Supporting Information

Juárez et al. 10.1073/pnas.1002866107

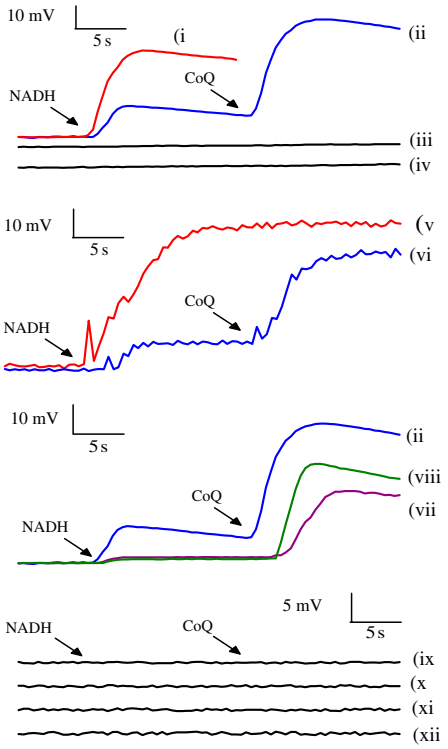

Fig. S1. ΔΨ formation by reconstituted wild type and mutant Na^+-NQR. The experiments were performed with wild type (traces i-viii) or mutant (ix-xii) Na^+-NQR reconstituted into proteoliposomes, using oxonol VI (traces i-iv and vii-xii) or with RH421 (v and vi), as described under *Materials and Methods*. Unless otherwise stated the reaction buffer contained 100 μM CoQ, 100 mM NaCl, 50 mM HEPES, 150 mM KCl, 1 mM EDTA, pH 7.5. CoQ was omitted for single turnover experiments (traces ii and vi) and the reaction was started by the addition of 100 μM NADH. Membrane potential was examined in wild type Na^+-NQR in the presence of NADH, CoQ and sodium (trace i and v), with 5 mM ETH-157 (trace iii) or with valinomycin (trace iv). Traces ii and vi show the results of adding NADH before CoQ. Traces vii and viii show the effect of $CoQH_2$ on membrane potential with 100 μM (trace vii) or 300 μM NADH (trace viii). These experiments were performed with the following mutants NqrB-T236Y (trace ix), NqrC-T225Y (trace x), NqrB-D346A (trace xi) and NqrB-D397A (trace xii).

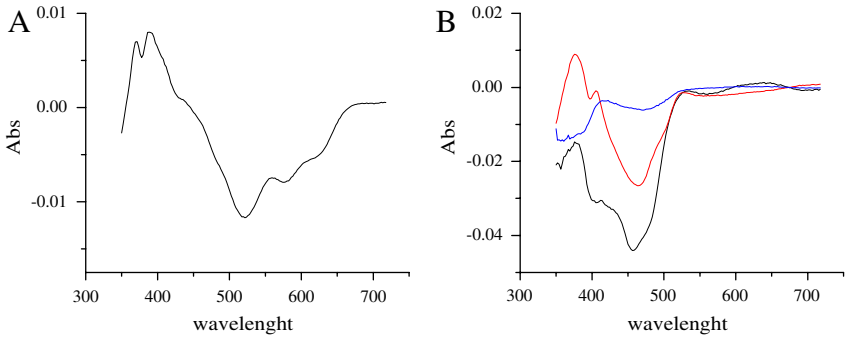

Fig. S2. Difference spectra of the phases of reduction of wild type Na^+-NQR with $CoQH_2$ (*A*) and NADH plus $CoQH_2$ (*B*). Black, first phase of reduction (FAD → $FADH_2$ plus F → $F^{\bullet -}$); red, second phase of reduction (F → $F^{\bullet -}$); blue, third phase of reduction ($F^{\bullet -}$ → FH_2).

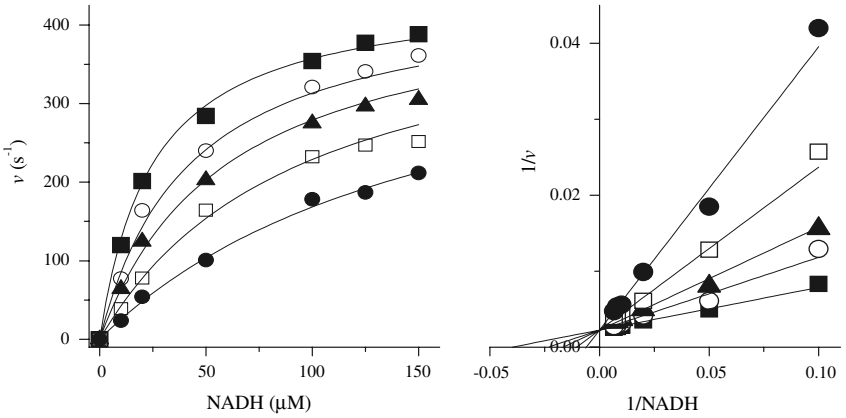

Fig. S3. Saturation kinetics by NADH of wild type Na^+-NQR in the presence of different concentrations of $CoQH_2$ (mM): ■; 0, ○; 0.5, ▲; 1, □; 2,•; 4.

