NMR Reveals Double Occupancy of Quinone-type Ligands in the Catalytic Quinone Binding Site of the Na\(^+\)-translocating NADH:Quinone Oxidoreductase from Vibrio cholerae

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Background: The Na\(^+\)-NQR is a respiratory Na\(^+\) pump found in prokaryotes.

Results: The NqrA subunit binds two quinone-type ligands in direct vicinity to each other.

Conclusion: Simultaneous binding of two quinones enhances catalytic efficiency of the final electron transfer step.

Significance: We provide the first direct experimental evidence of simultaneous quinone binding with relevance for the catalytic mechanism.

The sodium ion-translocating NADH:quinone oxidoreductase (Na\(^+\)-NQR) from the pathogen Vibrio cholerae exploits the free energy liberated during oxidation of NADH with ubiquinone to pump sodium ions across the cytoplasmic membrane. The Na\(^+\)-NQR consists of four membrane-bound subunits NqrBCDE and the peripheral NqrF and NqrA subunits. NqrA binds ubiquinone-8 as well as quinones with shorter prenyl chains (ubiquinone-1 and ubiquinone-2). Here we show that the quinone derivative 2,5-dibromo-3-methyl-6-isopropyl-(ubiquinone-1 and ubiquinone-2). Here we show that the quinone derivative 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), a known inhibitor of the bc\(_1\) and bc\(_{0}\) complexes found in mitochondria and chloroplasts, also inhibits quinone reduction by the Na\(^+\)-NQR in a mixed inhibition mode. Tryptophan fluorescence quenching and saturation transfer difference NMR experiments in the presence of Na\(^+\)-NQR inhibitor (DBMIB or 2-n-heptyl-4-hydroxyquinoline N-oxide) indicate that two quinone analog ligands are bound simultaneously by the NqrA subunit with very similar interaction constants as observed with the holoenzyme complex. We conclude that the catalytic site of quinone reduction is located on NqrA. The two ligands bind to an extended binding pocket in direct vicinity to each other as demonstrated by interligand Overhauser effects between ubiquinone-1 and DBMIB or 2-n-heptyl-4-hydroxyquinoline N-oxide, respectively. We propose that a similar spatially close arrangement of the native quinone substrates is also operational in vivo, enhancing the catalytic efficiency during the final electron transfer steps in the Na\(^+\)-NQR.

Vibrio cholerae is a marine-borne pathogen causing in 3–5 million disease cases an estimated 100,000–130,000 deaths per year (World Health Organization, 2010). This pathogen, like many other bacteria, harbors a unique respiratory enzyme complex, namely the sodium ion-translocating NADH:quinone oxidoreductase (Na\(^+\)-NQR),\(^5\) that is unrelated to the eukaryotic complex I on the level of primary structure yet serves a similar purpose in that it generates an electrochemical gradient across the cytoplasmic membrane that in turn drives many other cellular processes like H\(^+\)/Na\(^+\)-antiporters, solute uptake, and rotation of the flagellum (1).

Na\(^+\)-NQR is composed of six subunits NqrA–F and harbors at least five redox-active cofactors: a non-covalently bound FAD and a 2Fe-2S cluster in the NqrF subunit, two covalently bound FMNs in subunits NqrB and NqrC, and one non-covalently bound riboflavin in the subunit NqrB (2–5). Upon oxidation of NADH, electrons are transferred from NADH via FAD and the 2Fe-2S cluster on NqrF to FMN on NqrC, to FMN on NqrB, and finally to riboflavin on NqrB (6, 7). However, the final step of the reaction cycle, the reduction of the quinone substrate, and the coupling of redox chemistry to sodium ion translocation are still largely unclear.

Already in 1992 it was recognized that the resistance of Vibrio alginolyticus toward korormicin, a putative quinone analog, is brought about by two mutations in the NqrB subunit of its Na\(^+\)-NQR (8). Therefore, it was expected that the NqrB subunit would carry the active site for quinone binding and reduction. Instead, we have recently identified the NqrA subunit to bind ubiquinone-8 and to interact with short chain quinones in the context of the membrane-embedded/detergent-solubilized holo-Na\(^+\)-NQR enzyme complex as well as with the isolated, soluble NqrA subunit (9). On the other hand, Juárez et al. (10) have shown that the point mutations at glycine 140 and glycine 141 of the NqrB subunit would carry the active site for ubiquinone-8 and to interact with short chain quinones in the context of the membrane-embedded/detergent-solubilized holo-Na\(^+\)-NQR enzyme complex as well as with the isolated, soluble NqrA subunit (9).

