Sodium-Dependent Steps in the Redox Reactions of the Na^+-Motive NADH:Quinone Oxidoreductase from *Vibrio harveyi*†

Alexander V. Bogachev,‡ Yulia V. Bertsova,‡ Blanca Barquera,§ and Michael I. Verkhovsky*,||

Department of Bioenergetics, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia, Helsinki Bioenergetics Group, Department of Medical Chemistry, Institute of Biomedical Sciences and Biocentrum Helsinki, University of Helsinki, P.O. Box 8, FIN-00014 Helsinki, Finland, and Department of Biochemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

Received November 6, 2000

ABSTRACT: The Na^+-translocating NADH:ubiquinone oxidoreductase (Na^+-NQR) from *Vibrio harveyi* was purified and studied by EPR and visible spectroscopy. Two EPR signals in the NADH-reduced enzyme were detected: one, a radical signal, and the other a line around $g = 1.94$, which is typical for a [2Fe-2S] cluster. An E_m of −267 mV was found for the Fe−S cluster ($n = 1$), independent of sodium concentration. The spin concentration of the radical in the enzyme was approximately the same under a variety of redox conditions. The time course of Na^+-NQR reduction by NADH indicated the presence of at least two different flavin species. Reduction of the first species (most likely, a FAD near the NADH dehydrogenase site) was very rapid in both the presence and absence of sodium. Reduction of the second flavin species (presumably, covalently bound FMN) was slower and strongly dependent on sodium concentration, with an apparent activation constant for Na^+ of ∼3.4 mM. This is very similar to the K_m for Na^+ in the steady-state quinone reductase reaction catalyzed by this enzyme. These data led us to conclude that the sodium-dependent step within the Na^+-NQR is located between the noncovalently bound FAD and the covalently bound FMN.

Tokuda and Unemoto found that the NADH:quinone oxidoreductase in the marine bacterium *Vibrio alginolyticus* is a primary Na^+ pump capable of generating a transmembrane difference in Na^+ electrochemical potential ($\Delta\mu_{Na^+}$)[1] (*1*, *2*) This $\Delta\mu_{Na^+}$ is utilized to support osmotic work, i.e., uphill transport of solutes (*3*), rotation of flagella (*4*), and possibly ATP synthesis (*5*).

The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) of *V. alginolyticus* was purified and characterized (*6*) and was shown to consist of six subunits (*7*). The operon encoding this enzyme was cloned, sequenced, and found to be composed of six structural genes that correspond to the six subunits present in the Na^+-NQR complex (*7*−*9*). Three of the subunits (NqrA, NqrC, and NqrF) are relatively hydrophilic, while the other three subunits (NqrB, NqrD, and NqrE) are very hydrophobic (*7*). NqrF contains binding motifs for NADH, FAD, and an iron−sulfur center, and is therefore believed to be involved in the NADH dehydrogenase activity (*8*, *10*). Little is known about the function of the other subunits. Sequence alignments show no substantial homologies to any other proteins with related function. It has been demonstrated that the Na^+/e^- stoichiometry for Na^+-NQR is 1 (*11*), but the mechanism of sodium ion translocation by this enzyme remains unknown.

Studies on the purified Na^+-NQR from *V. alginolyticus* have found that it contains FAD and an Fe−S center, and tightly bound ubiquinone-8 in a ratio of approximately 1/1 with FAD (*12*). *Vibrio harveyi* has a closely homologous Na^+-NQR which also functions as an electrogenic sodium pump (*13*). Using this enzyme, it has been shown that the NqrC subunit contains a covalently bound flavin, which appears to be FMN (*13*). Since these various Na^+-NQRs are genetically similar, and thus may be expected to have the same cofactor composition, it can be concluded that the enzyme contains at least the following prosthetic groups: a covalently bound FMN, a noncovalently bound FAD, an [2Fe-2S] cluster, and a tightly bound ubiquinone.

Recently, it has been reported that both the NqrC and NqrB subunits of the Na^+-NQR from *V. alginolyticus* contain covalently bound flavins, tentatively identified as FMN (*14*). The data indicate that these flavins are attached to Thr-223 in NqrB and Thr-235 in NqrC. However, the cofactor composition of Na^+-NQR remains controversial. Further investigation is required to verify the organization of the

† This work was supported by grants from The Russian Foundation for Basic Research (99-04-49161 and 01-04-48147), The Academy of Finland, The Sigrid Jusèlius Foundation, The University of Helsinki, Biocentrum Helsinki, and the National Institutes of Health (HL 16101) to R. B. Gennis. A.V.B. is indebted to the Federation of European Biochemical Societies for the Short Fellowship, which supported his visit to the Helsinki Bioenergetics Group.

