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Impedance spectroscopy of bacterial membranes: Co-enzyme Q diffusion in a finite diffusion layer

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Abstract

The inner membrane of *Escherichia coli*, over-expressing an ubiquinol oxidase, cytochrome *bo*₃ (*cbo*₃), was tethered in a planar configuration to a gold electrode. Electron transfer to *cbo*₃ was achieved via native ubiquinol-8 or added ubiquinol-10 and impedance spectroscopy was used to characterise the diffusion properties of the ubiquinol/ubiquinone in the tethered membrane system. Spectra were obtained at varying DC potentials covering the potential window in which the voltammetric catalytic wave of *cbo*₃ is visible. These spectra were compared to those obtained after addition of a potent inhibitor of *cbo*₃, cyanide, and the difference in impedance was analysed using a derived equivalent circuit, which is similar to that of Open Finite-Length Diffusion (OFLD) or the finite Warburg circuit, but with the boundary conditions modified to account for the fact that ubiquinol reoxidation is limited by enzyme activity. Analysis of the impedance spectra of the tethered membrane system gave kinetic parameters that are consistent with values obtained using cyclic voltammetry. Importantly, the diffusion rate of ubiquinone (10^{-13} - 10^{-12} cm²/s) was found to be orders of magnitude lower than accepted values for lateral diffusion (10^{-8} - 10^{-7} cm²/s). It is hypothesised that this result represent perpendicular diffusion of quinone across the membrane, corresponding to a 'flip' time between 0.05 and 1 s.

Introduction

The function of coenzyme-Q (quinone) in electron and proton transport (the "redox loop") has been undisputed since the chemiosmotic theory was generally accepted, about a decade after it was initially proposed by Mitchell.¹ However, many properties of this lipophilic quinone, like its exact position and orientation in the membrane, remain debated. The same is true for our understanding of the anti-oxidant role that has more recently been attributed to quinones.² It is now generally accepted that the polyisoprene chains of quinones lie in the hydrophobic mid-plane or the fatty-acid chain region of the membrane,³⁻¹⁰ mostly parallel with the membrane surface. However, it is debated whether this is in a linear or bend conformation. The position of the polar headgroup is either located in the mid-plane^{10,11} or at the tail-headgroup interface of the phospholipids.¹² Alternatively, it has been suggested that the quinone ring oscillates between both sites¹³ while the isoprenoid chain anchors the co-enzyme to the mid-plane. The reported diffusion rates of quinones also vary. Fluorescent quenching techniques usually report values in the order of 10^{-7} cm²/s.¹³⁻¹⁶ Fluorescence

recovery after photobleaching (FRAP) and electrochemical techniques using hybrid bilayers give values one order of magnitude lower.^{4,17}

Apart from the properties of the quinones themselves, the interaction between quinones and quinone enzymes also pose many unanswered questions. Quinones are substrates for a wide variety of enzymes, most of which belong the class of oxidoreductases and are either integral or peripheral membrane enzymes. The lipophilic properties of both the co-enzymes and the enzymes increase the complexity of these questions. Traditional enzyme assays have often taken one of two approaches. Water soluble analogues of co-enzyme Q can be utilised. These offer experimental advantages as the substrate can be titrated into the assay solution to study the substrate concentration profile. However, these analogues have been shown to have different positions and orientations in the membrane^{3,13,18} and slightly higher diffusion constants.¹⁷ Additional complications arise when interpreting results of studies with detergent-solubilised enzymes. Alternatively, assays are performed with membrane extracts using the intrinsic quinone levels or the extracts are depleted from quinones using organic solvents. Here, the physiological conditions are more closely realised but the resulting kinetic analyses can show high degree of scatter. Finally, the interpretation of these results could be further complicated by the existence of enzyme-supercomplex in these extract. For a long time it was believed that quinone-enzymes have a random organisation in the membranes (e.g., ref.^{19,20}), but now the hypothesis of enzyme supercomplexes is favoured again.^{21,22}

We have recently developed a system in which the inner membrane of *Escherichia coli* is tethered to a gold electrode modified with cholesterol groups to bind the membrane in a planar configuration.²³ In this supported membrane system, the quinone pool in the membrane can be electrochemically oxidised and reduced²⁴ while the quinone still interacts with the oxidoreductases as was shown by the catalytic activity of cytochrome *bo*₃ (*cbo*₃), an ubiquinol oxidase of *E. coli*.²³ In this thin layer system the quinone diffusion in the finite layer can, in principle, be studied with impedance spectroscopy. Here, we describe a theoretical approach to describe an equivalent circuit for our system and have applied this theory to impedance spectra of the supported membranes.