\(^5\) The abbreviations used are: Na\(^+\)-NQR, sodium ion-translocating NADH:quinone oxidoreductase; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; HQNO, 2-n-heptyl-4-hydroxyquinoline N-oxide; DDM, n-dodecyl β-D-maltoside; Q, ubiquinone; STD, saturation transfer difference; ILOE, interligand Overhauser effect; INPHARMA, interligand NOEs for pharmacophore mapping.
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EXPERIMENTAL PROCEDURES

Purification of the Na⁺-NQR and Subunit NqrA—Full-length Na⁺-NQR complex linked to an N-terminal hexahistidine tag on the subunit NqrA was produced and purified as described previously (23). Subunit NqrA encoded on plasmid pBR322 (9) was produced in Escherichia coli BL21(DE3). Perdeuterated NqrA was produced in labeled M9 medium according to Marley et al. (24). The cells were grown in unlabeled LB medium at 37 °C with shaking at 150 rpm. At an A₅₀₀ of ~0.7, the cells were pelleted by centrifugation for 15 min at 5,000 × g at room temperature. The cells were then washed once with M9 medium in D₂O and pelleted again. Cells were resuspended in deuterated M9 medium that was supplemented with perdeuterated glucose and incubated for 1 h at 37 °C and 150 rpm. Subsequently, protein expression was started by addition of isopropyl-β-D-galactoside to a concentration of 1 mM. After 4 h of incubation, the cells were harvested. To purify His₆-NqrA, washed cells (25 g) were suspended in 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5% (v/v) glycerol. One spout of MgCl₂, DNase I (Roche Applied Science), and one tablet of protease inhibitor mixture (Roche Applied Science) were added to the cell suspension, which was passed twice through a French pressure cell at 7.58 megapascals. Cell lysate was centrifuged at 100,000 × g for 60 min. The supernatant was then filtered through a syringe filter with a 2.2-μm surfactant-free cellulose acetate membrane (Corning) and loaded onto a HisTrap 2-ml (GE Healthcare) column equilibrated with running buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5% (v/v) glycerol, 4 mM NaN₃) containing 20 mM imidazole. NqrA was eluted with running buffer containing 130 mM imidazole. All experiments were performed with monomeric NqrA, which was separated from NqrA aggregates on a Superdex 200 16/60 (GE Healthcare) column in 50 mM phosphate buffer, pH 8.0, 300 mM NaCl, 5% (v/v) glycerol, 4 mM NaN₃ (9).

Enzymatic Assays—NADH dehydrogenase activities of Na⁺-NQR were determined with NADH (AppliChem) (0.005–0.05 mM) and 0.1 mM Q₁ (MCAT GmbH) at pH 7.5 as described previously (3). Quinone reductase activities were determined with Q₁ (0–0.1 mM) or Q₂ (0–0.1 mM; Sigma–Aldrich) as electron acceptor at a fixed NADH concentration of 50 μM. Rates of NADH oxidation were monitored at 340 nm, and quinol formation was determined from the difference in absorption at 248 and 268 nm (Tables 1 and 2). Kinetic measurements were performed under anaerobic conditions with magnetic stirring on an HP 8452A diode array spectrophotometer (Agilent) in reaction buffer (20 mM Tris-Cl, 50 mM Na₂SO₄, pH 7.5, 0.05 mg/ml BSA (AppliChem), 0.05% DDM (Roche Applied Science)) at 25 °C. Kₘ and V max were calculated using the Michaelis–Menten equation. For enzyme inhibition studies, the Na⁺-NQR was incubated at 4 °C in reaction buffer containing DBMIB (Sigma–Aldrich) and Q₁ or Q₂ in <1% ethanol. The reaction was started by adding NADH.

Kₗ for inhibition of NADH dehydrogenase activity by DBMIB was calculated assuming an uncompetitive mode of inhibition (25). The correlation coefficients and kinetic constants of specific quinone reductase activities are listed in Table 2. The rates were background-corrected by the changes in absorbance aris-
Inhibition of Na\(^+\)-NQR by DBMIB at varying NADH concentrations described by the Michaelis-Menten formalism

The concentration of Q1 in the assays was 0.1 mM. Both NADH oxidation and quinol formation were recorded. Corresponding rates are presented in Fig. 2. For calculation of \( K_i \), the equation for uncompetitive inhibition was applied: \( V_0 = \frac{(V_{max} \cdot [S])}{(K_m + [S])} \) where \( \alpha = 1 + [I]/K_i \) (25).

| Activity recorded | 0 \( \mu \)M DBMIB | 10 \( \mu \)M DBMIB |
|-------------------|------------------|------------------|
| NADH oxidation   |                  |                  |
| \( R^2 \)         | 0.9998           | 0.9966           |
| \( V_{max} \) (\( \mu \)mol min\(^{-1}\) mg\(^{-1}\)) | 147.5 ± 1.7      | 63.1 ± 2.5       |
| \( K_m \) (\( \mu \)M) | 29.6 ± 0.6       | 15.1 ± 1.4       |
| \( K_m/V_{max} \) | 0.2              | 0.24             |
| \( K_i' \) (\( \mu \)M) | 7.5              |                  |
| Quinol formation |                  |                  |
| \( R^2 \)         | 0.9985           | 0.9904           |
| \( V_{max} \) (\( \mu \)mol min\(^{-1}\) mg\(^{-1}\)) | 38.9 ± 1.2       | 19.4 ± 1.0       |
| \( K_m \) (\( \mu \)M) | 24.5 ± 1.5       | 12.4 ± 1.7       |
| \( K_m/V_{max} \) | 0.63             | 0.64             |
| \( K_i' \) (\( \mu \)M) | 9.9              |                  |

