Redox-Dependent Sodium Binding by the Na^+-Translocating NADH:Quinone Oxidoreductase from *Vibrio harveyi*†

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ABSTRACT: Relaxation characteristics of the ^{23}Na nuclei magnetization were used to determine the sodium-binding properties of the Na^+-translocating NADH:quinone oxidoreductase from *Vibrio harveyi* (NQR). The dissociation constant of Na^+ for the oxidized enzyme was found to be 24 mM and for the reduced enzyme about 30 μM. Such large (3 orders in magnitude) redox dependence of the NQR affinity to sodium ions shows that the molecular machinery was designed to use the drop in redox energy for creating an electrochemical sodium gradient. Redox titration of NQR monitored by changes in line width of the ^{23}Na NMR signal at 2 mM Na^+ showed that the enzyme affinity to sodium ions follows the Nernst law for a one-electron carrier with E_m about −300 mV (vs SHE). The data indicate that energy conservation by NQR involves a mechanism modulating ion affinity by the redox state of an enzyme redox cofactor.

The Na^+-translocating NADH:quinone oxidoreductase (NQR)[1] generates a redox-driven transmembrane electrochemical Na^+ potential (*1*−*4*). The enzyme consists of six subunits (NqrA−F) (*5*) that correspond to the six genes of the *nqr* operon (*6*, *7*). Three of the subunits, NqrA, NqrC, and NqrF, are relatively hydrophilic, whereas the NqrB, NqrD, and NqrE subunits are very hydrophobic (*6*, *7*). NQR is thought to contain the following set of prosthetic groups: one 2Fe−2S cluster, one noncovalently bound flavin adenine dinucleotide (FAD), two covalently bound flavin mononucleotide (FMN) residues, and possibly also one ubiquinone-8 (*4*). Subunit NqrF possesses binding motifs for NADH, FAD, and 2Fe−2S cluster (*6*, *8*−*10*), whereas covalently bound FMN residues are attached by phosphoester bonds to threonine residues in subunits NqrB and NqrC (*11*−*13*).

The efficiency of Na^+ translocation by NQR was shown to be one Na^+/e^- (*14*), but the mechanism of energy conversion between the redox transitions and the transmembrane translocation of a sodium ion is still unknown for this enzyme. The simplest mechanism of redox−electrochemical coupling implies that the reduction of an NQR cofactor should be connected with the capture of the sodium ion from the cytoplasmic side of the membrane. The subsequent oxidation of this cofactor has to be accompanied by the ejection of the cation from the other side of the membrane. If this mechanism is used by NQR, the redox potential of at least one of its prosthetic groups should depend on the Na^+ concentration (*4*). It was earlier established that on NQR reduction by NADH, the rate of electron transport from FAD to FMN residues is strongly dependent on the sodium ion concentration (*15*, *16*). Thus it was assumed that one of these sodium-dependent transitions could be coupled with transmembrane Na^+ translocation (*4*). However, the midpoint potentials of all the redox transitions determined in the enzyme were found to be independent of Na^+ concentration (*17*). These results raise a question: How can the mechanism of coupling of electron transfer to ion translocation be built so that the electron affinity of NQR prosthetic groups does not depend on concentration of its coupling ion?

To answer this question, it was important to determine the Na^+-binding characteristics of NQR and to study their dependence upon reduction of the enzyme. In the present work, it is demonstrated that NQR reduction leads to about 1000-fold increase in affinity of the enzyme to its coupling ion.

EXPERIMENTAL PROCEDURES

Purification of NQR. NQR from *V. harveyi* cells was purified as described previously (*17*).

