# SUPPORTING INFORMATION

## Inhibition studies of cytochrome *bo*$_3$ (*cbo*$_3$) using a novel enzyme assay

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### Simulations, methods

Cyclic voltammograms were modeled using a finite difference procedure programmed in C according to Hirst et al.[1] The model is based on a kinetic scheme as shown in Figure SI1. Due to number of parameters in this already simplified model, we cannot obtained values for these parameters by fitting the cyclic voltammogram data nor can we use the experimental data to independently verify the whole of the model (but it is possible to verify parts of the model, see below). Instead, these models have been used to support the hypotheses made in the main article and parameters are fixed at physical relevant values.

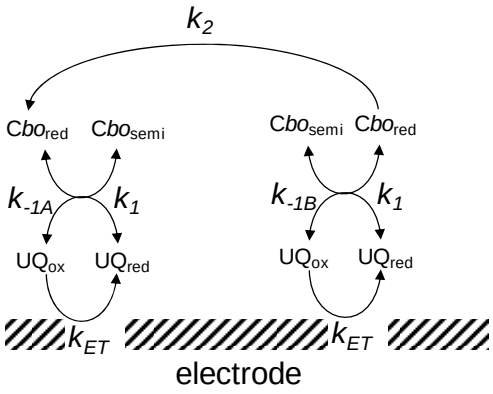


**Figure SI1**: Schematic representation of the model used to simulate the cyclic voltammograms. See text for more explanation.

*Rates and Parameters*

The interfacial electron transfer rate between the electrode and ubiquinol-10 (UQ-10) are modeled using the Butler-Volmer equations with the following parameters: electron transport rate at zero over-potential ($k_0$), reduction potential of UQ-10 ($E_0^{UQ}$) and $n_{app}$ to represent cooperativity of the two electron transfer process ($n$ = 1 for non-cooperative and $n$=2 for fully cooperative). The values used for these and other parameters are given in Table 1. Rate $k_1$ is calculated using Michaelis-Menten assuming the two consecutive reductions by ubiquinol – required for the reduction of single oxygen molecule – with equal rates. $K_M^{UQ}$ was estimated from the data shown in Figure 1B of the article and $k_1^{cat}$, like $k_2$ was set to an appropriate value for c*bo*$_3$ and varied to simulate inhibition. As all inhibition experiments were performed at > 10 times the apparent $K_M$ for oxygen, the oxygen reduction rate was assumed to be constant modeled with a single rate. The surface coverage of ubiquinol-10 ($\Gamma_{UQ}$) was varied as shown in the results below. The coverage of the enzyme ($\Gamma_{cbo}$) is fixed and given in Table 1. Note that for each reduction of ubiquinol-10 (UQ-10), 2 electrons are transferred and for each catalytic step ($k_2$) oxygen is thus reduced by 4 electrons to water.

| Parameter | Value |
|---|---|
| $k_0$ | 0.004 $s^{-1}$ |
| $k_1^{cat}$ | < 500 $s^{-1}$ |
| $k_2$ | < 250 $s^{-1}$ |
| $K_M^{UQ}$ | 5 pmol/cm$^2$ |
| $E_0^{UQ}$ | 0.05 V |
| $n_{app}$ | 1.33 |
| $\Gamma_{UQ}$ | Variable |
| $\Gamma_{cbo}$ | 50 fmol/cm$^2$ |

Table 1: Parameters used in the simulation

## Results

*Substrate inhibition*

Figure SI2A shows the simulated cyclic voltammograms for the model outlined above. In this simulation, in which no substrate or product inhibition is modeled, three observations can be made. First, the onset of the wave shifts to more positive potentials upon addition of UQ-pmol/cm$^2$, similar to the experimental results. The major increase in enzyme activity is seen when increasing the UQ-10 coverage till 20 pmol/cm$^2$, due to the chosen $K_M^{UQ}$ of 5 mol/cm$^2$. Finally, at UQ-10 coverages above 20 mol/cm$^2$, a quinone reduction peak becomes visible that appears superimposed on the catalytic wave.