Binding of DBMIB Determined by Tryptophan Fluorescence Spectroscopy—NqrA was diluted in 50 mM phosphate buffer, 300 mM NaCl, pH 8.0, 5% (v/v) glycerol to a concentration of 1 \( \mu \)M. DBMIB was added from an ethanol stock solution to final concentrations between 0 and 200 \( \mu \)M (final ethanol concentration, 1%). Fluorescence was analyzed with a Fluorolog 3 spectrophotometer (Horiba Scientific) using a temperature-controlled quartz cuvette. Measurements were performed in triplicate at 10 °C. The intensity of tryptophan fluorescence emission was determined at 338 nm (\( \lambda_{excitation} = 295 \) nm). The increase in quenching (\( \Delta F \)) of the tryptophan emission was normalized to values between 0 and 1 and plotted against the concentration of DBMIB. Non-linear regression analyses were performed using the equations for one-site binding and two-site binding in Origin 6.1.

NMR Spectroscopy—NqrA was transferred to D\(_2\)O containing 10 mM potassium phosphate, pH 8.0, 150 mM NaCl, 4 mM NaN\(_3\) (PBS buffer) by repeated (at least 5-fold) ultrafiltration with Ultrafree 4 membranes (Millipore; molecular mass cutoff, 10 kDa). Na\(^+\)-NQR was transferred to the same buffer as used for NqrA with addition of 0.05% DDM; Ultrafree 4 membranes with molecular mass cutoff of 100 kDa were used in this case. To 10 \( \mu \)M NqrA or Na\(^+\)-NQR, respectively, Q\(_1\) was added from 20 mM stock solutions in DMSO-d\(_6\) to a final concentration of 100 \( \mu \)M. DBMIB and HQNO were added from 20, 4, or 1 mM stock solutions in DMSO-d\(_6\) to obtain NqrA samples containing 0, 1, 5, 10, 20, 40, 60, 80, 100, 125, or 150 \( \mu \)M DBMIB or HQNO, respectively, and additionally 200 \( \mu \)M in the case of HQNO. Additional DMSO-d\(_6\) was added to keep its concentration constant within the series (1.5%, v/v). The STD NMR experiment with DBMIB alone was performed with 2.5 \( \mu \)M NqrA in PBS buffer. DBMIB was added from a 20 mM stock solution to a concentration of 100 \( \mu \)M. Controls were prepared in the same way without NqrA. The samples were transferred to 5-mm NMR tubes, and STD NMR experiments (26) were performed as described (9). The resonances of Q\(_1\) were assigned as described (9). Water suppression was achieved by excitation sculpting (27). Resonances of NqrA and Na\(^+\)-NQR, respectively, were saturated by applying a train of low power Gaussian-shaped pulses at 0.2 ppm with a total saturation time of 2 s. Off-resonance irradiation was set to 33 ppm. Under these conditions, percent STD in control experiments was below 3% for the most upfield methyl group of DBMIB (H-8) and below 1% for all other protons. On- and off-resonance spectra were acquired in an interleaved manner and subtracted after processing. Up to 4096 transients were collected at a spectral width of 12 ppm. Percent STD effects were determined within the multiple display mode by scaling the off-resonance spectrum down to superimpose with the signal of interest in the difference (off – on) spectrum. The influence of DBMIB or HQNO, respectively, on Q\(_1\) STD effects was plotted in Origin 8.1G and fitted to a one-site binding model: \( \Delta F_{STD} = \Delta F_{STD,max} \times c_{molar} / (k + c_{molar}) \).

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Analysis of the rates of quinol formation by Na\(^+\)-NQR at varying Q\(_1\) or Q\(_2\) concentrations in the absence or presence of DBMIB

The NADH concentration in the assays was 50 \( \mu \)M. Rates of Q\(_1\) and Q\(_2\) reduction were background-corrected by the rate of quinol formation when using DBMIB as the sole electron acceptor as shown in the bottom section of the table. For calculation of \( K_i \) and \( K_s \), the equation for mixed inhibition was applied: \( V_0 = \frac{(V_{max} \cdot [S])}{(K_m + [S])} \) where \( \alpha = 1 + [I]/K_i \) and \( \alpha = 1 + [I]/K_s \) (25).