NMR. The ^{23}Na NMR spectra were measured at 132.2 MHz on a Varian Unity INOVA 500 NMR spectrometer, using a single pulse NMR experiment. Acquisition time for FID was 50−250 ms, and the spectral width was 2−10 kHz. Recycle delay was 100−500 ms. For separate determination of two characteristic times of transverse relaxation T_2' and T_2'', the decay curve of transverse magnetization was approximated by the superposition of two exponentials using a multidimensional unconstrained nonlinear minimization

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[1] Abbreviations: CD, circular dichroism; DM, dodecyl maltoside; E_m, midpoint redox potential; FID, free induction decay; NQR, Na^+-translocating NADH:quinone oxidoreductase; NQR^{ox} and NQR^{red}, oxidized and reduced NQR, respectively; SHE, standard hydrogen electrode; $\Delta\bar{\mu}_{Na^+}$, the transmembrane difference in electrochemical potentials of the sodium ions.

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(Nelder−Mead) method run in Matlab (MathWorks Inc.) software.

Measurements were carried out at 20 °C in buffer containing 100 mM KCl, 20 mM Hepes−Tris (pH 7.5), 10% D_2O, 0.1% of dodecyl maltoside (DM), and varying concentrations of NaCl and NQR. To reduce the protein, the samples were supplemented with 5 mM potassium dithionite (potassium dithionite was prepared by passing 1 M sodium dithionite solution through a column with potassium form of Dowex 50 WX 4 under anoxic conditions).

Redox Titration of NQR as Monitored by Changes in Line Width of the ^{23}Na NMR Signal. NQR samples contained 100 μM of the protein in 100 mM KCl, 20 mM Hepes−Tris (pH 7.5), 10% D_2O, 0.1% DM, and 2 mM NaCl. To achieve anaerobic conditions, the samples were supplemented with 1.3 mg/mL glucose oxidase, 0.1 mg/mL catalase, and 10 mM glucose, and all manipulations were carried out under a stream of argon.

NQR was titrated by varying the NADH/NAD^+ ratio (0−10 mM of NAD^+ and 1−10 mM of NADH). The protein was also titrated by varying the lactate/pyruvate ratio (2 mM pyruvate and 0.2−20 mM L-lactate) in the presence of NAD^+ (5 mM) and lactate dehydrogenase (1 U). In all experiments, only Tris salts of lactate, pyruvate, NAD^+, and NADH were used.

Optical Spectra. Optical spectra of NQR were measured on an Agilent-8453 or a Hitachi-557 spectrophotometer. The spectra in the 310−800 nm range were measured in 100 mM KCl, 20 mM Hepes−Tris (pH 7.5), 0.1% DM, and 2.5 mg/mL NQR. Na^+ and K^+ were added as chloride salts.

Circular Dichroism Measurements. Circular dichroism (CD) spectra were recorded using a modified Jobin−Yvon Mark V dichrograph. The spectra in the UV range (215−252 nm) were measured in 100 mM KCl, 20 mM Hepes−Tris (pH 7.5), 0.1% DM, and 0.13 mg/mL NQR using a 0.1-cm quartz cuvette. Na^+ and K^+ were added as chloride salts.

Protein and Sodium Content. Protein content was determined by the bicinchoninic acid method using bovine serum albumin as standard. NQR concentration was determined using the following extinction coefficients (*17*): for oxidized NQR, $\epsilon_{465-520} = 29.1$ mM^{-1} cm^{-1}; for the reduced *minus* oxidized NQR spectrum, $\epsilon_{465-770} = 30.4$ mM^{-1} cm^{-1} and $\epsilon_{465-520} = 19.6$ mM^{-1} cm^{-1}.

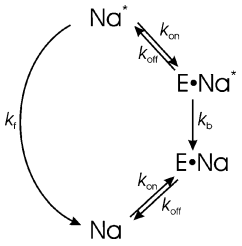
Sodium concentration was measured by flame photometry.

RESULTS

The investigation of sodium ion binding to NQR is not a trivial task for the common methods used for the determination of ligand binding. Being a substrate for NQR, Na^+ should be exchangeable with this protein on the time scale of the enzyme turnover, which is ≤3 ms (*18*). For such fast exchange, the usual methods of removal of free unbound ligand by washing, sedimentation, column chromatography, etc. cannot be used. However, ^{23}Na NMR spectroscopy was proven (*19*−*23*) to be a good technique for determining the ion-binding properties of macromolecules under conditions of very fast chemical exchange, and so we chose this method for our study.