* To whom correspondence should be addressed. Telephone: +358 9 191 8248. Fax: +358 9 191 8296. E-mail: michael.verkhovsky@helsinki.fi.

‡ Moscow State University.

§ University of Illinois at Urbana-Champaign.

|| University of Helsinki.

[1] Abbreviations: DM, *n*-dodecyl β-D-maltoside; E_m, midpoint potential (in millivolts); LDAO, lauryldimethylamine oxide; Na^+-NQR, Na^+-translocating NADH:ubiquinone oxidoreductase; SBP, sub-bacterial particles; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; $\Delta\mu_{Na^+}$, transmembrane Na^+ electrochemical potential difference.

10.1021/bi002545b CCC: $20.00
Published on Web 05/22/2001

redox center and its role in electron transfer and sodium translocation.

In this work, EPR and visible spectroscopy have been used to study the Na^+-NQR from *V. harveyi*. It was found that when NADH reduces the enzyme, the rate of reduction of one of the flavins is strongly dependent on sodium concentration. The data allow us to conclude that the sodium-dependent step within the Na^+-NQR is located between the noncovalently bound FAD and the covalently bound FMN.

MATERIALS AND METHODS

Purification of Na^+-NQR. V. harveyi cell growth and subbacterial particle (SBP) preparation were carried out as described previously (*13*). Membranes were solubilized with 1% LDAO as described previously (*12*). The extract was applied to a Fractogel TSK DEAE-650(S) column (26 mm × 60 mm) equilibrated with buffer 1 [100 mM KCl, 0.05 mM EDTA, 5% glycerol, 10 mM Tris-HCl, and 0.1% LDAO (pH 7.5)], and the column was washed with buffer 1 containing 150 mM KCl. The bound Na^+-NQR was then eluted with a KCl gradient, linearly increasing from 150 to 260 mM. Fractions containing Na^+-NQR activity were diluted 1.5 times with buffer 2 [10 mM Tris-HCl, 0.05 mM EDTA, 5% glycerol, and 0.05% DM (pH 7.5)] and applied to a DEAE Sepharose CL-6B column (16 mm × 80 mm) equilibrated with buffer 2 supplemented with 100 mM KCl. The column was washed with 3 bed volumes of the latter buffer, and the Na^+-NQR was eluted with buffer 2 supplemented with 450 mM KCl. The purified enzyme was concentrated and used for EPR and optical spectroscopy experiments or further purified by centrifugation on a sucrose density gradient. For this, the Na^+-NQR was loaded onto 12 mL centrifuge tubes with an 8 to 18% (w/v) gradient of sucrose in buffer 2 containing 100 mM KCl. After centrifugation for 16 h at 100000*g*, the NADH dehydrogenase and Q_1 reductase activities were found in a yellow band in the middle of the gradient. The enriched fraction with Na^+-NQR was also used for EPR and visible spectroscopy experiments

EPR Spectroscopy. *V. harveyi* Na^+-NQR was studied with EPR spectroscopy on a Bruker ESP-300 X-band EPR spectrometer. The field modulation frequency was 100 kHz. The temperature of the sample was controlled with an ESR 900 liquid helium cryostat with an ITC4 temperature controller (Oxford Instruments). The frequency was calibrated with an HP X532B microwave frequency meter. All EPR data were corrected by subtracting the blank (EPR signal of tube with buffer). The radical per Fe−S cluster ratio was determined by dividing the values from direct double integration of the corresponding lines in the dithionite-reduced Na^+-NQR spectrum obtained with a microwave power of 100 μW where the signals of both cofactors are not saturated.

Redox Titration of the Fe−S Cluster in V. harveyi Na^+-NQR. Samples (3.1 mg/mL) in 50 mM Hepes/Tris (pH 7.5), 200 mM NaCl or KCl, and 0.05% DM were titrated under strictly anaerobic conditions by varying the NADH/NAD^+ ratio. The protein was mixed with NAD^+ at concentrations ranging from 0 to 5 mM and NADH (from 0 to 10 mM), and after incubation for 1 min on ice, the samples were frozen and used for EPR measurements. The protein was also titrated by varying the lactate/pyruvate ratio (from 1 to 10 mM pyruvate and from 1 to 16 mM lactate) in the presence of NAD^+ (5 mM) and lactate dehydrogenase (1 unit). In all the experiments, only Tris salts of NAD^+ and NADH were used.