| Q\(_1\) reductase                           | Michaels-Menten formalism | No DBMIB | 10 \( \mu \)M DBMIB | 25 \( \mu \)M DBMIB |
|-------------------------------------------|---------------------------|----------|-------------------|-------------------|
| R\(^2\)                                   | 0.9963                    | 0.9817   |                   |                   |
| V\(_{max}\) (\( \mu \)mol min\(^{-1}\) mg\(^{-1}\)) | 25.8 ± 0.6                | 22.6 ± 1.4 | 15.2 ± 1.1        |                   |
| K\(_m\) (\( \mu \)M)                      | 14.0 ± 0.9                | 19.4 ± 3.1 | 39.5 ± 6.0        |                   |
| K\(_i\) (\( \mu \)M)                      | 35.9                      | 38.7     |                   |                   |
| K\(_i\) (\( \mu \)M)                      | 70.6                      | 35.8     |                   |                   |

TABLE 3

| NqrA/Q\(_1\)/DBMIB | Na\(^+\)-NQR/Q\(_1\)/DBMIB | NqrA/Q\(_1\)/HQNO |
|--------------------|-----------------------------|-------------------|
| R\(^2\)            | 0.97                        | 0.83              |
| k                  | 26.69 ± 2.59                | 28.52 ± 11.18     |
| \( \Delta F_{STD,max} \times c_{molar} / (k + c_{molar}) \) | 50.97 ± 8.69 |
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d all samples, DMSO-d₆ was added to a final concentration of 5% in the sample. Two samples for control experiments were prepared in the same manner but excluding NqrA or quinone analogs, respectively. The two-dimensional NOESY experiments were performed with water suppression achieved by low power presaturation of the water signal during relaxation delay and mixing time (28, 29). The mixing time was set to 600 ms. Up to 1024 increments with 48 scans in each increment were collected at a spectral width of 10 ppm. Selective one-dimensional NOESY experiments based on the double pulsed field gradient spin-echo method were performed using Bruker’s pulse program SELNOGP (30). Resonances of interest were selectively excited with a low power Gaussian-shaped pulse. The mixing time was set to 600 ms. Up to 8192 scans were collected at a spectral width of 20 ppm.

All NMR experiments were acquired at 300 K on a Bruker AVANCE III 600-MHz spectrometer equipped with a cryogenic 5-mm TCI-H/C/N triple resonance probe with actively shielded z-gradient. Spectra were processed and analyzed with the software TopSpin (Bruker; v3.1).

RESULTS

**Interaction of Na⁺-NQR with Q₁ and DBMIB Monitored by the Electron Transfer Activities**—As DBMIB is an analog and antagonist of ubiquinone, it was used in this work to obtain information on quinone binding by the Na⁺-NQR. First, we questioned whether DBMIB has an influence on NADH oxidation by the Na⁺-NQR. The effect of DBMIB on NADH oxidase activity was assayed by maintaining a constant concentration of the artificial electron acceptor Q₁, while varying the concentration of the substrate NADH. Both NADH oxidation and quinol formation rates were recorded. Determined Vₘₐₓ and Kₘ values for NADH oxidase activity were 147.5 ± 1.7 μmol min⁻¹ mg⁻¹ and 29.6 ± 0.6 μM, respectively, when quantifying NADH oxidation and 38.9 ± 1.2 μmol min⁻¹ mg⁻¹ and 24.5 ± 1.5 μM, respectively, when analyzing quinol formation (Table 1). The difference between the two rates is caused by reaction of ubisemiquinone with oxygen as described (31). Preincubation of the enzyme with 10 μM DBMIB led to a decrease in the NADH oxidation rate Vₘₐₓ as well as in the Michaelis constant Kₘ with similar manifestations on the NADH oxidation and quinol formation rates (Fig. 2).

Varying the ubisemiquinone-1 concentration of the buffer while starting the assay always by adding 50 μM NADH allowed for characterization of the quinone reductase activity of the Na⁺-NQR. Assays in the absence of DBMIB displayed a clearly defined Michaelis-Menten-like behavior of the enzyme with a Vₘₐₓ value of 25.8 ± 0.6 μmol min⁻¹ mg⁻¹ and a Kₘ value of 14.0 ± 0.9 μM for quinol formation (Table 2). If quinone reductase activity was measured in the presence of 10 or 25 μM DBMIB (Fig. 3, upper panel), a decrease of Vₘₐₓ and an increase of Kₘ values were observed (Table 2).

We also performed enzymatic assays with ubiquinone-2, decylubiquinone, and ubiquinone-10. With Q₁₀, similar quinol formation rates were observed as with Q₁; however, the measured rates show a sigmoidal dependence on the concentration of Q₁₀ and did not yield meaningful kinetic parameters (Fig. 3, lower panel, and Table 2). This observation is in accordance with earlier reports (32) and is most likely caused by diffusion and desolvation processes involving the DDM micelle that become rate-limiting under these conditions. In the case of decylubiquinone and Q₁₀, significantly lowered rates compared with Q₁ were observed (data not shown). This precludes inhibition studies with DBMIB because, under these conditions, DBMIB itself will be reduced much faster than decylubiquinone or Q₁₀.