Methods

Materials

EO3-cholesterol was made as previously described.²⁵ 6-mercaptohexanol (6MH, Sigma), 2-propanol, methanol (HPLC grade, Fisher), *E. coli* polar lipid extract were used as received. All electrochemical experiments were performed in 20 mM (3-(N-Morpholino)propanesulfonic acid (MOPS) buffer (Sigma) with 30 mM Na₂SO₄ adjusted to pH 7.4 with NaOH at 20°C. Vesicles were prepared by mixing 5 mg lipid with 0.05 mg ubiquinone-10 (Sigma) in 50/50 methanol/chloroform and drying for 2 hours under nitrogen, vortexing in 1 mL buffer and extruding 11 times through 200 nm track-etched nucleopore membranes (Avanti).

Gold electrodes

Template-stripped gold (TSG) surfaces were prepared according to Stamou et al.²⁶ In short, 150 nm of gold (Goodfellow's) was evaporated on a polished silicon wafer (Rockwood Riddings Wafer Reclaim) using Edwards Auto 306 evaporator at $< 2 \times 10^{-6}$ mBar. The gold surfaces were then glued with EPO-TEK 377 to glass slides and cured for 120 min at 120°C. After cooling, the slides were detached from the silicon wafers to expose the TSG surface. SAMs were formed by directly immersing the substrate in 0.11 mM EO3-Cholesterol and 0.89 mM 6MH solution, in propanol, for 16 h. The samples were rinsed with propanol and

methanol, dried under nitrogen and used within several hours. It was shown previously that these surfaces contain ~60% EO3-Cholesterol and ~40% 6MH by area.²⁷

Supported membranes

Inner membranes from the *E. coli* strain, GO105/pJRhisA, lacking cytochrome *bd* and over-expressing cytochrome *bo*₃,²⁸ were isolated using a continuous-step sucrose gradient [35 – 55% (w/w)].²⁹ The protein content in the inner membranes was determined with a Schaffner-Weissman assay.³⁰ 50 μ L (5 mg/mL protein) of the inner membranes were mixed with 450 μ L *E. coli* polar lipid extract vesicles (5 mg/mL) and freeze/thawed three times using liquid nitrogen. This mixture was extruded another 11 times through 200 nm track-etched nucleopore membranes, stored at 4° C and used within 2 days. The mixed vesicles were added to the cholesterol modified TSG electrodes together with 10 mM CaCl₂ and incubated for an hour. The solution was rinsed thoroughly using 1 mM EDTA to remove the Ca(II). The electrode surface remained under fluid at all times.

Instrumentation

Cyclic voltammetry (CV) and Electrochemical Impedance Spectra (EIS) were obtained using a thermostatted electrochemical cell of all-glass construction held at 20° C, with the main compartment housing the gold working electrode (imbedded in a PTFE holder with a rubber O-ring), a platinum wire counter electrode and a saturated calomel reference electrode (SCE, Radiometer) or Ag/AgCl reference electrode (Radiometer). All potentials are quoted versus Standard Hydrogen Electrode (SHE). EIS was recorded using an Autolab electrochemical analyzer (Eco-chemie, Utrecht, The Netherlands) equipped with PGSTAT30 potentiostat and FRA2 frequency analyser. The cell was enclosed in a Faraday cage to minimize electrical noise and purged with Argon to remove oxygen. Typically, the oxygen was removed for 10 minutes after which the Argon was stopped while continuously recording CVs and monitoring the enzymatic activity of *cho*₃. Once the activity reached a maximum value and did not increase further, Argon was again blown over the electrolyte. We have previously established that increasing the flux to the electrode's surface by stirring the electrolyte solution did not further change the CV,²³ indicating that under these conditions, effects due to oxygen diffusion can be neglected. After the CV measurements, the EIS were recorded at varying DC potentials while recording CVs between each EIS to check enzyme activity remained unchanged. We have varied the DC potential either from high to low and from low to high potential and no difference was observed indicating prolonged exposure to DC potentials does not significantly change the supported membrane system. After these measurements 1 mM NaCN was added to the electrolyte which completely inhibited *cho*₃ as was confirmed with CV. The EIS experiments were then repeated as described in the Result section. Finally, experiments were also performed in aerobic conditions which gave identical results except that prolonged exposure to low potentials increased the non-enzymatic oxygen reduction wave at potentials below ≈ -0.15 V vs. SHE suggesting that in ambient aerobic conditions some damage can occur to the supported membrane.

The fits were performed by a program that uses a Levenberg-Marquardt procedure and the errors in the parameters were analysed using a Monte-Carlo bootstrap method. These errors are included in the Figures of the Result section, but usually lie within the size of the data-points, while differences between experiments are usually much larger.

Theory

The theory presented here only takes the electrochemical reduction of the quinone into account because the quinol oxidation can be neglected for the electrochemical potentials used in this work. However, the theory can easily be extended to include oxidation too.