Stopped-Flow Kinetic Measurements. Na^+-NQR (1−3 mg/mL) in buffer A [100 mM KCl, 10 mM HEPES/Tris (pH 7.5), and 0.05% DM] was rapidly mixed with buffer A containing 40 μM NADH and various concentrations of NaCl (from 0 to 120 mM). Rapid mixing experiments were carried out using a stopped-flow spectrophotometer equipped with a diode array detector capable of recording spectra at a rate of up to one per millisecond (Unisoku Instruments). The experiments were carried out at 3.5 or 25 °C. In the absence of added NaCl, the background sodium concentration in the mixture was 25 μM.

Data Analysis. Basic data matrix manipulations and analysis were carried out with MATLAB (The Mathworks, South Natick, MA). Decomposition of data surfaces was achieved using SPLMOD (*15*), a global multiexponential fitting program, run under a MATLAB interface (*16*).

Na^+-NQR NADH dehydrogenase and Q_1 reductase activities were measured at 30 °C in buffer containing 10 mM Tris-HCl, 100 mM KCl, 40 mM NaCl, and 0.05% DM (pH 7.5) as described previously (*13*). SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (*17*) at a gel concentration of 15%. Protein concentrations were determined by means of the bicinchoninic acid method using bovine serum albumin as a standard. Sodium concentrations were measured by flame photometry.

RESULTS

Purification of the Sodium-Motive NADH:Quinone Oxidoreductase from V. harveyi. Na^+-NQR was purified using one of two different detergents, LDAO or DM. The LDAO preparation was found to oxidize NADH even in the absence of oxygen. Replacing LDAO in the purification procedure with DM eliminated this NADH oxidation, which suggests that the LDAO, or impurities in the detergent, can accept electrons from Na^+-NQR.

Replacement of LDAO with DM also resulted in a more stable preparation, which allowed us to use a sucrose density gradient centrifugation step to further purify the enzyme. In this study, two types of enzyme preparation were used: (1) a partially purified preparation consisting of the active fractions collected from the DEAE-Sepahrose column (specific NADH dehydrogenase activity of 50−60 μmol min^{-1} mg^{-1}; Q_1 reductase activity of 19−23 μmol min^{-1} mg^{-1}) and (2) a more highly purified preparation obtained after the sucrose density gradient (NADH dehydrogenase activity of 100−110 μmol min^{-1} mg^{-1}; Q_1 reductase activity of 38−42 μmol min^{-1} mg^{-1}). The results of SDS−PAGE of the partially and highly purified Na^+-NQR preparations are shown in Figure 1.

EPR Characterization of the Purified Na^+-NQR. Both partially purified and highly purified Na^+-NQR preparations were studied by EPR spectroscopy. In agreement with ref *12*, two different EPR signals were detected in the NADH-reduced Na^+-NQR at 45 K: a strong line at $g = 2.00$ and another line around $g = 1.94$, the latter most likely originating from a [2Fe-2S] cluster (Figure 2b). Scanning at

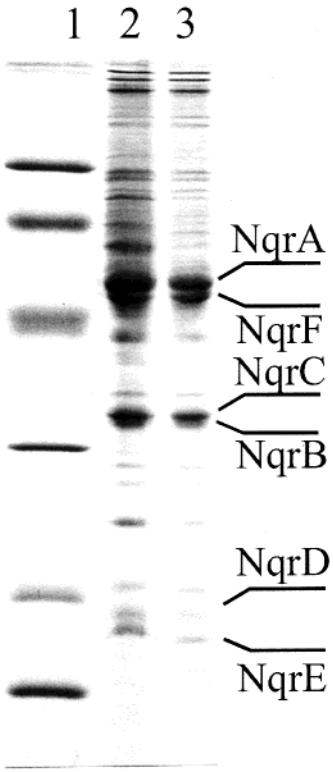

FIGURE 1: Coomassie-stained SDS−PAGE of partially purified (lane 2) and highly purified (lane 3) Na^+-NQR preparations. Lane 1 contained protein standards with molecular masses of 94.6, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa. The labels indicate the preferable localization of the Na^+-NQR subunits according to ref *7*.