**Binding of DBMIB to the NqrA Subunit**—We have recently shown that subunit NqrA of the Na⁺-NQR harbors a Q binding site (9). We now asked whether NqrA also interacts with DBMIB. NqrA contains three tryptophan residues. Measurement of tryptophan fluorescence during DBMIB titration
enabled us to detect changes in the microenvironment of the binding site. Incubation of NqrA with DBMIB led to quenching of the fluorescence. Saturation of the quenching was observed at concentrations >100 μM DBMIB with 6% of the original fluorescence remaining, indicating that at least two of the three tryptophan residues of the protein are closely affected by DBMIB binding. The data were fitted to one-site and two-site binding models (Fig. 4). The goodness of fit was slightly higher with the two-site model, yielding an apparent \(k_D\) value of 3.08 ± 1.11 μM and a second \(K_D\) value of 33.24 ± 34.10 μM (Table 4).

As a control, we performed the same titration with Q1 (data not shown); however, Q1 is apparently not as well suited for fluorescence quenching studies as DBMIB as documented by fluctuating data points at low concentrations. Therefore, we refer to the \(K_D\) values reported previously (9).

STD NMR confirmed the binding of DBMIB to NqrA with, at a given ligand-to-protein-ratio, STD effects that were even stronger (maximum, 25%) than with Q1 (maximum, 8.5%) (data not shown). Furthermore, signals in the proton NMR spectrum of DBMIB became broad and shifted upon binding to NqrA, which is indicative of a binding kinetic at the intermediate NMR time scale. To circumvent problems caused by exchange, broadening STD effects were quantified at a higher excess of DBMIB (40:1) as compared with experiments with Q1. The strongest STD signals originated from the isopropyl group of DBMIB (methyl groups, 25.0%; CH group, 24–25%). The methyl group bound directly to the quinone ring showed an STD effect of 16.1%. This suggests that the isopropyl group of DBMIB is in closest contact to NqrA. 

**TABLE 4**

| Binding of DBMIB to the NqrA subunit according to tryptophan fluorescence quenching |
|-----------------------------------------------|---|
| **One-site binding model:** \(y = B_{max} \times x/(k_1 + x)\) | **Two-site binding model:** \(y = B_{max1} \times x/(k_1 + x) + B_{max2} \times x/(k_2 + x)\) |
| \(R^2\) | 0.99645 |
| \(B_{max1}\) | 0.36 ± 0.17 |
| \(B_{max2}\) | 0.71 ± 0.20 |
| \(k_1\) (μM) | 33.24 ± 34.10 |
| \(k_2\) (μM) | 3.08 ± 1.11 |

**FIGURE 4.** Binding of DBMIB to NqrA monitored by quenching of tryptophan fluorescence. Upper panel, fluorescence emission spectra of, from top to bottom (using 338 nm as reference), 1 μM NqrA titrated with 0, 0.5, 1, 2.5, 5, 7.5, 10, 20, 30, 50, 100, and 200 μM DBMIB. The bottom-most spectrum is buffer only (50 mM Tris-HCl, 300 mM NaCl, pH 8, 5% glycerol). The excitation wavelength was 295 nm. Samples were measured in triplicates; one spectrum of each condition is shown. DBMIB exhibits no fluorescence in the analyzed wavelength range (data not shown). Lower panel, the increase in quenching (ΔF) of the tryptophan emission at 338 nm depicted in the upper panel was normalized (norm) to values between 0 and 1 and plotted against the concentration of DBMIB. Non-linear regression analysis using a one-site binding model (solid line) and a two-site binding model (dashed line) was performed. Error bars represent S.D.

**FIGURE 5.** Saturation transfer difference NMR spectroscopy of Q1 and DBMIB in the presence of NqrA. A reference (upper panel) and three STD NMR (three lower panels) spectra of Q1 at increasing DBMIB concentration in the presence of NqrA. The reference spectrum was scaled to 37% of its original intensity. Signals of the same intensity in the STD spectrum correspond to an STD effect of 37%. The panel shows growing STD effects of Q1 signals with increasing DBMIB concentration. Impurities and solvent signals are marked with asterisks. One-dimensional. \(I_x\), changes of saturation transfer difference NMR effects of Q1, depending on the concentration of DBMIB. Experimental data points were fitted to a one-site binding model (\(y = l_{max} \times x/(k + x)\); solid lines); the constant \(k\) was fitted globally for all curves.
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FIGURE 6. Saturation transfer difference NMR spectroscopy of Q1 and DBMIB in the presence of Na\textsuperscript{+}-NQR. Changes of saturation transfer difference NMR effects of Q1 depending on the concentration of DBMIB are shown. Experimental data points were fitted to a one-site binding model \( y = I_{\text{max}} \times x/(k + x); \) solid lines; the constant \( k \) was fitted globally for all curves.