Charge transfer for cyclic voltammetry

The equilibrium rate current (I_{DC}) is dependent on the interfacial ET rate to the quinone (k_r), quinone diffusion and on the enzyme turnover. As is discussed in the Result section, it is not possible to distinguish between quinone diffusion and enzyme turnover using the data obtained with cyclic voltammetry. For the purpose of this model, the quinone diffusion from the electrode to the enzyme across the distance δ_e is therefore not specifically defined but combined with the enzyme turnover in a single rate constant, k_2 . Using a membrane thickness of δ_m the following equilibrium rate can thus be defined,

$$\frac{\partial c_O}{\partial t} = 0 = k_2 c_R(\delta_e) - k_r c_O(0) \quad (1)$$

$$\delta_m c_O(\delta_e) = \delta_m c_O(x) = \delta_m c_O(0) = \Gamma_O \quad (2)$$

$$\delta_m c_R(\delta_e) = \delta_m c_R(x) = \delta_m c_R(0) = \Gamma_R \quad (3)$$

$$\Gamma_O + \Gamma_R = \Gamma, \quad (4)$$

in which c_O is the concentration of oxidised quinone, c_R the concentration of reduced quinone and $c_O(x)$ and $c_R(x)$ their relative concentration at distance x from the electrode surface. Γ , Γ_O and Γ_R are the two-dimensional surface coverages of the total, oxidised and reduced quinones, respectively. Figure 1 shows a scheme of the tethered membrane system in which the distances δ_m and δ_e are indicated. Eqs. 1 - 4 can be rewritten as:

$$\Gamma_O = \frac{k_2 \Gamma}{k_r + k_2}, \quad (5)$$

and thus the current can be given by³⁴

$$I_{DC} = -nFA\Gamma \frac{k_2 k_r}{k_2 + k_r}, \quad (6)$$

in which n is the number of electron transferred (2 for quinones), F the Faraday constant and A the electrode area. If the electron-transfer kinetics obey the Butler-Volmer equation then k_r for a 2-step electron transfer reaction is given by³³

$$k_r = k_0 e^{-(\gamma+1-\alpha)\frac{F}{RT}(E-E^0)}, \quad (7)$$

in which $\gamma = 1$ for a 2-electron reaction, α is charge transfer coefficient, k_0 the exchange rate and the remaining symbols have their usual meaning.

Charge transfer for impedance spectroscopy

To model the impedance of the Faradaic electrochemical system we follow the approach of Girault³⁴ combined with concepts introduced by Boillot.³⁵ In contrast to I_{DC} , we assume that the current for impedance spectroscopy (I_{AC}) is dependent on the diffusion of the quinone through the membrane and the diffusion effects thus have to be incorporated in the model. If the electron-transfer kinetics obey the Butler-Volmer equation then the current can be given by

$$I_{AC} = -nFAc_O(0)k'_r \quad (8)$$

$$k'_r = k'_0 e^{-(\gamma+1-\alpha)\frac{F}{RT}(E-E^0)} \quad (9)$$

Note that to compare rates (k) used in Eqs. 1-7 with those from Eqs. 8-9 we need to approximate

$$\Gamma_O k \approx c_O(0)k' \quad (10)$$

$$k \approx \frac{k'}{\delta_m} \quad (11)$$

Concentration and diffusion for impedance spectroscopy, Model 1

After a Laplace transform of the expression of the current as a function of the potential and the interfacial concentration, the following definition for the Faradaic impedance, Z_f , can be obtained:³⁴

$$Z_f = \frac{\Delta \bar{E}}{\Delta I_{AC}} = \left(\frac{\partial E}{\partial I_{AC}} \right) - \left(\frac{\partial I_{AC}}{\partial c_O(0,t)} \right) \left(\frac{\partial E}{\partial I_{AC}} \right) \frac{\Delta \bar{c}_O(0,s)}{\Delta \bar{I}_{AC}(E)} \quad (12)$$

Z_f is the sum of two impedance terms (three if oxidation had been included). The first term is the resistance of electron transfer and its reciprocal can be obtained by differentiating eq. (8) with respect to the potential,

$$R_{ET}^{-1} = \frac{\partial I_{AC}}{\partial E} = \frac{nF^2}{RT} A(\gamma+1-\alpha)c_O(0)k'_r \quad (13)$$

The second term of eq. 12 is the concentration impedance and consist of three parts. The second part is identical to R_{ET}^{-1} (Eq. 13) and the first part is obtained by differentiating Eq. 8 with respect to the interfacial concentration,

$$\frac{\partial I_{AC}}{\partial c_O(0,t)} = -nFAk'_r \quad (14)$$

Finally, the third and last part can be obtained by solving the Fick diffusion equation,

$$\frac{\partial c_{O(x,t)}}{\partial t} = D_O \frac{\partial^2 c_O(x,t)}{\partial x^2}, \quad (15)$$

with the boundary conditions,

$$c_O(x,0) = c_O \quad (16)$$

$$\frac{\partial c_o(\delta_e, t)}{\partial t} = -\frac{I_{DC}}{nFA} \quad (17)$$