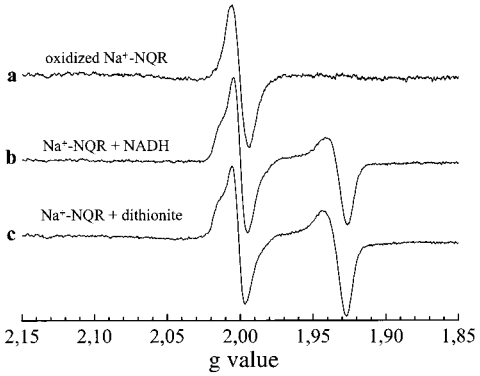

FIGURE 2: EPR spectra show the two signals in Na^+-NQR from *V. harveyi*: (a) air-oxidized, (b) NADH-reduced, and (c) dithionite-reduced. Sample conditions were as follows: 50 mM Hepes/Tris (pH 7.5), 200 mM NaCl, and 0.05% DM with a protein concentration of 3.1 mg/mL, (b) 5 mM NADH, and (c) 5 mM dithionite. EPR conditions were as follows: microwave frequency, 9.421 GHz; microwave power, 2 mW; temperature, 45 K; and modulation amplitude, 0.789 mT.

temperatures as low as 5 K revealed no additional signals. It is thus unlikely that there are [4Fe-4S] clusters in the Na^+-NQR preparation. Addition of dithionite also revealed no new signals.

To determine the redox properties of these two signals, the enzyme was titrated under strictly anaerobic conditions by varying the NADH/NAD^+ or lactate/pyruvate ratios (the latter in the presence of NAD^+ and lactate dehydrogenase). A Nernstnian fit to the data (Figure 3) gives an E_m value of −267 mV ($n = 1$) for the [2Fe-2S] cluster, which was independent of sodium concentration (data not shown).

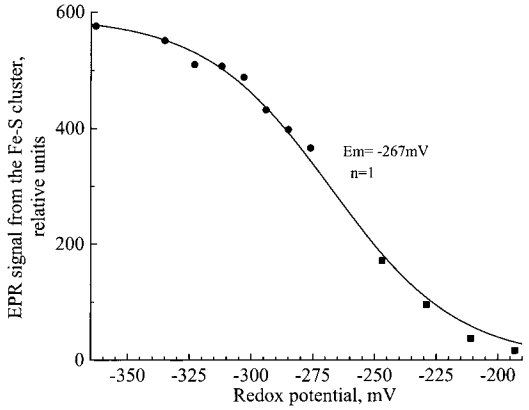

FIGURE 3: Redox titration of the Fe−S cluster of *V. harveyi* Na^+-NQR. Protein (3.1 mg/mL) was titrated under strictly anaerobic conditions by varying the NADH/NAD^+ ratio (●) or the lactate/pyruvate ratio, in the presence of NAD^+ and lactate dehydrogenase (■). For details, see Materials and Methods. The solid line represents the theoretical curve for an $n = 1$ component with an E_m of −267 mV.

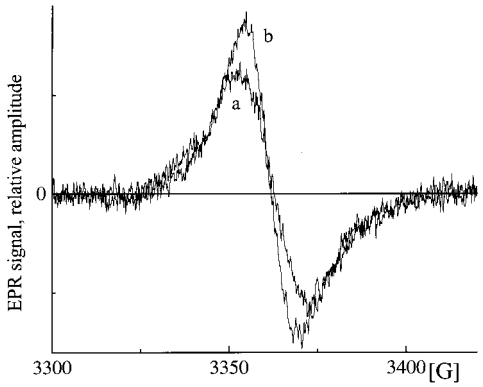

FIGURE 4: EPR spectra of the radical signal in (a) air-oxidized and (b) dithionite-reduced Na^+-NQR of *V. harveyi*. Sample conditions were the same as those described in the legend of Figure 2. EPR conditions were as follows: microwave frequency, 9.424 GHz; microwave power, 5 μW; temperature, 100 K; and modulation amplitude, 0.789 mT.

Addition of dithionite caused an increase in the amplitude of the [2Fe-2S] signal beyond what NADH could produce (Figure 2c). This could be due to the existence of a pool of partially denatured protein that cannot be reduced by the natural substrate. Alternatively, the Fe−S cluster might interact with a nearby redox center, giving rise to two waves during the redox titration. An interaction of this kind is well-documented in cytochrome *c* oxidase (*18*).