FIGURE 7. Saturation transfer difference NMR spectroscopy of Q1 and HQNO in the presence of NqrA. Changes of saturation transfer difference NMR effects of Q1 depending on the concentration of HQNO are shown. As with DBMIB, experimental data points were fitted to a one-site binding model \( y = I_{\text{max}} \times x/(k + x); \) solid lines; the constant \( k \) was fitted globally for all curves.

levelled off at DBMIB concentrations above 80–100 \( \mu M \) in both cases. STD changes of signals of Q1 were fitted globally to a one-site binding model \( y = I_{\text{max}} \times x/(k + x); \) solid lines; the constant \( k \) was fitted globally for all curves.

An identical experiment was also done with Q1 and HQNO in the presence of NqrA. The influence of HQNO on STD effects of Q1 was very similar to the influence of DBMIB (Fig. 7). STD changes of signals of Q1 were fitted globally to a one-site binding model \( y = \Delta I_{\text{STD,max}} \times x/(k + x) \), which provided an \( EC_{50} \) value of 51.0 \( \mu M \) (Table 3).

The influence of DBMIB on STD effects of Q1 was also analyzed with regard to the binding mode of Q1 (Table 5). For this purpose, a ratio between the STD effect of Q1 at maximal concentration of DBMIB and in the absence of DBMIB was calculated. These ratios were then normalized to the minimal ratio (for H-10) that was set to 1. Rather than increasing or decreasing all STD effects of Q1 homogeneously, the quinone analog DBMIB affected the STD intensities of H-5’ and H-7 more strongly than the remaining signals of Q1. Our analysis shows that STD effects of H-5’ and H-7 increased disproportionately upon addition of DBMIB, suggesting that these protons are brought into closer contact to the binding site in the presence of the inhibitor.

The Inhibitors DBMIB and HQNO and Ubiquinone Q1 Occupy an Extended Quinone Binding Site—The fact that, according to STD NMR, DBMIB and HQNO, respectively, do not displace Q1 from the binding site but rather change the binding mode of the native substrate led us to surmise whether both pairs, Q1/DBMIB and Q1/HQNO, interact with NqrA in direct vicinity to each other.

To test this, we recorded NOESY spectra of both pairs of ligands in the presence of NqrA (Figs. 8 and 9). Indeed, we observed ILOEs between Q1 and DBMIB and between Q1 and HQNO (33). These correlations were only observed in the presence of NqrA. A mixture of both ligands in the absence of NqrA did not show ILOEs (Figs. 8 and 9) nor did NqrA show cross-peaks at these positions in the absence of ligands. It is important to note that the observation of ILOEs critically depends on the intrinsic signal-to-noise ratio and not from protein-mediated effects.

Further evidence for a critical role of NqrA for observing ILOEs is that, in the absence of NqrA, Q1 and DBMIB did not mutually perturb their chemical shifts. This indicates that both quinone analogs do not form complexes in solution.

**TABLE 5**

| Signal | No DBMIB | 150 \( \mu M \) DBMIB | Ratio 150 \( \mu M/0 \mu M \) DBMIB | Relative ratio (ratio[H-10] = 1) |
|--------|----------|-----------------|----------------|-----------------|
| H-11 | 6.0 | 26.3 | 4.38 | 1.07 |
| H-10 | 7.5 | 30.7 | 4.09 | 1.00 |
| H-5’ | 5.1 | 25.8 | 5.06 | 1.24 |
| H-7 | 3.4 | 20.0 | 5.88 | 1.44 |
| OCH\textsubscript{3} | 8.5 | 34.9 | 4.11 | 1.00 |

**DISCUSSION**

The kinetic characterization of the Na\textsuperscript{+}-NQR complex suggests that quinone reduction is best described by the classic Michaelis-Menten formalism, indicating a single catalytically active quinone binding site. However, recent surface plasmon resonance study of Q1 binding to the isolated NqrA subunit provided hints for the presence of a second quinone binding site with lower affinity (9). Furthermore, numerous studies report uncompetitive inhibition of the Na\textsuperscript{+}-NQR by inhibitor molecules that are supposed to interact with the quinone binding site (8, 34). It is also noteworthy that enzyme complexes catalyzing related chemical reactions (bc\textsubscript{1} complex and bc\textsubscript{f} complex) were indirectly shown to have spacious quinone binding sites that can accommodate more than 1 quinone eq at a time at least according to inhibitor studies by high resolution magic angle spinning NMR spectroscopy (21) and according to EPR experiments (20, 22).

Most recently, crystal structures of the alternative NADH dehydrogenase Ndi1 from yeast were reported (35, 36). Indeed,
Feng et al. (35) localized two ubiquinone-4 molecules in the binding site of Ndi1. One Q4 is located close to FAD, and the other appears to be only loosely bound. This scenario might be very close to the situation of the Na\(^{+}\)-NQR characterized here.