The latter boundary condition describes the fact that after diffusion for distance δ_e the quinone is reoxidised by the enzyme with a rate that is measured using cyclic voltammetry (or I_{DC} , see Eq. 6). Transforming Eq. 15 and 17 into Laplace space gives

$$\bar{c}_o(x, s) = \frac{c_o}{s} + A(s) e^{-\sqrt{\frac{s}{D_o}}x} + B(s) e^{\sqrt{\frac{s}{D_o}}x} \quad (18)$$

$$\bar{c}_o(\delta_e, s) = -\frac{I_{DC}}{nFA s^2} + \frac{c_o}{s^2} \quad (19)$$

On either side of the diffusion layer we then have,

$$\bar{c}_o(0, s) = \frac{c_o}{s} + A(s) + B(s) \quad (20)$$

$$\bar{c}_o(\delta_e, s) = -\frac{I_{DC}}{nFA s^2} + \frac{c_o}{s^2} = \frac{c_o}{s} + A(s) e^{-\sqrt{\frac{s}{D_o}}\delta_e} + B(s) e^{\sqrt{\frac{s}{D_o}}\delta_e} \quad (21)$$

which can be used to calculate expressions for $A(s)$ and $B(s)$ (with $\bar{c}_o(0, s)$ still as a variable). The current is given by

$$\frac{-I_{AC}}{nFA} = D_o \left[\frac{\partial \bar{c}_o(x, s)}{\partial x} \right]_{x=0} = [B(s) - A(s)] \sqrt{s D_o} \quad (22)$$

Using Eqs 22 to solve $\bar{c}_o(0, s)$ and thus the expressions for $A(s)$ and $B(s)$, the expression of $\bar{c}_o(x, s)$ (Eq. 18) follows which can be used to obtain, finally,

$$\frac{\Delta \bar{c}_o(0, s)}{\Delta I_{AC}} \approx \frac{\tanh\left(\sqrt{\frac{s}{D_o}}\delta_e\right)}{nFA \sqrt{s D_o}} \quad (23)$$

This term is exactly the same as the equation for concentration impedance for a diffusion layer of fixed thickness (the OFLD - Open Finite-Length Diffusion element). The total Faradaic impedance is therefore given by (Eqs. 12, 13, 14 and 23)

$$Z_f = R_{ET} \left(1 + \frac{k'_r}{\sqrt{D_o}} \frac{\tanh\left(\sqrt{\frac{s}{D_o}}\delta_e\right)}{\sqrt{s}} \right) \quad (24)$$

Concentration and diffusion for impedance spectroscopy, model 2

At very low frequency, the concentration of the oxidised and reduced species at distance δ_e from the surface could start to become influenced by the AC fluctuations. To include this possibility in the model the boundary condition given in Eq. 17 needs to be modified to

$$\frac{\partial c_o(\delta_e, t)}{\partial t} = -\frac{I_{AC}}{nFA} \quad (25)$$

If the same derivation as above is followed, Eq. 23 is modified to

$$\frac{\Delta \bar{c}_o(0, s)}{\Delta \bar{I}_{AC}} \approx \frac{-1}{nFA s^2 \cosh\left(-\sqrt{\frac{s}{D_o}} \delta_e\right)} + \frac{\tanh\left(\sqrt{\frac{s}{D_o}} \delta_e\right)}{nFA \sqrt{s D_o}} \quad (26)$$

Comparison of Eq. 23 with Eq. 26 shows that Eq. 26 contains an extra term. However, for values of $\delta_e/\sqrt{D_o} > 0.1$, $D_o < \times 10^{-7} \text{ cm}^2/\text{s}$ and impedance frequency $\omega > 0.01 \text{ Hz}$ ($\omega = sj$ with $j^2 = -1$), the additional term found in Eq. 26 can be neglected. Eq. 23, which has three adjustable parameters (R_{ET} , $\delta_e/\sqrt{D_o}$ and $k'_r/\sqrt{D_o}$), is thus used to analyse the data obtained here.

Finally, we note that this circuit could also be used to model Faradaic impedance of a membrane-covered electrode in which the passage of the redox species through the membrane is rate limiting.

Results

A typical cyclic voltammogram of the supported membrane system, which includes the ubiquinol oxidase, cytochrome *bo*₃ (*cbo*₃), is shown in Figure 2. Oxygen is catalytically reduced by *cbo*₃ using the ubiquinol-10 as a native-like electron mediator. The oxygen (substrate) concentration is sufficiently high to saturate the enzyme and any contribution by oxygen diffusion can be neglected (see Weiss et al.²³ for more details). The latter point is illustrated clearly by the fact that stirring the electrolyte solution under these oxygen conditions does not change the cyclic voltammograms.²³ Furthermore, the supported membrane contains a known fixed concentration of ubiquinone ($\sim 8 \text{ pmol/cm}^2$ or $> 10 \text{ mM}$ assuming a 5 nm thick membrane), which is determined electrochemically at the end of the experiments by addition of the enzyme inhibitor, cyanide (Figure 2).