Theoretical Considerations. The theory of NMR spectroscopy of quadrupolar nuclei with spin of $^3/_2$ is well-established (*19*, *24*, *25*). Here we will briefly mention only a part of it relevant for the current investigation.

Scheme 1



For free sodium ions in aqueous solution, where rotation of the ion is very fast, the condition of so-called extreme narrowing, $\omega^2\tau_c^2 \ll 1$ (where ω is the angular Larmor frequency of the nuclei and τ_c is the correlation time), is satisfied. In this case, the relaxation of transverse magnetization is characterized by a single-exponential decay with a time constant T_{2free} that corresponds to a simple Lorentzian line in the ^{23}Na NMR spectrum with line width $\Delta\nu_{1/2}^0$

$$\Delta\nu_{1/2}^0 = \frac{1}{\pi T_{2free}} = \frac{2\pi}{5}\chi^2\tau_c \qquad (1)$$

where χ is the quadrupolar coupling constant.

When sodium ions are bound to a large molecule such as a protein, their tumbling rate is decreased, and they are subjected to a large electric field gradient due to the lowered symmetry of the binding environment. This results in significant broadening of the ^{23}Na NMR spectrum. If the chemical exchange rate between bound and free Na^+ is fast, then 100% of the intensity is observed in the central resonance.

For such a bound, ion the conditions of extreme narrowing are not satisfied ($\omega^2\tau_c^2 \gg 1$) and relaxation of transverse magnetization is characterized by a two-exponential decay (*24*). The fast component of the decay with 60% of the amplitude has relaxation time T_{2b}':

$$\frac{1}{T_{2b}'} = \frac{\pi^2}{5}\chi^2\left(\tau_c + \frac{\tau_c}{1+\omega^2\tau_c^2}\right) \qquad (2)$$

and the slow component representing the remaining 40% of the signal has relaxation time T_{2b}'':

$$\frac{1}{T_{2b}''} = \frac{\pi^2}{5}\chi^2\left(\frac{\tau_c}{1+4\omega^2\tau_c^2} + \frac{\tau_c}{1+\omega^2\tau_c^2}\right) \qquad (3)$$

These two relaxation time constants correspond to an NMR spectrum with superposition of two Lorentzian lines with different line widths but the same resonance frequency.

In the presence of an enzyme (E) capable of binding sodium ions when [E] ≪ [Na^+], the relaxation kinetics of transverse magnetization of ^{23}Na nuclei after a 90° pulse can be described by Scheme 1where Na* and Na are the nonequilibrium and equilibrium states of the sodium nuclei, respectively, k_f is the relaxation rate constant for free sodium, k_{on} and k_{off} are the rate constants of sodium binding and release from the enzyme, and k_b is the relaxation rate constant of bound ^{23}Na.

When $k_{off} \gg k_b$ the apparent rate constant of transverse relaxation $1/T_2$ (or k_{app}) (*19*) is

$$\frac{1}{T_2} = k_f + \frac{k_b[E_{tot}]}{K_D + [Na_{tot}]} = \frac{1}{T_{2free}} + \frac{1}{T_{2b}} \frac{[E_{tot}]}{K_D + [Na_{tot}]} \quad (4)$$

or

$$\frac{1}{T_2} - \frac{1}{T_{2free}} = \frac{k_b[E_{tot}]}{K_D + [Na_{tot}]} = \frac{1}{T_{2b}} \frac{[E_{tot}]}{K_D + [Na_{tot}]} \quad (5)$$

where $[E_{tot}]$ and $[Na_{tot}]$ denote the total enzyme and sodium ion concentrations, respectively, and K_D is the dissociation constant for the Na^+−enzyme complex. The rate constant of relaxation for bound sodium, k_b or $1/T_{2b}$, consists of two components (k_b' and k_b'') defined by eqs 2 and 3. In this case, the dependence of apparent relaxation time on the sodium concentration lets us calculate such parameters as K_D, k_b', and k_b''.