It is noteworthy, however, that almost simultaneously Iwata et al. (36) published a structure of Ndi1 with only one Q2 mol-
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eule in the binding site bound in a rather different way as compared with the study of Feng et al. (35). This controversy awaits clarification by independent experimental methods, e.g. NMR.

Here we show that the NqrA subunit of the Na\textsuperscript{+}-NQR binds two quinone-type ligands adjacent to each other in an extended binding site. We used the quinone analog and antagonist DBMIB, a well known inhibitor of electron transfer complexes, to serve as a second quinone-type ligand that can easily be distinguished from Q\textsubscript{1} by NMR spectroscopy. In addition, we found very similar results with another well known inhibitor, namely HQNO, which had been proposed to interact with the aliphatic chain, suggesting that the alkyl chain of HQNO is and kinetic studies to Q\textsubscript{2} as a ligand was unsuccessful probably because of unfavorable kinetics and interference caused by the detergent DDM (32).

In the current study, we provide (to our knowledge for the first time in the case of quinone-binding enzymes) direct experimental evidence that two quinone analog molecules are situated in immediate vicinity to each other in the binding pocket of NqrA. This result was obtained by measuring ILOEs between ubiquinone-1 and the inhibitors DBMIB and HQNO, respectively. NOEs between both molecules could in principle derive from several situations. If Q\textsubscript{1} and DBMIB or HQNO formed stable complexes in solution one would expect intermolecular NOEs. However, in the absence of NqrA, we did not detect intermolecular NOEs (see control experiment shown in Figs. 8 and 9). Alternatively, NOEs between Q\textsubscript{1} and inhibitors could have been mediated by protons of the binding site of NqrA. Such intermolecular NOEs, called INPHARMA effects, could build up even in a purely competitive binding situation (37). However, for INPHARMA effects to develop, it is relevant that the magnetization has to be “stored” on the receptor during the ligand exchange process. This transfer is not possible if the experiment is carried out with a perdeuterated receptor, and thus, INPHARMA effects should be absent or at least strongly reduced under these conditions. We performed the same experiment with protonated as well as perdeuterated NqrA (\textsuperscript{2}H labeling \textgreater 95% according to mass spectrometry) and saw no significant reduction in the intensity of the ILOE cross-peaks (Fig. 8). The only plausible interpretation is simultaneous binding of both ligands directly adjacent to each other within an extended quinone binding site of NqrA.

In the case of DBMIB, the precise relative arrangement of Q\textsubscript{1} and DBMIB within the binding site cannot be determined to great accuracy from the ILOEs because of the generally low signal intensity and because essentially all protons of Q\textsubscript{1} show ILOEs to all protons of DBMIB with slightly varying intensities. Interestingly, in the case of HQNO, the strongest ILOEs toward protons of Q\textsubscript{1} originate from protons located at the terminus of the aliphatic chain, suggesting that the alkyl chain of HQNO is oriented toward Q\textsubscript{1} during simultaneous binding to NqrA (Fig. 9A). Furthermore, we could exploit the high sensitivity of selective one-dimensional double pulsed field gradient spin-echo NOESY experiments to show that H-3 is the only aromatic proton of HQNO that displays a weak ILOE effect to the methoxy groups of Q\textsubscript{1} (Fig. 9B). Taking all observed (and absent) ILOEs into account, we propose a plausible arrangement of the two ligands, Q\textsubscript{1} and HQNO, adopted during simultaneous binding to the NqrA subunit (Fig. 9C), noting, however, that this should not be considered as a unique solution.

The fact that DBMIB does not displace Q\textsubscript{1} from the binding site in a competitive manner is furthermore supported by an STD NMR titration. In a competitive binding situation, the STD effects of Q\textsubscript{1} should decrease upon titrating in DBMIB because DBMIB would displace Q\textsubscript{1} from the binding site, lowering the fraction of bound Q\textsubscript{1} and, thus, lowering the amount of saturation transferred from NqrA to Q\textsubscript{1}. However, we observed exactly the opposite. Increasing the concentration of DBMIB led to a markedly increasing STD effect of Q\textsubscript{1}. In addition, in the presence of DBMIB, the ratio of STD effects of individual protons differed from the situation without inhibitor. This suggests that DBMIB alters the binding mode of Q\textsubscript{1}, which in turn leads to a more efficient saturation transfer and therefore stronger STD effects. The EC\textsubscript{50} of this interaction was 27 \(\mu\text{M}\). A similar effect was observed in the case of HQNO with an EC\textsubscript{50} of 51 \(\mu\text{M}\). This finding, in addition to the presence of ILOEs between Q\textsubscript{1} and both DBMIB and HQNO, respectively, suggests that simultaneous binding of quinone analog ligands could be a general mechanism.