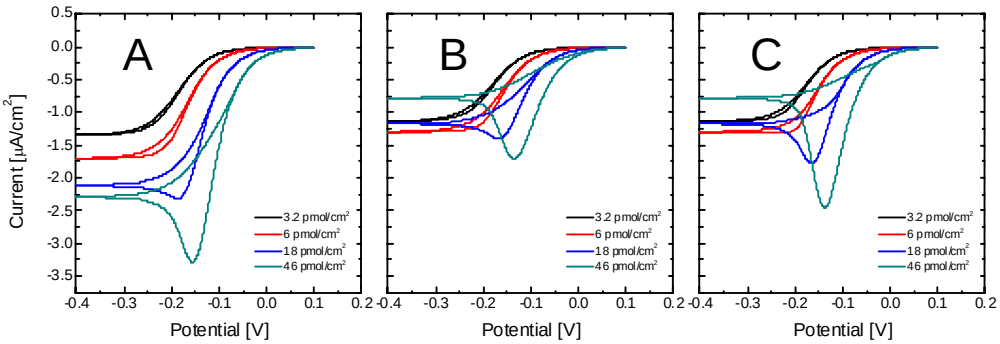


**Figure SI2:** Simulations of cyclic voltammograms using the models explained in detail in the text. (A) No inhibitions, (B) Substrate and product inhibition, (C) Substrate inhibition. Parameters used: $k_1^{cat}$ = 500 s$^{-1}$; $k_2$ = 250 s$^{-1}$; $\Gamma_{UQ}$ as indicated in the graphs.

In order to simulate the observed inhibition at high UQ-10 coverage (Figure 1 of the article), two models have been evaluated for their effectiveness to qualitatively describe the experimental data. In the first model, the UQ-10 oxidation activity ($k_1$) was inhibited as a function of the total UQ-10 concentration (oxidised or reduced) with a $K_I$ of 12.5 pmol/cm$^2$. Rate $k_1$ is thus multiplied by $K_I/(K_I + \Gamma_{UQ})$. This simulation is shown in Figure SI2B and the inhibition effect is clearly visible, as expected. Comparing Figure SI2A and SI2B shows that the 'superimposed' peaks at high UQ-10 coverage, due to the reduction of UQ-10 on the surface, are similar in magnitude. The (normalised) peak area is thus $\leq \Gamma_{UQ}$. In contrast, the experimental results indicate that the peak area exceeds $\Gamma_{UQ}$. To explain this, the simulation was adjusted so that catalytic rate ($k_1$) is only inhibited by reduced UQ-10 (substrate inhibition, Figure SI2C). In this case it is observed that the area underneath the peak at high $\Gamma_{UQ}$ exceeds $\Gamma_{UQ}$ supporting the hypothesis in the article that *cbo*$_3$ is inhibited by its ubiquinol (substrate), but not ubiquinone (product).

*Other inhibitors*

Figure SI3 shows simulations performed to model the inhibitory effects of NaCN, 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO) and Zn(II) ions. In Figure SI3A, the NaCN behaviour is modelled by decreasing the rate $k_2$ as NaCN binds to the binuclear active site of *cbo*$_3$ and impairs oxygen reduction. In the bottom row of Figure SI3, the first derivatives of the simulations are shown which can be compared to the experimental data in the article (Figure 2). Similar to the experimental data, the midpoint potential (peak in the first derivative) of the catalytic wave shifts to higher potential upon lowering the oxygen reduction rate, $k_2$.