If $k_{off} \ll k_b$, the scheme can be simplified (*19*, *23*) as shown in Scheme 2 and

$$\frac{1}{T_2} = k_f + \frac{k_{off}[E_{tot}]}{K_D + [Na_{tot}]} = \frac{1}{T_{2free}} + \frac{1}{\tau_{off}} \frac{[E_{tot}]}{K_D + [Na_{tot}]} \quad (6)$$

or

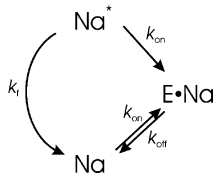
$$\frac{1}{T_2} - \frac{1}{T_{2free}} = \frac{k_{off}[E_{tot}]}{K_D + [Na_{tot}]} = \frac{1}{\tau_{off}} \frac{[E_{tot}]}{K_D + [Na_{tot}]} \quad (7)$$

This relaxation would be monoexponential, unlike the previous case, and the dependence of apparent transverse relaxation time on the Na^+ concentration yields K_D, k_{on}, and k_{off}.

In other words, whether the magnetization decay is monoexponential or two-exponential depends on the rate of chemical exchange between bound and free Na^+.

Sodium-Binding Properties of Oxidized NQR. To avoid nonspecific ion binding to the enzyme, all measurement were performed in the presence of high K^+ concentration (100 mM KCl). Under these conditions, in the absence of the protein, the relaxation of transverse magnetization for free Na^+ is a monoexponential process with characteristic time $T_2 \approx 40$ ms at all used sodium concentrations (2−400 mM). The ^{23}Na NMR spectrum of NaCl solution was represented by a single Lorentzian line with the width at half-height $\Delta\nu_{1/2}^0 \approx 8$ Hz (see Figure 1, dashed line). However, in samples containing 120 μM oxidized NQR (NQR^{ox}) the decay of transverse magnetization of ^{23}Na is significantly faster and appears to be a sum of two exponential processes with amplitude ratio 0.6:0.4 (as predicted by theory, see above). The ^{23}Na NMR spectrum in this case was broadened and could be represented as the sum of two Lorentzian lines with different line widths but the same resonance frequency (Figure 1, dotted line). It is noteworthy that the values of the integrals of ^{23}Na NMR spectra for NaCl solutions in the absence as well as in the presence of NQR^{ox} were identical. All these data mean that in the presence of NQR^{ox} sodium ions are in free as well as in protein-bound states, and there is very fast chemical exchange between these two populations of Na^+ (*19*).

Scheme 2



To determine the affinity of NQR^{ox} to Na^+, the decay of transverse magnetization of ^{23}Na was investigated at various NQR/Na^+ ratios. The gradual increase in sodium concentration from 4 to 400 mM at constant enzyme concentration (120 μM) narrowed the broad ($\Delta\nu_{1/2}'$, data not shown) as well as the narrow ($\Delta\nu_{1/2}''$, Figure 2A) components of the ^{23}Na NMR spectrum. Fitting these data to eq 5 gave $K_D^{Na} \approx 24$ mM and rate constants for transverse magnetization $k_b' \approx 1.6 \times 10^5$ s^{-1} and $k_b'' \approx 8.2 \times 10^3$ s^{-1}. Thus, our results show that NQR^{ox} binds Na^+ with relatively low affinity. It is important to stress that the value of 24 mM is a characteristic of the binding site with the highest affinity in this state of the enzyme. Because the dependence of the excess line width of the ^{23}Na NMR spectrum presented in Figure 2A does not approach zero at high ion concentrations, it is clear that NQR^{ox} has one or several other binding site-(s) with lower affinity.