For the purpose of the discussion, it is useful to separate the observed catalytic wave in a low and high potential window. In the low potential region - where the over-potential for ubiquinol reduction is larger - the interfacial electron transfer kinetics between the gold and ubiquinol (k_r) will be relatively fast. In this region the reduction rate is thus either limited by the turn-over rate of *cbo*₃ or by ubiquinone diffusion kinetics. In the high potential region, in principle all three steps could be rate limiting (interfacial electron transfer, enzyme or ubiquinol diffusion kinetics). However, when the CV is compared to a CV taken when the enzyme catalysis is inhibited, the onset of the catalytic wave overlaps with the onset of the reduction peak of the ubiquinone. This indicates that in the high potential region enzyme kinetics are not rate limiting. The potential rate limiting steps are schematically shown in Figure 2.

Analysis of the CV is thus not able to fully describe the rate-limiting steps in the relatively complex supported membrane systems. In order to further investigate the electrode kinetics, electrochemical impedance spectra (EIS) were measured at varying DC potentials. The EIS data at high frequency reflect the RC element of the electrode with the solution resistance (R) in series with the double layer capacitance (C) of the electrode/membrane surface. We have previously described^{27,36} that the double layer capacitance of the supported membrane system shows a certain degree of frequency dispersion due to a Maxwell-Wager effect.^{37,38} In this study we are primarily concerned with the Faradaic reactions and thus the following approach has been adopted. EIS data were measured in the absence and presence of the inhibitor cyanide. With the inhibitor present, only the RC element contributes to the EIS data and the frequency dispersion of the double layer capacitance was modelled using a dispersion model as described previously.^{27,36} An additional resistor ('background resistance'), parallel to the capacitor, was added to the equivalent circuit (a) to model the increased conductivity of the supported membrane observed at low potentials (see³⁶ for more information) and (b) to model the non-enzyme catalysed oxygen reduction. This model and its parameters were then fixed and used to model the data measured without the inhibitor, placing the Faradaic circuit element parallel to the double layer capacitance. This provides an accurate platform with which the contribution of the Faradaic elements due to the enzymatic catalysis could be analysed.

Figure 3 shows a set of impedance spectra obtained at varying DC potentials with and without enzyme inhibitor. Analysis of data in the high potential region shows that the Faradaic component can be modelled using a single resistor (R_{ET}^{EIS}). When the values of R_{ET}^{EIS} are compared to resistance values measured from the CVs (R_{ET}^{CV} taken from the reciprocal first derivative of the CV), the values are similar (see Figure 4, Top). This confirms that R_{ET}^{EIS} represents the Faradaic current and indicates that in the high potential region the current is only limited by the interfacial electron transfer kinetics to the quinone (see Figure 1). In other words, in the high potential region the ubiquinol diffusion and turn-over rate of *cbo*₃ are fast compared to the interfacial electron transfer kinetics.

When the EIS in the low potential region is analysed, the catalytic current could not be modelled anymore with just a resistor. This is as expected when the DC current becomes limited by either the quinone diffusion or enzyme turn-over. In the Theory section we have derived Eq. 24 for the impedance of a redox active probe (ubiquinol) with diffusion constant, D_O , fixed in a thin membrane of thickness δ_m and which is continuously reoxidised by the enzyme after diffusion through the membrane with distance δ_e . Eq. 24 has four variables with three adjustable parameters, R_{ET} , $\delta_e/\sqrt{D_O}$ and $k'_t/\sqrt{D_O}$. R_{ET} is the resistance for the interfacial Faradaic electron transfer for ubiquinone reduction and k'_t the interfacial electron transfer rate to ubiquinone. R_{ET} and k'_t are, of course, related to each other and dependent on the DC potential (see Eqs. 8 and 13).

In Figure 4 all the fitted parameters are shown for three different sets of experiments and as a function of DC potential. As already mentioned, in the high potential window only R_{ET}^{EIS} can be given. We note, however, that also in the high potential region a fit could be obtained using Eq. 24. In this case $R_{ET} = R_{ET}^{EIS}$, but no reliable values for $\delta_e/\sqrt{D_O}$ and $k'_t/\sqrt{D_O}$ can be determined, as expected. Also at the very low potentials (- .25 V vs. SHE), no reliable values can be obtained because the EIS data is very similar with and without inhibitor and thus large errors in the fitted parameters were observed (Figure 3).

Discussion

We have considered other models to explain and fit the EIS data of our supported membrane system. Besides the thin film diffusion elements described in the Theory section, we considered simpler diffusion models like Warburg elements, but these were not able to describe the impedance data. Another option that was considered is that not the quinone, but *cho*₃ is responsible for the observed impedance behaviour. *Cho*₃ is a proton pump and the pumping of charged protons through the membrane could change the impedance spectra by dynamically changing the resistance of the membrane. However, we have performed the same experiments with *E. coli* inner membranes which only express cytochrome *bd* – an ubiquinol oxidase that is not a proton pump – and found the same behaviour. Furthermore, by directly comparing impedance spectra of the supported membrane system with and without inhibitor, all properties not related to the Faradaic impedance should not influence our analysis. In summary, we conclude that the analysis performed in this work must represent the Faradaic impedance of the quinone pool in the supported membrane system. We have thus analysed the impedance data using a model that is based on the Open Finite-Length Diffusion (OFLD) element which analyses the quinone diffusion along the direction normal to the electrode. The experimental system is more complex than this model and contains a mixture of diffusion elements normal and parallel to the surface (see Figure 1). To account for this fact two length parameters, δ_e and δ_m , are introduced. Solving the Fick equation for cylindrical diffusion would produce a more realistic model, but lies outside the scope of the present work. Furthermore, the latter model would include more parameters and it is likely that the impedance data is not able to establish the values of all parameters.

