Electron transfer between cytochrome c and the binuclear center of cytochrome oxidase.

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Highlights
- A simplified *in-silico* kinetic model of Cytochrome Oxidase is developed
- The model includes Cytochrome c binding, oxidation and reduction of oxygen
- It reproduces the experimental reduction of Cu₆, heme a and heme a₃ during turnover
- It predicts that the off rate constants for oxidized and reduced Cytc can be unequal
- Disequilibrium between Cytc and Cu₆ results in the non-Nernst response of heme a
Electron transfer between cytochrome c and the binuclear center of cytochrome oxidase.

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Running title: Electron transfer between Cytc and the BNC

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Abstract

The Minnaert model, which can account for the reaction kinetics between cytochrome oxidase and cytochrome c (Cytc), has been used to justify equal binding rate constants for reduced and oxidized Cytc. Here we extend the model beyond reversible binding of Cytc and its irreversible oxidation to include CuA, heme a and the oxidation cycle of the binuclear center. The model reproduces the experimental reduction of CuA and heme a during turnover and the low population of the ferryl and ferrous heme a3. It predicts that the off rate constants for reduced and oxidized Cytc can be unequal and that the non-Nernst response of CuA and heme a is due to disequilibrium between free Cytc and CuA rather than redox anti-cooperativity.

Keywords: Cytochrome oxidase, enzyme kinetics, enzyme modeling, midpoint potential, redox anti-cooperativity and proton pumping.
Introduction

Cytochrome aa3 oxidase (CytOx) is the terminal proton pumping enzyme of the mitochondrial electron transport chain and uses electrons delivered by intermembrane cytochrome c (Cytc) to reduce oxygen to water. Electrons from bound Cytc are first passed to the CuA center just beneath the Cytc binding site and then onto heme a buried in the complex at a depth of \( \approx 1/3 \) of the membrane thickness [1]. From there, they are passed parallel to the membrane to the binuclear center (BNC), consisting of heme a3 and CuB, where oxygen is reduced to water using protons from the matrix. Conrad and Smith [2] found that the turnover number was strictly first order with reduced Cytc as reduced Cytc was consumed and oxidized Cytc was produced during catalytic turnover. They also found that CytOx follows Michaelis-Menten kinetics [3] (first proposed by Henri, see [4]) so that the turnover must be given by:

\[
v = \frac{k_{\text{cat}} [c^{2+}]}{[c^{2+}] + [c^{3+}] + K_M}
\]

where \([c^{2+}]\) and \([c^{3+}]\) are the concentration of reduced and oxidized Cytc, respectively. Later, Minnaert [5] developed a model where both oxidized and reduced Cytc can reversibly bind to CytOx, and the bound reduced Cytc is oxidized by the downstream oxygen reaction (fig. 1a). This model can account for the so-called Smith-Conrad kinetics of eqn. 1 if (1) the binding rate constants of reduced and oxidized Cytc are the same and (2) the oxidation of bound Cytc is unidirectional (irreversible). Since the midpoint potentials of Cytc and CuA are similar (260 [6] and 250mV [7], respectively) and the rate constants for electron transfer are large (>100,000 s\(^{-1}\) [8]), the CuA center would have to be held in a fully oxidized state by the oxygen reaction at the BNC in order to ensure unidirectional electron transfer between bound Cytc and the CuA center. Interestingly, the recent measurement of the CuA and heme a oxidation states simultaneously with turnover [6] have shown that both CuA and heme a are considerably reduced at high turnover. This lead to the presumption that Cytc, CuA and heme a remain in close equilibrium, contrary to the requirements of the Minnaert model. However the reduction state of the centers did not follow a simple n=1 Nernst function and it was concluded that there must be considerable redox anti-cooperativity between CuA, heme a and CuB to account for this discrepancy. Redox anti-cooperativity occurs between redox centers and causes the midpoint potential of one centre to become lower when the other center is reduced (see [9] for a description of the redox anti-cooperativity between heme a and a3). It often occurs due to electrostatic repulsion between reduced centers and is always reciprocal in nature so the lowering of the midpoint potential of one redox center due to the reduction of a second

**Figure 1.** Graphical representations of the 4 models describing binding of Cytc to CytOx and electron transfer. Both reduced Cytc (c\(^{2+}\)) and oxidized Cytc (c\(^{3+}\)) bind reversibly in all models. In the a) Minnaert model: bound reduced Cytc is oxidized by the BNC in a single irreversible step. In the b) One-center model: the electron from reduced Cytc is accepted reversibly by a single center (C1) before being irreversibly oxidized by the BNC. In the c) Two-center model, the electron passes reversibly through two centers (C1 and C2) before being passed irreversibly to the BNC. Finally, in the d) BNC model, the electron is passed through the CuA and heme a centers before being passed to the BNC, which cycles through the 5 reductions states (P, F, O, E and R) before oxygen binding (A) and reduction of oxygen to regenerate (P). Blue bi-arrows depict reversible Cytc binding and black bi-arrows/arrows denote reversible and irreversible electron transfers, respectively.
center is equal to the lowering of the midpoint potential of the second center due to reduction of the first. In the case of CytOx where the midpoint potential of CuA and heme a are similar, redox anti-cooperativity would make it more difficult to reduce both redox centers at low redox potentials so both would be more oxidized than would be predicted by a simple n=1 Nernst function, as observed experimentally. However, as low Cytc redox potentials result in high turnover in the system studied, and any disequilibrium (deviation from equilibrium) is expected to be greater at high turnover, it is possible that the greater than expected oxidation at low redox potentials could be due to the loss of equilibrium between free Cytc and bound Cytc rather than redox anti-cooperativity.