The EPR signal at $g = 2.00$ was also studied. The *g* value, saturation properties, and temperature dependence are all in agreement with ref *12*, and indicate that this signal arises from a radical. However, in contrast with the previous report, we found that this signal was present under all the redox conditions that were tested; the radical signal was present in the air-oxidized enzyme (Figure 2a), in the dithionite-reduced enzyme (Figure 2c), and when Na^+-NQR was incubated with either ferricyanide or hexaammine ruthenium-(III). The spin concentration of the radical in oxidized Na^+-NQR was found to be the same as in the dithionite-reduced enzyme (Figure 4). Small redox-dependent variations in line shape and saturation properties were, however, observed. The peak of the radical signal is sharper in the reduced Na^+-NQR than in the oxidized enzyme (line widths of 1.5 and

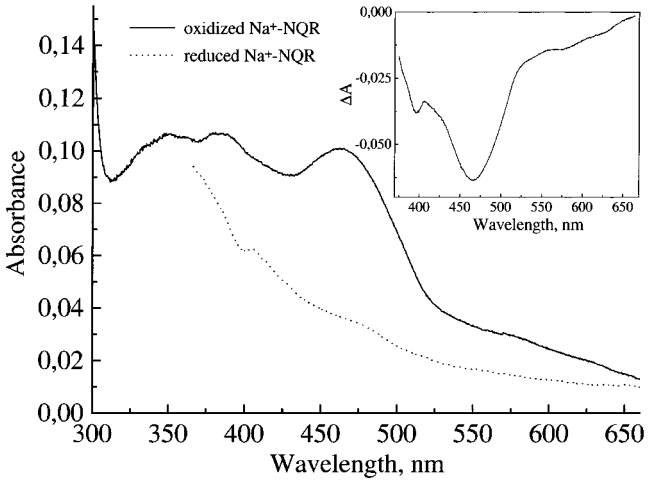

FIGURE 5: Absorption spectra of oxidized and reduced Na^+-NQR: (—) air-oxidized and (•••) dithionite-reduced. The inset shows the difference spectrum (reduced *minus* oxidized). Sample conditions were as follows: 50 mM Hepes/Tris (pH 7.5), 200 mM NaCl, 0.05% DM, and 1 mM dithionite (in the dotted spectrum), with a purified protein concentration of 0.34 mg/mL.

2.1 mT, respectively; see Figure 4), and in the reduced enzyme, the radical signal saturates at a slightly lower power ($P_{1/2}$ values of the radical signal in the reduced and oxidized enzyme were 0.13 and 0.36 mW, respectively). The radical could be reduced by dithionite only after Na^+-NQR denaturation by treatment with 3 M guanidine hydrochloride. Such behavior may indicate that the radical is inaccessible to redox agents. In all of our Na^+-NQR preparations that were tested, the radical was present under reduced or oxidized conditions and in an ~1/1 ratio with the amount of Fe−S center in the dithionite-reduced samples. The same results were obtained using *V. harveyi* sub-bacterial particles; the radical signal was present in air-oxidized SBP as well as in the NADH- or dithionite-reduced SBP.

Studies of the Kinetics of Na^+-NQR Reduction by Optical Spectroscopy. The optical spectra of oxidized and dithionite-reduced Na^+-NQR are shown in Figure 5. The oxidized *minus* reduced difference spectrum exhibits two peaks at ~464 and ~395 nm (spectral features characteristic of protein-bound flavins, an assignment consistent with the presence of flavins in Na^+-NQR) and several shoulders that are likely to belong to the Fe−S cluster. The same spectral changes could also be obtained using NADH as the reductant. The kinetics of the reduction process was studied by mixing Na^+-NQR with NADH in a stopped-flow apparatus under anaerobic and aerobic conditions. Spectra were recorded at intervals of 1 ms. The dependence of this process on sodium concentration was studied by including various amounts of sodium in the NADH solution prior to mixing. In the presence of sodium, reduction of the enzyme was very rapid and almost monophasic, whereas in the absence of added sodium (the background sodium concentration was ~25 μM), it was much slower and three distinct phases could be resolved. The spectra of these kinetic phases (Figure 6) were obtained using a global multiexponential fit to the absorbance (as a function of wavelength and time) data surface (see Materials and Methods). The spectrum of the slowest process (phase 3) had troughs at 460−465 and 395 nm, indicative of flavin reduction, and did not show any significant absorbance change above 550 nm. Spectral changes characteristic of flavin reduction were also observed with very similar kinetics in the 250−290 nm region (data not shown).