In order to simulate the inhibitory effect of HQNO, the rate $k_1$ was systematically reduced (Figure SI3B). In contrast to the experimental data, this did not produce a set of cyclic

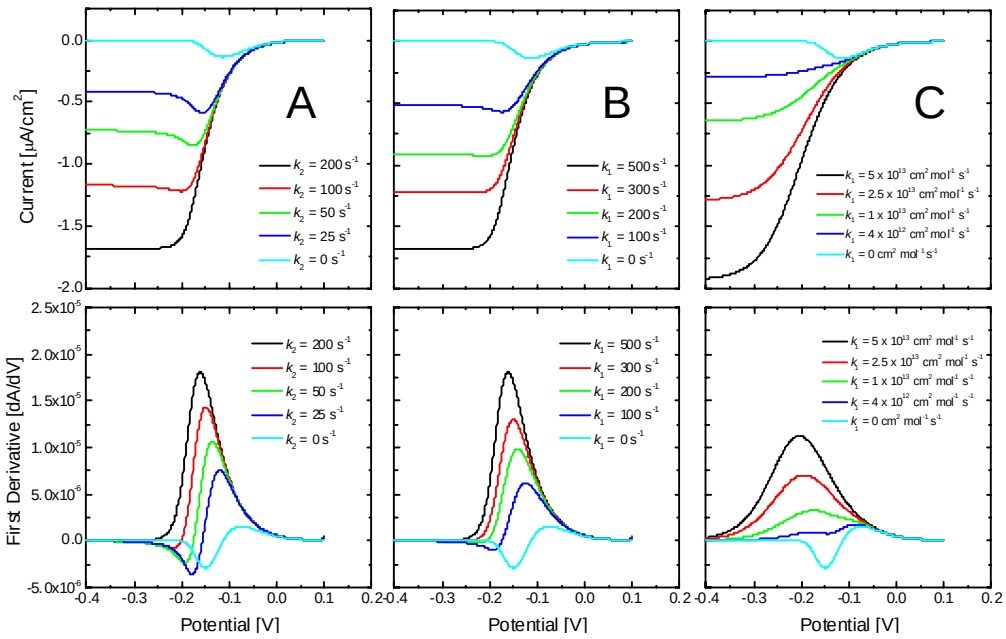


**Figure SI3:** Simulations of cyclic voltammograms (top) and the first dirivatives (bottom) using the models explained in detail in the text. (A) Parameters as shown in Table 1, $k_1$ = 500 $s^{-1}$, $\Gamma_{UQ}$ = 8 pmol/$cm^2$, $k_2$ as indicated. (B) Parameters as shown in Table 1, $k_2$ = 200 $s^{-1}$, $\Gamma_{UQ}$ = 8 pmol/$cm^2$, $k_1$ as indicated. (C) Alternative model in which rate $k_1$ is modelled as a reversible second order rate with rate $k_1$ as given and rate $k_{-1A}$ and $k_{-1B}$ (Figure SI1) calculated using the equilibrium using the reduction potential $E_0^{cbo3A}$ = 0.025 V and $E_0^{cbo3B}$ = 0.075 V, respectively.

voltammograms in which the midpoint of the catalytic wave remained largely unchanged. Detailed analysis of the modelling data indicated that this is due to the fact that the Michaelis-Menten kinetics does not take the reverse reaction into account (i.e., enzyme reduction by ubiquinone). We therefore repeated the calculations assuming the quinone/ubiquinol redox reactions are reversible assuming reduction potentials of the enzyme close to that ubiquinone/ubiquinol ($\Delta E$ =0.025 and 0.075 V for the first and second redox reaction respectively). This second model is shown in Figure SI3C, which now more closely follows the experimental observations in that the midpoint of the wave remains largely unaltered upon reducing the rate $k_1$. Another feature that changes is that the catalytic wave is significantly broadened, more closely representing the experimental data. The exact wave shape of the latter model is dependent on many parameters used for the simulations, but is generally a consequence of the fact that the enzyme's oxidation state is in equilibrium with the redox state of the ubiquinol/one pool. Finally, we have repeated all the simulations shown in Figure SI2 and SI3 using the reversible quinol/quinone reaction and the conclusions made above are still valid.

[1] Hirst, et al. (1998) *Anal Chem* 70, 5062