The validity of the model is shown by analysing the values obtained after fitting the data. In line with expectations, the parameter $\delta_e / \sqrt{D_O}$ is independent on DC potential and consistently lies between 2 and 4 s^{1/2} after analysing three sets of EIS data (see Figure 4; The weighted mean of $\delta_e / \sqrt{D_O}$ is 2.4 ± 0.2 s^{1/2}). Similarly and again in line with expectations, the parameter $k'_r / \sqrt{D_O}$ is exponentially dependent on the DC potential (see Eq. 8 and 9).

We now have two parameters, with three variables of which two can be estimated (δ_e and k'_r). δ_e will depend on the enzyme coverage on the surface which is estimated to be about 20 fmol/cm² based on the observed catalytic activity ($\sim 1.4 \mu\text{A}/\text{cm}^2$) and known maximum activity for *cho*₃ in solution (540 e⁻/s at 25° C²⁸). This means that the average distance between a location on the electrode surface and an enzyme is in the order of 0.01 - 0.02 μm . At the high potential region, where I_{DC} is limited by the electron transfer kinetics, k_r can

also be estimated independently from the CV data using Eqs. 6 and 11. In Figure 5, $\frac{k_2 k_r}{k_2 + k_r}$ values are shown from the cyclic voltammetry data (at high potentials $\frac{k_2 k_r}{k_2 + k_r}$ is equal to k_r) and k_r from the EIS analysis using $\delta_e = 0.015 \mu\text{m}$. As is clear from Figure 5(Top) the exponential trend of k_r obtained from CV and EIS line up when $\delta_e = 0.015 \mu\text{m}$. It is important to realise, however, that to convert k'_r obtained from the EIS data into k_r , Eq. 11 is used with $\delta_m = 5 \text{ nm}$. Thus, the actual parameter that determines how k_r from the EIS

analysis relates to $\frac{k_2 k_r}{k_2 + k_r}$ from e CV is $\frac{\delta_e}{\delta_m} \simeq 3$. To confirm the thickness of the supported membrane (δ_m), surface plasmon resonance (SPR) measurements were performed indicating a membrane thickness of $7.6 \pm 0.8 \text{ nm}$, slightly thicker than the expected 5 nm. It is furthermore important to realise that the quinone in the supported membrane acts as a single

pool, confirming the membrane is continuous and in a planar orientation. The electrochemical response is thus not due a small fraction of micron-sized adsorbed vesicles.

Importantly, with δ_e in the tens of nanometer range as shown above, D_O lies between 10^{-13} and 10^{-12} cm²/s, which is more than 4 orders of magnitude lower than accepted values of lateral ubiquinone diffusion in the lipid membrane (10^{-8} – 10^{-7} cm²/s) and indeed lateral diffusion rates for most low-molecular weight membrane components. This clearly indicates that the D_O value estimated here is not directly related to lateral diffusion of ubiquinone in a lipid membrane. We hypothesise that D_O in this study is thus representative of perpendicular diffusion. This is consistent with the model used which only takes the quinone diffusion normal to the electrode into account. To our knowledge, no perpendicular diffusion constant has been reported, but MD simulations over several nanoseconds have shown that the transition from the lipid headgroup region to the lipid mid-plane is a much slower process than lateral diffusion.⁶

The quinone is reoxidised obligatorily by the enzyme (as electrode oxidation of the quinone is neglectably slow) and this process will take a certain amount of time. Interestingly, the maximum DC current of $\sim 1 \mu\text{A}/\text{cm}^2$ (Figure 2) corresponds with a ubiquinol turn-over of 5 pmol/s/cm² and, as the quinol coverage is about ~ 8 pmol/cm², a turn-over time about 1.5 seconds per quinol is calculated. Combining this with the obtained D_O of about 5×10^{-13} cm²/s gives a diffusion length of $L_d = \sqrt{4Dt} = 17$ nm, which is very similar to δ_e . With the diffusion length similar to δ_e it cannot be excluded that the electron transfer from the electrode to the enzyme is limited by the diffusion rate of the ubiquinol. It can thus be argued that ubiquinone needs ~ 0.67 s to cross the membrane (assuming ubiquinone has to cross the membrane to reach the active site of *cbo3*).