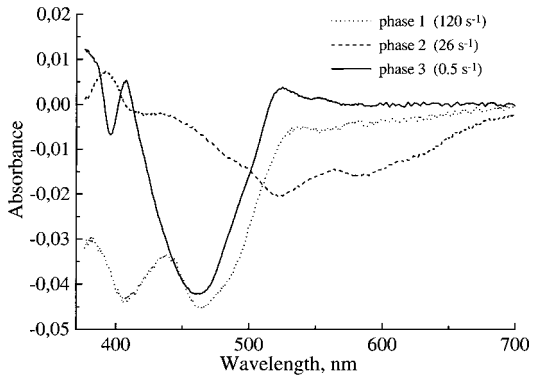

FIGURE 6: Spectra of kinetic phases during reduction of Na^+-NQR: (•••) 120, (- - -) 26, and (—) 0.5 s^{-1}. Stopped-flow mixing conditions were as follows: 1.7 mg/mL protein, in buffer A [100 mM KCl, 10 mM HEPES/Tris (pH 7.5), and 0.05% DM] in syringe I and 40 μM NADH in buffer A in syringe II. After mixing, spectra were recorded once every millisecond, at 3.5 °C. Spectra of phases were obtained by a global multiexponential fit (see Materials and Methods).

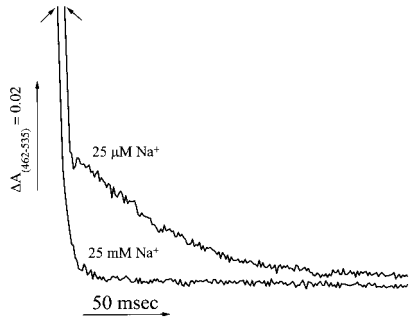

FIGURE 7: Kinetic time course at 462−535 nm showing the reduction of flavin at high and low sodium concentrations during the reaction of Na^+-NQR with NADH. Individual wavelength traces were obtained from measurements of the type described in the legend of Figure 6. Conditions were like those described in the legend of Figure 6, except the protein concentration was 1.3 mg/mL and the temperature was 25 °C. For the high-sodium trail, the NADH solution in syringe II was supplemented with 50 mM NaCl to obtain a final concentration of 25 mM; the background concentration of Na^+ was 25 μM. Arrows indicate the time of mixing.

The spectrum of the fastest process (phase 1) also exhibited a main trough at 460−465 nm, indicative of flavin. The spectrum of this phase had an additional trough at 410 nm (see Figure 6); this suggests the presence of a heretofore-unidentified cofactor in the enzyme. Unfortunately, the phase is very fast, and it is hard to resolve even at 3.5 °C. It remains unclear whether phase 1 consists of two separate redox events with slightly different rate constants, or the concerted reduction of two sites.

The spectrum of the middle phase exhibits a broad trough at ~400−500 nm with several shoulders in the region of 550−650 nm.

Absorbance changes at 462 nm *minus* 535 nm show the reduction of flavins without possible interference from the Fe−S cluster. Figure 7 shows the time course of the reduction of Na^+-NQR by NADH at two different sodium concentrations at this wavelength pair. The traces are derived from full-spectrum surface data like those given above. The reduction of flavin clearly takes place in two phases with a

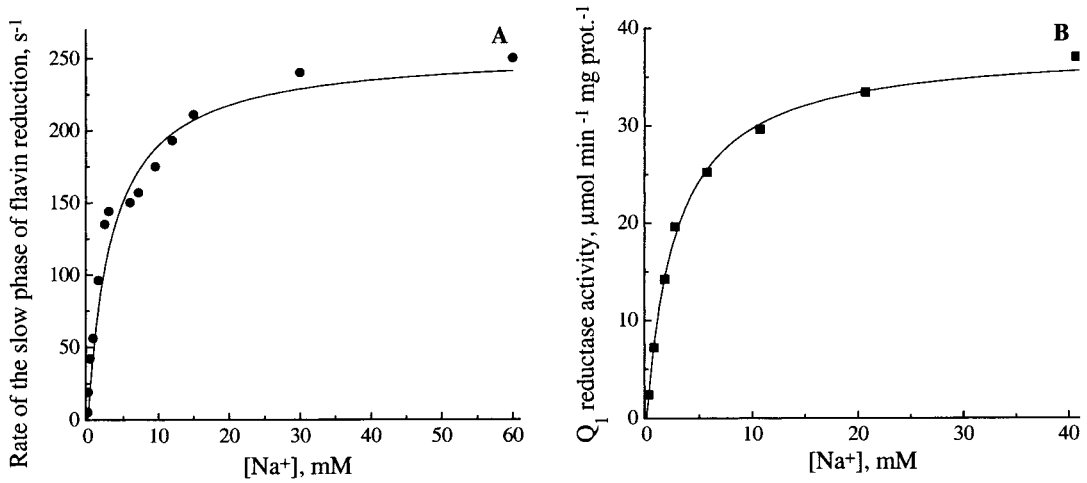

FIGURE 8: Na^+ concentration dependence of (A) the slow phase of flavin reduction in Na^+-NQR by NADH (conditions as described in the legend of Figure 7) and (B) Q_1 reductase activity catalyzed by purified Na^+-NQR.