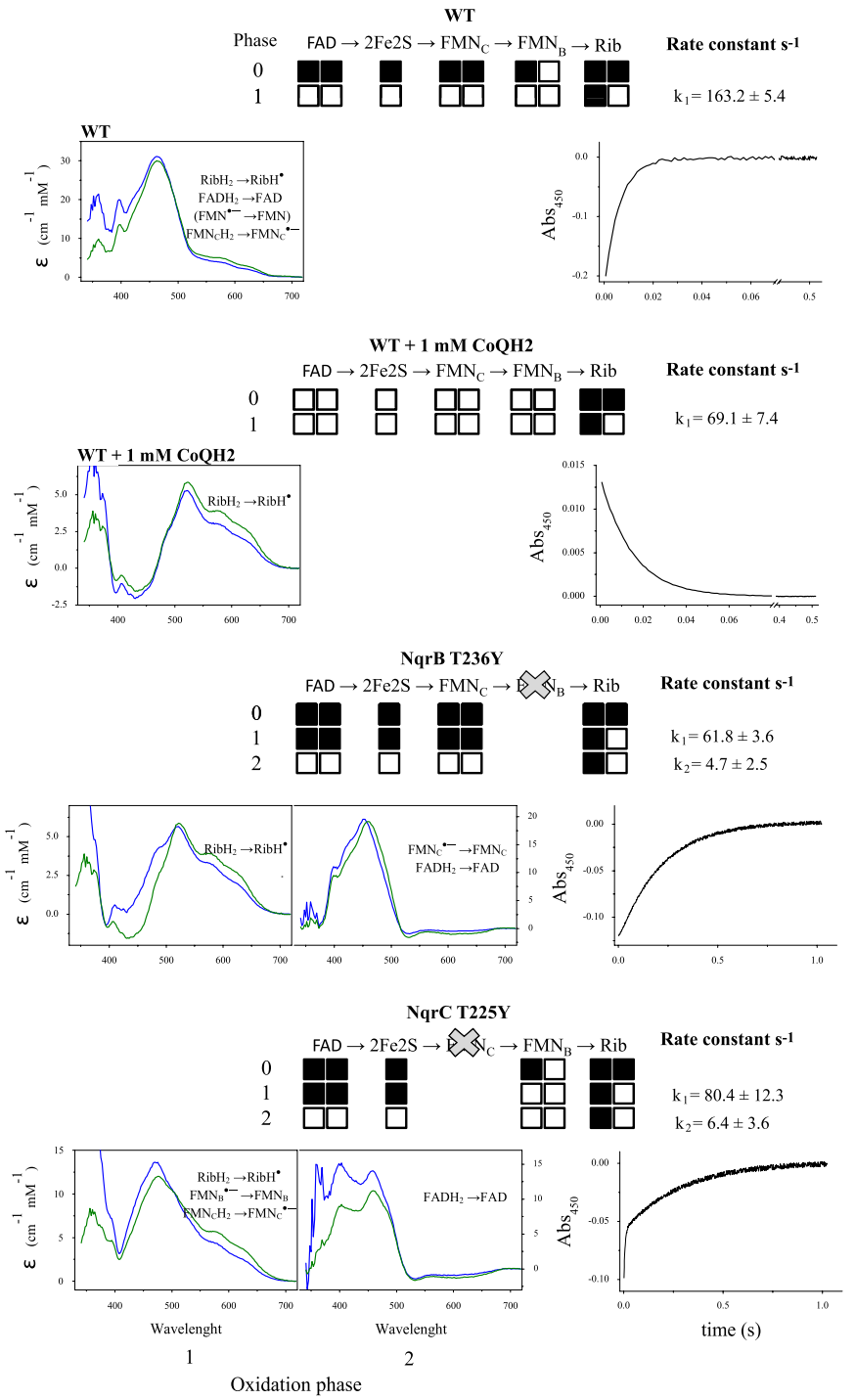

Fig. S4. Oxidation phases of wild type, wild type preincubated with 1 mM $CoQH_2$, NqrC-T225Y and NqrB-T236Y. Blue lines represent the differential spectrum of the components associated with each transition. Green lines represent the difference spectra of the assigned redox steps, using the transitions found in wild-type Na^+-NQR. The kinetics of the absorbance at 450 nm is shown in the right panels. The upper part of each data series represents the observed redox transitions. The open squares indicate the oxidized state of the cofactor, and the black squares indicate the reduced form.