Having obtained estimates for k'_r (which is dependent on the estimate of $\frac{\delta_e}{\delta_m}$), the interfacial concentration of ubiquinone can be calculated using Eq. 13. It is important to note that the absolute values of $c_O(0)$ vary strongly with small changes to δ_e , but the qualitative trend is

much less sensitive. Figure 5 shows the values of $c_O(0)$ for $\frac{\delta_e}{\delta_m} = 3$ and is in line with expectations. In the high potential window, $c_O(0)$ is similar to the total concentration of ubiquinol/ubiquinone in the membrane as determined independently by CV (this is, in fact,

an independent indication that the estimate for $\frac{\delta_e}{\delta_m}$ is correct). When the DC potential is lowered, the concentration drops accordingly until all the ubiquinol exist only in its reduced state.

Conclusions

A supported membrane system of 5-10 nm thick was prepared from inner membrane extracts from *E. coli*. A concentration impedance for diffusion in a thin layer was derived which takes into account that the quinone is catalytically reoxidised by *cbo3*. Within the parameter space relevant in this work, this new element is equal to that of a finite Warburg element, sometimes designated as 'O' and commonly used to analyse data from rotating disk

electrodes (RDE). The analysis of the impedance data showed that $\frac{\delta_e}{\sqrt{D_O}} = 2.4 \pm 0.2 \text{ s}^{\frac{1}{2}}$. δ_e can confidently be assumed to be in the tens of nanometer range and thus $10^{-13} < D_O < 10^{-12}$ cm²/s, which is orders of magnitude lower than *lateral* diffusion of quinone in lipid membranes.

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Abbreviations

Cbo₃	Cytochrome <i>bo₃</i>
CV	Cyclic Voltammetry
EIS	Electrochemical Impedance Spectroscopy
UQ	UbiQuinol

References

- [1]. Mitchell P. *Nature*. 1961; 191:144–148. [PubMed: 13771349]
- [2]. Bentinger M, Brismar K, Dallner G. *Mitochondrion*. 2007; 7S:S41–S50. [PubMed: 17482888]
- [3]. Ulrich EL, Girvin ME, Cramer WA, Markley JL. *Biochemistry*. 1985; 24:2501–2508. [PubMed: 3839413]
- [4]. Chazotte B, Wu ES, Hackenbrock CR. *Biochim. Biophys. Acta-Bioenerg.* 1991; 1058:400–409.
- [5]. Hauss T, Dante S, Haines TH, Dencher NA. *Biochim. Biophys. Acta-Bioenerg.* 2005; 1710:57–62.
- [6]. Sderhll JA, Laaksonen A. *J. Phys. Chem. B*. 2001; 105:9308–9315.
- [7]. Lenaz G. *J. Membr. Biol.* 1988; 104:193–209. [PubMed: 2850362]
- [8]. Samori B, Lenaz G, Battino M, Marconi G, Domini I. *J. Membr. Biol.* 1992; 128:193–203. [PubMed: 1501247]
- [9]. Lenaz G. *FEBS Lett.* 2001; 509:151–155. [PubMed: 11741580]
- [10]. Gomez-Fernandez JC, Llamas MA, Aranda FJ. *Eur. J. Biochem.* 1999; 259:739–746. [PubMed: 10092859]
- [11]. Castresana J, Alonso A, Arrondo JLR, Goni FM, Casal H. *Eur. J. Biochem.* 1992; 204:1125–1130. [PubMed: 1551391]
- [12]. Fiorini R, Ragni L, Ambrosi S, Littarru GP, Gratton E, Hazlett T. *Photochem. Photobiol.* 2008; 84:209–214. [PubMed: 18173722]
- [13]. Lenaz G, Samori B, Fato R, Battino M, Castelli GP, Domini I. *Biochem. Cell Biol.* 1992; 70:504–514. [PubMed: 1449717]
- [14]. Di Bernardo S, Fato R, Casadio R, Fariselli P, Lenaz G. *FEBS Lett.* 1998; 426:77–80. [PubMed: 9598982]
- [15]. Blackwell MF, Gounaris K, Zara SJ, Barber J. *Biophys. J.* 1987; 51:735–744. [PubMed: 3593871]
- [16]. Blackwell MF, Whitmarsh J. *Biophys. J.* 1990; 58:1259–1271. [PubMed: 19431774]
- [17]. Marchal D, Boireau W, Laval JM, Moiroux J, Bourdillon C. *Biophys. J.* 1998; 74:1937–1948. [PubMed: 9545054]
- [18]. Roche Y, Peretti P, Bernard S. *Biochim. Biophys. Acta-Biomembr.* 2006; 1758:468–478.
- [19]. Lenaz G, Fato R. *J. Bioener. Biomembr.* 1986; 18:369–401.
- [20]. Chazotte B, Hackenbrock CR, Faseb J. 1993; 7:A1106–A1106.
- [21]. Boekema EJ, Braun HP. *J. Biol. Chem.* 2007; 282:1–4. [PubMed: 17102127]
- [22]. Lenaz G, Genova ML. *Am. J. Phys.-Cell Phys.* 2007; 292:C1221–C1239.
- [23]. Weiss, S.; Bushby, R.; Evans, SD.; Henderson, P.; Jeuken, LJC. 2008. Submitted
- [24]. Jeuken LJC, Bushby RJ, Evans SD. *Electrochem. Comm.* 2007; 9:610–614.
- [25]. Boden N, Bushby RJ, Clarkson S, Evans SD, Knowles PF, Marsh A. *Tetrahedron*. 1997; 53:10939–10952.

