & Unemoto, T. (1995) *FEBS Lett. 363*, 75−77.
Hayashi, M., Miyoshi, T., Sato, M., & Unemoto, T. (1992) *Biochim. Biophys. Acta 1099*, 145−151.
Kröger, A. (1978) *Methods Enzymol. 53*, 579−591.
Leif, H., Sled, V. D., Ohnishi, T., Weiss, H., & Friedrich, T. (1995) *Eur. J. Biochem. 230*, 538−548.
Michelson, A. M. (1978) *Methods Enzymol. 57*, 385−406.
Pfenninger-Li, X. D., & Dimroth, P. (1992) *Mol. Microl. 6*, 1943−1948.
Pfenninger-Li, X. D., & Dimroth, P. (1995) *FEBS Lett. 369*, 173−176.
Pos, K. M., Bott, M., & Dimroth, P. (1994) *FEBS Lett. 347*, 37−41.

Redfearn, E. R. (1967) *Methods Enzymol. 10*, 381−384.
Rich, P., Meunier, B., & Ward, F. B. (1995) *FEBS Lett. 375*, 5−10.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 386−379.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.
Takada, M., Ikenoya, S., Yazuriha, T., & Katayama, K. (1984) *Methods Enzymol. 105*, 147−155.
Tokuda, H., & Kogure, K. (1989) *J. Gen. Microbiol. 135*, 703−709.
Tokuda, H., & Unemoto, T. (1981) *Biochem. Biophys. Res. Commun. 102*, 265−271.
Tokuda, H., & Unemoto, T. (1983) *J. Bacteriol. 156*, 636−643.
Unemoto, T., & Hayashi, M. (1979) *J. Biochem. 85*, 1461−1467.
Unemoto, T., & Hayashi, M. (1989) *J. Bioenerg. Biomem. 21*, 649−662.
Walker, J. E. (1992) *Q. Rev. Biophys. 25*, 253−324.
Weidner, U., Geier, S., Ptock, A., Friedrich, T., Leif, H., & Weiss, H. (1993) *J. Mol. Biol. 233*, 109−122.
Yagi, T. (1986) *Arch. Biochem. Biophys. 250*, 302−311.
Yagi, T. (1991) *J. Bioenerg. Biomembr. 23*, 211−225.
Yagi, T. (1993) *Biochim. Biophys. Acta 1141*, 1−17.
Yagi, T., Hon-Nami, K., & Ohnishi, T. (1988) *Biochemistry 27*, 2008−2013.
Yagi, T., Xu, X., & Matsuno-Yagi, A. (1991) *Biol. Chem. Hoppe-Seyler 372*, 555.

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