Our current results show that DBMIB, at the accessible concentration of 400 \(\mu\text{M}\), is not able to displace Q\textsubscript{1} from the binding site as we saw the highest STD effects of Q\textsubscript{1} at this concentration of DBMIB. Also the presence of substantial ILOEs under these conditions indicates that apparently the “mixed situation” with 1 eq of Q\textsubscript{1} and 1 eq of DBMIB residing at the same time in the binding site is favored over double occupancy by 2 eq of DBMIB. However, the two-dimensional NOESY experiments provide evidence for double occupancy of two Q\textsubscript{1} molecules within the extended binding site of NqrA. Rather strong NOE correlations were observed between methyl groups of the isoprenyl chain of Q\textsubscript{1} and the methoxy groups of Q\textsubscript{1}. Within the same Q\textsubscript{1}, these protons are separated by 8–10 Å. Therefore, it is unlikely that these correlations originate from intramolecular dipolar interactions. Thus, we propose that these NOE contacts are indeed ILOEs between two individual Q\textsubscript{1} molecules. The fact that these cross-peaks showed up in the presence of protonated as well as deuterated NqrA further supports that these are true ILOEs and not due to relay or spin diffusion effects mediated by the protein.

Can these results, obtained with the isolated NqrA subunit, be transferred to the holo-Na\textsuperscript{+}-NQR complex? To gain insight into the physiological relevance, we performed the same STD NMR titration described above with Q\textsubscript{1} and DBMIB in the presence of holo-Na\textsuperscript{+}-NQR. This experiment provided an EC\textsubscript{50} value of 28.5 \(\mu\text{M}\), which is virtually identical to the EC\textsubscript{50} value for NqrA.

To further confirm this result, we performed an enzyme inhibition experiment with the entire enzyme complex. In the presence of DBMIB, the rate of NADH oxidation (\(V_{\text{max}}\)) was decreased, whereas \(K_{m}\) was lowered. This is characteristic of an competitive mode of inhibition typical for multisubstrate enzymes. That the catalytic site of NADH oxidation was not perturbed by DBMIB is indicated by the fact that the ratios of \(V_{\text{max}}/K_{m}\) in the presence or absence of DBMIB remain constant (Table 1). First, this confirms the notion that DBMIB dis-
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rupts the electron pathway further downstream, presumably at the quinone reductase site due to the structural resemblance to ubiquinone (Fig. 1). Second, this ascertainsthat NADH oxidase and quinone reductase sites of the Na\textsuperscript{+}-NQR are coupled in our experimental setup and no electrons short circuit to quinone at the level of the NADH oxidase. This needs to be considered because Q\textsubscript{1} can serve as an artificial electron acceptor when assaying the isolated NADH dehydrogenase domain (38). For quinone reduction, we observed a mixed mode of inhibition in the presence of DBMIB as documented by the rates of quinol formation.

Evaluating our experiments according to a Michaelis-Menten model yielded an average inhibition constant ($K_a$) of 53.2 $\mu$M. This $K_a$ value is strikingly similar to the $EC_{50}$ values determined in the STD NMR experiments with the NqrA subunit and the holo-Na\textsuperscript{+}-NQR complex (26.7 $\pm$ 2.6 and 28.5 $\pm$ 11.2 $\mu$M, respectively). In summary, this strongly indicates that the interaction of the inhibitor DBMIB with the extended binding site of the NqrA subunit is causing the inhibition of enzymatic turnover measured with the holoenzyme complex.

Recently, Juárez et al. (10) investigated in V. cholerae Na\textsuperscript{+}-NQR two mutations that had been discovered earlier in the Na\textsuperscript{+}-NQR of V. alginolyticus. These mutations in subunit NqrB confer resistance to the antibiotic korormicin in V. alginolyticus (8). Mutations of glycine residues Gly-140 and Gly-141, confer resistance to the antibiotic korormicin in the authors to propose that the NqrB subunit carries the site of reduced ubiquinone to the ubisemiquinone and subsequently to ubiquinol, which is then released by the enzyme. A schematic view of the Na\textsuperscript{+}-NQR complex with the two binding sites for quinones and quinone analogs located on the NqrA subunit is shown in Fig. 10.

The presence of two quinones in the binding site could enhance the efficiency of catalysis in at least two ways. One quinone ligand could serve essentially as a cofactor that remains tightly bound to the enzyme and switches between the quinone and the semiquinone state, whereas the other quinone ligand is much less tightly bound, reduced in two steps to the quinol, and then released from the enzyme. Alternatively, the second, low affinity binding site could constitute a waiting position for the next ubiquinone substrate to enter the site of reduction, leading to a faster supply of fresh substrate and, thus, accelerated enzymatic turnover. Which of these mechanisms is finally operational in the Na\textsuperscript{+}-NQR will be addressed in future studies.

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