- [26]. Stamou D, Gourdon D, Liley M, Burnham NA, Kulik A, Vogel H, Duschl C. *Langmuir*. 1997; 13:2425–2428.
- [27]. Jeuken L, Daskalakis NN, Han X, Sheikh K, Erbe A, Bushby R, Evans S. *Sens. Actuators B*. 2007; 124:501–509.
- [28]. Rumbley JN, Nickels EF, Gennis RB. *Biochim. Biophys. Acta-Protein Struct. Molec. Enzym.* 1997; 1340:131–142.
- [29]. Schnaitman CA. *J. Bact.* 1970; 104:890–901. [PubMed: 4099097]
- [30]. Schaffner W, Weissmann C. *Anal. Biochem.* 1973; 56:502–514. [PubMed: 4128882]
- [31]. Hirst J, Armstrong FA. *Anal. Chem.* 1998; 70:5062–5071. [PubMed: 9852788]
- [32]. Armstrong FA. *Curr. Opin. Chem. Biol.* 2005; 9:110–117. [PubMed: 15811794]
- [33]. Bockris, JO.; Reddy, AKN.; Gamboa-Aldeco, M. *Modern electrochemistry 2A: Fundamentals of electrochemistry*. Second edition ed. Kluwer Academic; New York: 1998. p. 1175-1180.
- [34]. Girault, HH. *Analytical and physical electrochemistry; Fundamental Sciences, Chemistry*. EPFL Press; Lausanne: 2004.
- [35]. Boillot M, Didierjean S, Lapique F. *J. Appl. Electrochem.* 2004; 34:1191–1197.
- [36]. Jeuken LJC. *Biophys. J.* 2008; 94:4711–4717. [PubMed: 18326663]
- [37]. Schönhals, A.; Kremer, F. *Broadband Dielectric Spectroscopy*. Kremer, F.; Schönhals, A., editors. Springer; Berlin: 2003. p. 59-98.
- [38]. Daniel, VV. *Dielectric relaxation*. Academic Press; London: 1967.

Figure 1.

A schematic representation of the tethered membrane system in which the electrochemical reduction, quinone diffusion/catalytic oxidation rates (k_r and k_2 , respectively) and two distances, δ_e and δ_m are indicated. UQ_{OX} and UQ_{RED} indicate the oxidised and reduced quinone species, respectively.

Figure 2.

(Top) Typical CV (10 mV/s) of the supported membrane system prepared from *E. coli* inner membranes overexpressing *cbo3* without (solid line) and with (dashed line) the inhibitor cyanide. The voltammogram with inhibitor was pre-poised for 15 seconds at 0.441 V vs SHE to oxidise all quinone in the membrane. The membrane proteins from the inner membrane are ‘diluted’ with *E. coli* lipid extract (1:9 membrane protein:lipid extract (w/w)) as described in the Methods section. Additional ubiquinol-10 was added to the *E. coli* lipid extract (1 weight percent). The “low” and “high” potential windows refer to the explanation given in the text. (Bottom) A scheme indicating the possible rate limiting steps of the interfacial electron transfer rate as observed by CV and after analysis of the EIS data. See text for more explanation.

Figure 3.

Electrochemical impedance data in the form of Bode plots of the supported membrane system at the indicated DC potential before (closed symbols) and after (open symbols) addition of the *cbo*₃ inhibitor cyanide. Squares = $|Z_0|$, left axis; Circles = θ , right axis. The lines show the fits as explained in the text.

Figure 4.

Parameters obtained from fitting three different sets of EIS data (shown by different colours). (Top) Surface normalised resistances obtained from the EIS data before ($R_{ET}=R_{ET}^{EIS}$, closed symbols, thick solid lines) and after ('background' resistance, thin lines) addition of the cbo_3 inhibitor cyanide. Resistances obtained from the cyclic voltammogram are also shown (R_{ET}^{CV} , open symbols, dashed line). (Middle and bottom) $\delta_e/\sqrt{D_o}$ and $k'_r/\sqrt{D_o}$ obtained from the EIS data measured without cyanide.

Figure 5.

(Top, open symbols) k_r determined from the parameters shown in Figure 4 and using Eq. 11

to convert k'_r into k_r (assuming $\frac{\delta_e}{\delta_m}=3$). (Top, closed symbols) $\frac{k_2 k_r}{k_2 + k_r}$ determined from CV

data using Eq. 6. (Bottom) $c_O(0)$, given as a ratio, $\frac{c_O(0)}{c}$, as determined from EIS data using

Eq. 13 with $\frac{\delta_e}{\delta_m}=3$.