Sodium-Binding Properties of Reduced NQR. Reduction of the enzyme by dithionite does not change the value of the spectrum integral but results in further broadening of the ^{23}Na NMR line in comparison with the oxidized state of NQR (Figure 1, solid line). This phenomenon can be due either to enhanced relaxation of the bound ion transverse magnetization or to an increase in the affinity of NQR to sodium ions upon the reduction of the enzyme (see eq 5). To determine which model is correct, the relaxation time of transverse magnetization was measured at various Na^+/NQR^{red} ratios. Increasing the Na^+ concentration from 4 to 400 mM at constant protein concentration (120 μM) resulted in *strong* narrowing of the ^{23}Na resonance (Figure 2B). The fit shows that K_D^{Na} is much lower than any ion concentration used in this experiment. This is why the value of the dissociation constant is not well defined from these data, and we can only conclude that $K_D^{Na} \ll 1$ mM.

To be able to determine K_D^{Na} for NQR^{red}, experiments with the reduced enzyme were also performed under different experimental conditions. The 100 μL volume of the sample

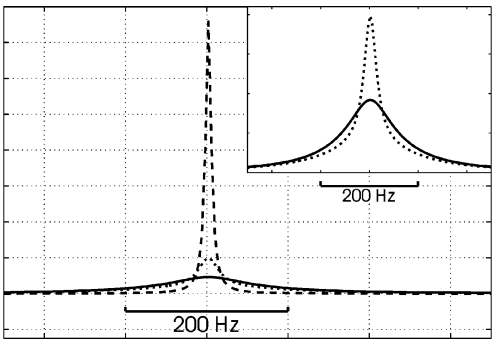


FIGURE 1: ^{23}Na NMR spectra of 4 mM NaCl solutions in the absence (− − −) or in the presence of 120 μM of oxidized (···) or reduced (—) NQR. The inset shows an enlarged representation of the ^{23}Na NMR spectra in the presence of oxidized and reduced NQR.

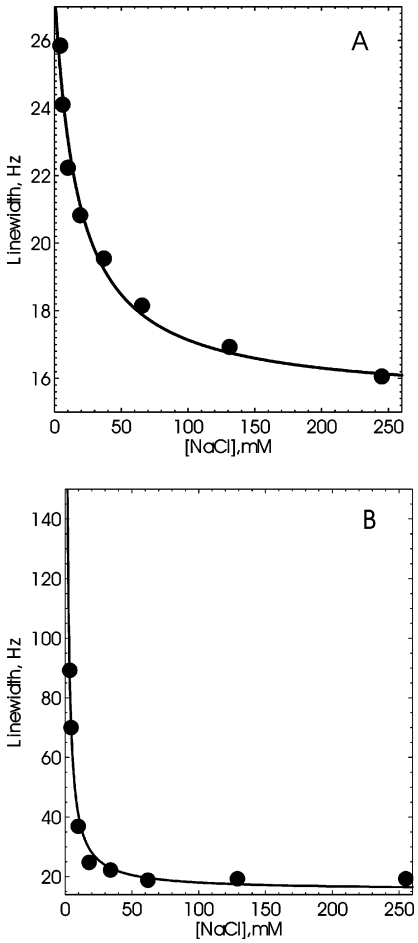


FIGURE 2: Line width excess $\Delta\nu''_{ex}$ ($\Delta\nu''_{ex} = \Delta\nu''_{1/2} - \Delta\nu_{1/2}$) of the narrow component of the ^{23}Na NMR signal at 4−400 mM Na^+ concentrations in the presence of 120 μM *oxidized* (A) and *reduced* (B) NQR. For small sodium concentrations during the acquisition, data were averaged from 1024 scans, and for the larger concentrations, 64 scans were averaged.

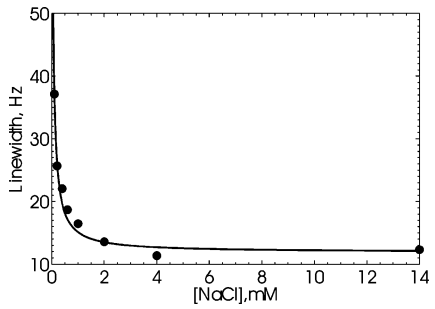


FIGURE 3: Line width excess, $\Delta\nu_{ex}$, of the ^{23}Na NMR signal at 0.1−14 mM Na^+ concentrations in the presence of 10 μM *reduced* NQR.