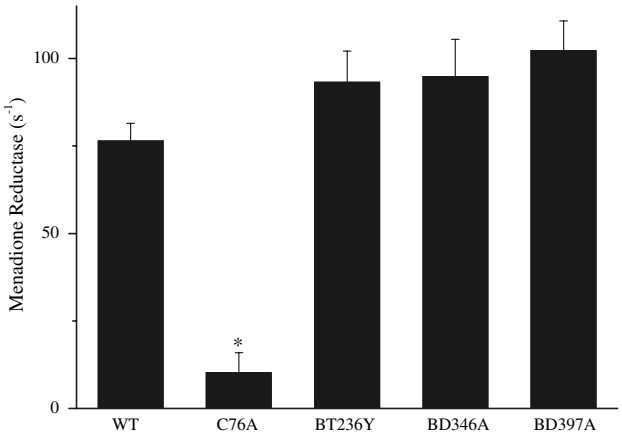

Fig. S5. Menadione reductase activity of wild type Na^+-NQR, NqrB-C76A, NqrB-T236Y, NqrB-D346A and NqrB-D397A. Menadione reductase activity was measured as indicated under *Materials and Methods*. *, $p < 0.005$.

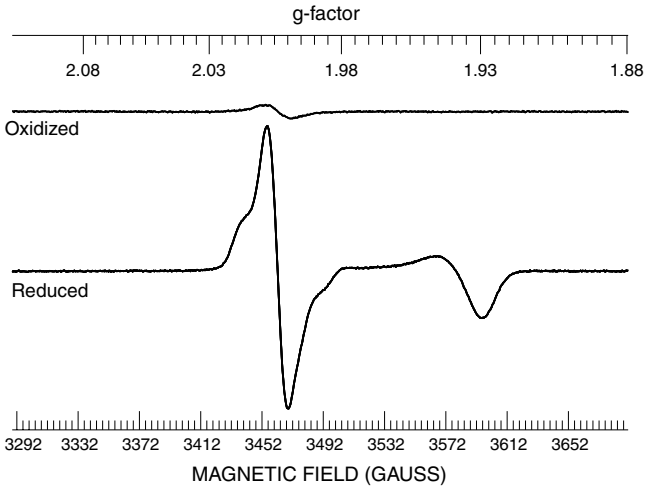

Fig. S6. X-band EPR spectra of NqrB-D346A mutant oxidized and reduced with 60 mM dithionite. Experiments were performed as described previously (1).

1 Barquera B, Ramirez-Silva L, Morgan JE, Nilges MJ (2006) A new flavin radical signal in the Na^+-pumping NADH:quinone oxidoreductase from *Vibrio cholerae*: An EPR/ENDOR investigation of the role of the covalently bound flavins in subunits B and C. *J Biol Chem* 281:36482–36491.

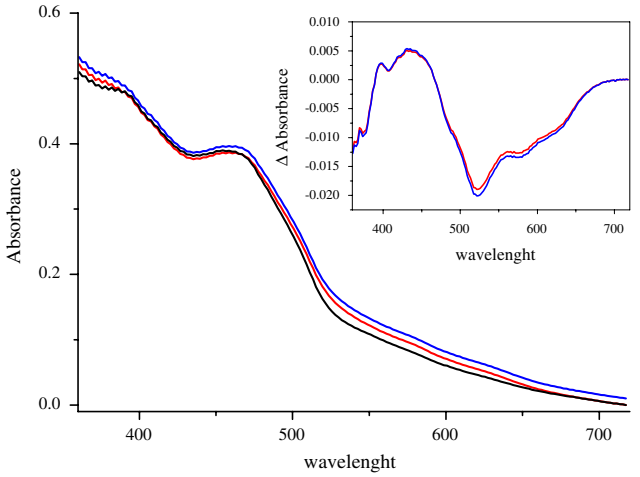

Fig. S7. Visible spectra of NqrB-D346 mutant. Black line: Oxidized spectrum (as prepared). The mutant enzyme was reduced using 60 mM dithionite, concentrated and washed twice with buffer containing Tris-HCl 50 mM pH 8.0, EDTA 1 mM, 5% glycerol, 0.05% dodecyl maltoside. Spectra were recorded after 1 hour (red) and 2 hour (blue) incubation in aerobic conditions at 4 °C. Inset shows the difference spectra of the sample after the dithionite reduction and washing, which is consistent with the reduction of the neutral radical (2). It is evident that the neutral radical formed during the reduction of the sample is stable during several hours under aerobic conditions.

2 Juarez O, Nilges MJ, Gillespie P, Cotton J, Barquera B (2008) Riboflavin is an active redox cofactor in the Na^+-pumping NADH:quinone oxidoreductase (Na^+-NQR) from *Vibrio cholerae*. *J Biol Chem* 283:33162–33167.

Table S1. Reduction steps of wild -type Na^+-NQR with $CoQH_2$ and NADH plus $CoQH_2$ in the presence of 100 mM NaCl

		rate constant (s^{-1})		
Reductant	Preincubation	K_1	K_2	K_3
1 mM $CoQH_2$		0.02 $RibH^{\bullet} \rightarrow RibH_2$		
100 μM NADH		170.4 $FAD \rightarrow FADH_2$ $RibH^{\bullet} \rightarrow RibH_2$	23.7 $2(F \rightarrow F^{\bullet -})$	0.45 $F^{\bullet -} \rightarrow FH_2$
100 μM NADH	1 mM $CoQH_2$	166.4 $FAD \rightarrow FADH_2$ $F \rightarrow F^{\bullet -}$	16.4 $F \rightarrow F^{\bullet -}$	0.3 $F^{\bullet -} \rightarrow FH_2$