1/1 stoichiometry, the second of which is strongly dependent on sodium concentration. These two phases, which correspond to phases 1 and 3 in the global fit (above), almost certainly reflect two different flavins in the enzyme structure. The faster phase is likely to be the reduction of a molecule FAD in the NqrF subunit, while the slower phase is probably the reduction of a covalently bound FMN (see the Discussion). The first phase (phase 1 above) is very rapid and can be resolved only at temperatures as low as 3.5 °C. It is essentially sodium independent. In contrast, the slower phase (phase 3 above) depends strongly on sodium concentration. At 25 °C, the rate constant increased from 4−6 s^{-1} at 25 μM Na^+ to 180−250 s^{-1} at 50 mM Na^+. The dependence of this rate constant upon the sodium concentration reveals an apparent activation constant for Na^+ of 3.4 ± 0.6 mM (Figure 8A). This value is close to the apparent K_m for sodium under steady-state turnover conditions, determined either for the reduction of Q_1 by the purified Na^+-NQR (3.3 ± 0.4 mM, Figure 8B) or for deaminoNADH oxidation by *V. harveyi* SBP (3.5 ± 0.5 mM, data not shown).

The same results could be obtained using a partially purified Na^+-NQR or a highly purified enzyme.

DISCUSSION

The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) of *V. harveyi* was purified, and its redox and kinetic properties were studied using EPR and optical spectroscopy.

The general characteristics of the EPR spectra presented here are very similar to those previously described for the *V. alginolyticus* enzyme (*12*). The same two signals were detected, one assigned to a radical and the other to an [2Fe-2S] cluster. In the *V. alginolyticus* Na^+-NQR, the spin concentration of the radical was 6−14 times larger than that of the Fe−S cluster (*12*), whereas in our case, the radical/Fe−S cluster stoichiometry was close to 1/1. This discrepancy may be due to the presence of LDAO in the previously used preparations of the enzyme (*12*). As described above, LDAO can cause oxidation of the enzyme, but this can be eliminated by substituting DM for LDAO as the detergent used during the purification of the enzyme.

The signal from the Fe−S cluster was titrated by varying the NAD^+/NADH ratio, and an E_m of −267 mV ($n = 1$) was obtained; this E_m value was essentially independent of sodium concentration. Surprisingly, in contrast to ref *12*, the radical signal was largely the same under all the redox conditions that were tested. The spin concentration of the radical was the same in the oxidized Na^+-NQR and the dithionite-reduced enzyme. Furthermore, the spin concentration in the radical signal does not vary across sample preparations, across different fractions collected during a given preparation, or with redox changes, and is present in an ~1/1 ratio with the amount of Fe−S observed in the dithionite-reduced samples. These observations are evidence that the radical does not arise from contaminants in the preparation; however, its true nature remains to be found.

Some aspects of the Fe−S cluster reduction remain unclear. It is well-known that NADH is a potent two-electron donor. Single-electron reduction of the Fe−S cluster should therefore be tightly coupled to one-electron reduction of another site in the enzyme, since there is only one Fe−S cluster per Na^+-NQR monomer. The primary candidates for this other site are flavins and ubiquinone. However, the EPR spectra did not reveal any *increase* in the population of radicals coupled to the Fe−S cluster reduction by NADH. It would also be possible to explain single-electron reduction of the Fe−S cluster by NADH if this process were coupled to the reduction of the observed radical, but there is no *decrease* in the concentration of the radical upon addition of NADH. These discrepancies suggest that some artifactual process, such as ubisemiquinone dismutation or partial enzyme oxidation, may occur during the incubation of high concentrations of Na^+-NQR with NADH prior to freezing (the incubation time before freezing was 60 s). Additional experiments for studying the reduction of Na^+-NQR by EPR with better time resolution are necessary to clarify this issue.

To find the sodium-dependent step, or steps, during turnover of Na^+-NQR, we also investigated the kinetics of Na^+-NQR reduction by NADH at different sodium concentrations. This study is a logical extension of our previous observation of two different flavin species in the enzyme from *V. harveyi*, a noncovalently bound FAD and a covalently bound FMN in NqrC (*13*). Flavin reduction takes place in two distinct phases, which almost certainly reflect the two different flavin cofactors. The first phase is rapid and sodium-independent. It almost certainly reflects the

reduction of the noncovalently bound FAD; the amino acid sequence shows an FAD binding motif in NqrF, the same subunit where the NADH binding motif is found. The slower phase is strongly Na^+ dependent, being at least 40 times faster at high than at low sodium levels. This phase presumably reflects the reduction of a covalently bound FMN (see below).