(in a 5-mm o.d. NMR tube) was increased to 1.5 mL (in a 10-mm o.d. NMR tube). This change was enough to make measurements with good signal-to-noise ratio down to 100 μM Na^+. As can be seen in Figure 3, *strong* narrowing of the ^{23}Na signal occurred at very low sodium concentrations. Fitting of the data according to eq 6 gave a relatively stable solution with $K_D^{Na} \approx 30$ μM and apparent $k_b \approx 1.2 \times 10^3$ s^{-1}. This result shows that reduction of NQR increases the affinity of the enzyme to sodium ions by about 3 orders of magnitude. As in the case with the oxidized enzyme, the dependence of the ^{23}Na line width excess on sodium concentration does not approach zero level at high Na^+ concentration (Figure 2B and Figure 3). This fact indicates that the reduced enzyme also has one or more additional sodium-binding site(s) with lower affinity.

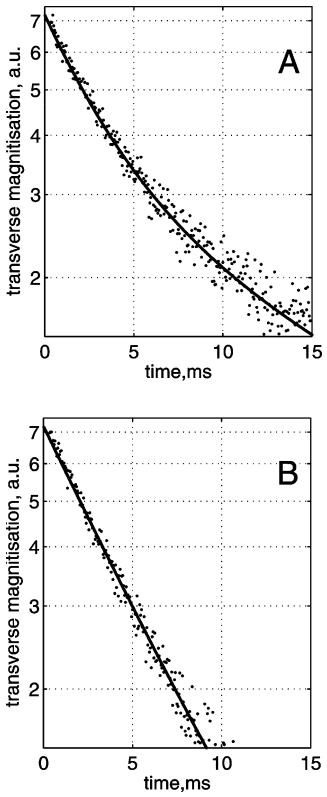


FIGURE 4: Relaxation of transverse magnetization of ^{23}Na in 2 mM NaCl solutions containing 100 μM of oxidized (A) or reduced (B) NQR. Dots are the experimental data, and solid lines represent the theoretical curves for two-exponential (A) and single-exponential (B) decay.

Determination of Kinetic Parameters of Sodium Binding by NQR. As mentioned above, the relaxation of transverse magnetization of ^{23}Na nuclei in the presence of oxidized enzyme consists of two components with amplitudes 0.6 for the fast and 0.4 for the slow one (see Figure 4A). The ratio of the rate constants for these components (k'_b/k''_b or so-called Bull ratio (*19*)) was 19.5. From eqs 2 and 3, this ratio gave the correlation time (τ_c) of ∼36 ns. These data according to eqs 2 and 3 also yield a quadrupolar coupling constant for ^{23}Na bound to oxidized NQR of $\chi \approx 1.5$ MHz, which is close to those reported for ^{23}Na bound to ionophores and proteins (*20*, *26*).

The inverse of the correlation time is the sum of the inverse mean times for each of the rate processes that would cause the nucleus to experience a fluctuating electric field gradient:

$$\frac{1}{\tau_c} = \frac{1}{\tau_R} + \frac{1}{\tau_{off}} + \frac{1}{\tau_i} + ...$$

where τ_R characterizes the rotation of the NQR−Na^+ complex and τ_i describes the internal motion of the sodium binding site relative to the overall motion of the enzyme (*19*). It means that $\tau_c < \tau_{off}$. The existence of two components of the transverse magnetization relaxation for *oxidized* enzyme suggests that $\tau_{off} < T_{2b}'$ and T_{2b}'' (*20*). These inequalities let us estimate the rate constant of sodium dissociation from oxidized enzyme as $2.8 \times 10^7 > k_{off} > 1.6 \times 10^5$ s^{-1} and, taking into account the value of $K_D^{Na} = 24$ mM, the