Recently, it was shown that the *V. alginolyticus* Na^+-NQR contains two covalently bound flavins attached to the NqrC and NqrB subunits (*14*), in addition to the noncovalently bound FAD. On this basis, it was suggested that there are three flavin species in Na^+-NQR. The results presented here do not support this conclusion, as we detected only two different flavins during reduction of the enzyme. However, these data may not be contradictory (*14*), but rather may indicate that only two flavins can be reduced rapidly by the two electrons from NADH. The fact that the putative third flavin was invisible in our assay may indicate that it is not involved in the electron-transfer mechanism of the enzyme. One possibility is that that the third flavin is the source of the radical observed in this enzyme. If that were the case, reduction of the third flavin would be a single-electron process with spectral characteristics different from those investigated in this study.

The sodium concentrations required to activate reduction of the second flavin are very similar to those needed to promote quinone reductase activity in this enzyme. This indicates that the rate-limiting step in enzyme turnover at low sodium concentrations is the reduction of FMN. We conclude that the sodium-dependent step within the Na^+-NQR turnover is located between the noncovalently bound FAD and a covalently bound FMN, and it is likely that this step is the one coupled to sodium translocation by Na^+-NQR.

ACKNOWLEDGMENT

We thank Joel Morgan for helping with the EPR measurements and his comments on the manuscript. We are also indebted to Prof. M. Wikström for helpful discussions and to Dr. M. Verkhovskaya and Mr. A. Shestopalov for critical reading of the manuscript. B.B. thanks Professor R. B. Gennis for discussions and support.

REFERENCES

1. Tokuda, H., and Unemoto, T. (1981) *Biochem. Biophys. Res. Commun. 102*, 265−271.
2. Tokuda, H., and Unemoto, T. (1982) *J. Biol. Chem. 257*, 10007−10014.
3. Tokuda, H., Sugasawa, M., and Unemoto, T. (1982) *J. Biol. Chem. 257*, 788−794.
4. Dibrov, P. A., Kostyrko, V. A., Lazarova, R. L., Skulachev, V. P., and Smirnova, I. A. (1986) *Biochim. Biophys. Acta 850*, 449−457.
5. Dibrov, P. A., Lazarova, R. L., Skulachev, V. P., and Verkhovskaya, M. L. (1986) *Biochim. Biophys. Acta 850*, 458−465.
6. Hayashi, M., and Unemoto, T. (1984) *Biochim. Biophys. Acta 850*, 449−457.
7. Nakayama, Y., Hayashi, M., and Unemoto, T. (1998) *FEBS Lett. 422*, 240−242.
8. Rich, P. R., Meunier, B., and Ward, F. B. (1995) *FEBS Lett. 375*, 5−10.
9. Hayashi, M., Hirai, K., and Unemoto, T. (1995) *FEBS Lett. 363*, 75−77.
10. Tan, K., Beattie, P., Leach, D. R., Rich, P. R., Coulson, A. F., and Ward, F. B. (1996) *Biocem. Soc. Trans. 24*, 12S.
11. Bogachev, A. V., Murtazina, R. A., and Skulachev, V. P. (1997) *FEBS Lett. 409*, 475−477.
12. Pfenninger-Li, X. D., Albracht, S. P. J., van Belzen, R., and Dimroth, P. (1996) *Biochemistry 35*, 6233−6242.
13. Zhou, W., Bertsova, Y. V., Feng, B., Tsatsos, P., Verkhovskaya, M. L., Gennis, R. B., Bogachev, A. V., and Barquera, B. (1999) *Biochemistry 38*, 16246−16252.
14. Nakayama, Y., Yasui, M., Sugahara, K., Hayashi, M., and Unemoto, T. (2000) *FEBS Lett. 474*, 165−168.
15. Provencher, S. W., and Vogel, R. H. (1983) in *Progress in Scientific Computing* (Deuflhard, P. and Hairer, E., Eds.) pp 304−319, Birkhauser, Boston.
16. Morgan, J. E., Verkhovsky, M. I., Puustinen, A., and Wikström, M. (1995) *Biochemistry 34*, 15633−15637.
17. Laemmli, U. K. (1970) *Nature 227*, 680−685.
18. Wikström, M., Krab, K., and Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, New York.

BI002545B