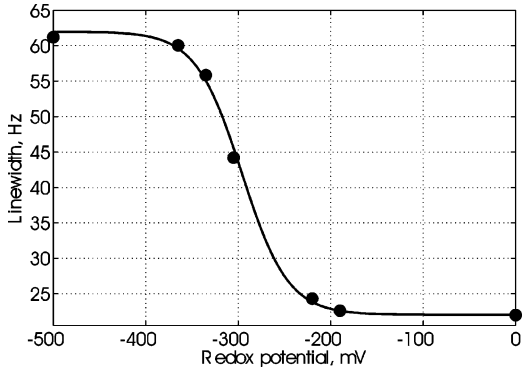


FIGURE 5: Redox titration of NQR (100 μM) at 2 mM NaCl monitored by changes in line width of the narrow component of the ^{23}Na NMR signal. The solid line represents the theoretical titration curve with $n = 1$ and $E_m = -300$ mV (vs SHE). For each data point, 8192 scans were averaged.

bimolecular rate constant of sodium binding as $1.2 \times 10^9 > k_{on} > 7 \times 10^6$ M^{-1} s^{-1}.

In contrast to the oxidized enzyme, the relaxation of the ^{23}Na transverse magnetization in the presence of the *reduced* NQR is monoexponential (see Figure 4B). Such pattern shows that in this case the kinetics of transverse magnetization relaxation are limited by the ion off rate (see theoretical considerations). In the experiments with the reduced enzyme, the apparent relaxation rate constant for bound ^{23}Na was found to be $\sim 1.2 \times 10^3$ s^{-1}, and with the assumption that the enzyme binds one sodium ion at this site, we can conclude that this value is defined by the k_{off} from the Na^+–NQR^{red} complex. Such a slow off rate taken together with the earlier defined K_D^{Na} (30 μM) gives very reasonable bimolecular rate constant for sodium entry into the enzyme of $\sim 3.6 \times 10^8$ M^{-1} s^{-1}.

Redox Titration of ^{23}Na Transverse Magnetization in the Presence of NQR. The strong increase in enzyme affinity to Na^+ described above was found upon complete NQR reduction, when all its redox prosthetic groups were transferred from the oxidized to the reduced state. To identify the reduction of which particular cofactor is responsible for this phenomenon, we performed "redox titration" of the transverse magnetization rates at sodium concentration of 2 mM and NQR concentration of 100 μM. The redox titration was achieved by varying NADH/NAD^+ ratio (the low ratios were buffered by the lactate/pyruvate couple in the presence of lactate dehydrogenase). As seen from Figure 5, the dependence of the half-width of the narrow ^{23}Na signal ($\Delta \nu'_{1/2}$) can be described by the Nernst equation with $E_m \approx -300$ mV (vs SHE) and $n = 1$. It is important to note that the maximal value of the half-width can be achieved by the addition of NADH as well as by the addition of dithionite in the absence of pyridine adenine dinucleotides. This fact indicates that the change in sodium affinity is not connected to another ligand binding to NQR (like NADH) but rather reflects one-electron reduction of the enzyme cofactor, which has $E_m \approx -300$ mV at 2 mM Na^+.

Spectrophotometric Studies of the Interaction between NQR^{ox} and Na^+. It was also of interest to investigate the effect of sodium binding on secondary structure of NQR and on spectral properties of its prosthetic groups. Unfortunately, this task can be done only with oxidized NQR due to very high UV absorption of compounds needed for NQR reduction (NADH or dithionite) and due to very low absorption of the reduced forms of NQR prosthetic groups.

To investigate the effect of the Na^+ binding on the secondary structure of NQR^{ox}, the change in UV–CD spectrum of the protein upon addition of 50 mM NaCl or KCl was studied. In these spectra, no significant sodium-specific change in molar ellipticity was observed (see Supporting Information, Figure S1).

To investigate the effect of a possible interaction of Na^+ with the NQR cofactors, absorption spectra of the oxidized protein in the visible region (310–800 nm range) were measured in the presence of 0.04–200 mM NaCl or KCl. Again, no significant sodium-specific change in the visible absorption spectra was observed (see Supporting Information, Figure S2). These results indicate that the Na^+ binding to NQR^{ox}, which we have demonstrated to occur by NMR, does not significantly perturb the secondary structure of the enzyme and the visible spectral characteristics of its prosthetic groups.

DISCUSSION

The mechanism of energy conversion of the NQR-catalyzed redox reaction (NADH:quinone oxidoreduction) into sodium transmembrane potential is still unknown. The simplest coupling mechanism of a redox-dependent ion pump implies that reduction of some NQR cofactor is accompanied by the capture of the sodium ion from the cytoplasmic side of the membrane. Consequent oxidation of this cofactor has to be coupled to ejection of Na^+, but from the other side of the membrane (*4*). If NQR uses just this mechanism of energy conservation together with efficient kinetic control of the reactions, the affinity of the protein to sodium ions must be dependent on the redox state of the enzyme. Moreover, the difference in K_D^{Na} values for fully oxidized and reduced NQR forms has to be at least 1000-fold because $\Delta\bar{\mu}_{Na^+}$ on the bacterial membrane can be as high as 180 mV. According to this model, in the present work it has been demonstrated that NQR reduction leads to significant (about 1000-fold) increase in affinity of the enzyme to its coupling ion. Thus, the data indicate that energy conservation by NQR involves a mechanism providing modulation of ion affinity by the redox state of a redox cofactor of the enzyme.

However, the values of the binding constant obtained in this work for reduced and oxidized NQR do not quite fit with such a model. It is unclear why the reduced enzyme has such high affinity for sodium ($K_D^{Na} = 30$ μM) if the cytosolic concentration of this ion in marine *Vibrio* species is 10–40 mM (*27*) It is possible that the binding pocket for these ions is located deep in the membrane dielectric. In this case, the delivery of the coupling ion from cytoplasm to the binding site should come against the electric field. This field hampers ion binding effectively, decreasing the *in vivo* affinity of NQR^{red} to Na^+.

The obtained value of K_D^{Na} for the oxidized enzyme (24 mM) also seems too low, because according to the simple mechanism described above the oxidized enzyme should release Na^+ to the outer space, which is seawater for marine bacteria, with sodium concentration about 500 mM. To overcome this problem, we have to propose that the enzyme has some additional mechanism for releasing sodium ions to the outside media from the oxidized enzyme or, what is

more likely, that NQR has several *different* binding sites. In the latter case, the binding site that we have found in the oxidized enzyme can be located in any part of the protein and play the role of, for example, selectivity or gating sites, and does not have direct relation with sodium release to the outside media. Such proposal for possible multiple sites is supported by our Na^+/NQR titrations, which showed additional binding site(s) with lower affinity for reduced as well as oxidized enzyme forms.

According to the simple model of sodium ion translocation coupled to oxidoreduction of the enzyme cofactor, it is important to determine the reduction of which of them is coupled with increase in the affinity. Formally, such a mechanism requires that the midpoint redox potential of this cofactor must be dependent on sodium concentration, and in a trivial case it should increase by 60 mV per decade increase in sodium concentration. However, as we have demonstrated earlier (*17*), there is no such dependence for any optically detectable NQR cofactors. Furthermore, in the present work we established that at 2 mM Na^+ the increase in NQR affinity to sodium followed the Nernst law with $E_m \approx -300$ mV (vs SHE). However, none of the optically visible NQR redox components was reported to have such low midpoint redox potential (*17*).

To explain these observations, we need to propose that NQR contains some additional redox prosthetic group having no or negligible absorption in the visible region but the expected sodium dependence of its E_m. Further studies are needed to clarify this question.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Sodium concentration dependence in molar ellipticity and in the visible absorption spectra are shown in Figures S1 and S2, respectively. These materials are available free of charge via the Internet at http://pubs.acs.org.

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