Here we develop a more realistic model of Cytc binding and electron transfer than the simple Minnaert model to explore the possible disequilibrium between free and bound Cytc, and between bound Cytc and the downstream redox centers. We first generate a model with a single redox center which accepts electrons from bound Cytc and donates electrons to oxygen in an irreversible step (fig. 1b), that can be solved analytically. We then generate a second model with two redox centers downstream of Cytc (fig. 2c) which is solved numerically. Finally, we develop a model in which electrons are transferred via CuA and heme a to the BNC which cycles through its 5 oxidation states (P, F, O, E and R) and binds and reduces molecular oxygen (fig. 1d).

Materials and Methods

The four models depicted in fig. 1 were solved in the steady state by assembling the chemical master equation of a state model. The Minnaert model has 3 states defined by the Cytc binding site, which can be either empty (E), bound with reduced Cytc (2) or bound with oxidized Cytc (3).

\[
\begin{align*}
\frac{d}{dt} \begin{pmatrix} E \\ 2 \\ 3 \end{pmatrix} &= \begin{pmatrix} -k_{12} & k_{2} & k_{3} \\ k_{12} & -k_{2} - k_{3} & 0 \\ k_{13} & k_{b} & -k_{3} \end{pmatrix} \begin{pmatrix} E \\ 2 \\ 3 \end{pmatrix}
\end{align*}
\]

Equation 2

where \(k_{2}\) and \(k_{2}'\) are the on and off rate constants for reduced (Fe^{2+}) Cytc, \(k_{3}\) and \(k_{3}'\) are likewise for oxidized (Fe^{3+}) Cytc, \([e^{2+}]\) and \([e^{3+}]\) are the concentration of reduced and oxidized Cytc, and \(k_{b}\) is the rate constant for oxidation of bound reduced Cytc.

The one-center model has six states defined by the Cytc binding site and the oxidation state of the redox center (O for oxidized, R for reduced). The chemical master equation is given by:

\[
\begin{align*}
\frac{d}{dt} \begin{pmatrix} EO \\ 2O \\ 3O \\ ER \\ 2R \\ 3R \end{pmatrix} &= \begin{pmatrix} -k_{12}' & k_{2} & k_{3} & k_{b} & 0 & 0 \\ k_{12}' & -k_{2} - k_{3} & 0 & 0 & k_{b} & k_{c} \\ 0 & 0 & -k_{2} - k_{3} & 0 & 0 & k_{b} \\ 0 & 0 & k_{c} & 0 & k_{12}' & -k_{2} - k_{3} \\ 0 & 0 & 0 & k_{12}' & -k_{2} - k_{3} & 0 \\ 0 & 0 & 0 & 0 & -k_{3} - k_{c} & k_{b} \end{pmatrix} \begin{pmatrix} EO \\ 2O \\ 3O \\ ER \\ 2R \\ 3R \end{pmatrix}
\end{align*}
\]

Equation 3

where \(k_{c}\) and \(k_{c}'\) are the forward and reverse rate constants for electron transfer from bound Cytc to the redox center and here \(k_{b}\) is the rate constant for oxidation of the redox center. Finally, the two center model has 12 states, because the second redox center can also be oxidized or reduced, and the BNC model has 72 states as the BNC cycles through 6 states. The chemical master equation was then solved in the steady state by matrix inversion either analytically or numerically using a fixed total CytOx concentration.

The turnover data and oxidation states of CuA, heme a and the 655nm band were digitized from Mason et al. [6] which used 6.5μM of bovine CytOx and 10μM of horse Cytc in 100mM potassium phosphate buffer with 0.1% lauryl maltoside at pH 7.4 and 30°C. The reductant was 40mM
ascorbate and Cytc reduction state was varied by changing the concentration of N,N,N’,N’, tetramethyl-p-phenylenediamine (TMPD).

Results

The general solution of the Minnaert model is given by:

\[ v_e = \frac{k_2k_3k_5c^{2+}}{(k_2 + k_b)c^{2+} + (k_2 + k_b)c^{3+} + k_3(k_2 + k_b)} \]

Equation 4

where \( v_e \) is the electron flux. This expression only follows Smith-Conrad kinetics if \( k_{+2}=k_{+4} \) and \( k_{-2}=k_{-3}, \) that is, if the on rate constant \( (k_{on}) \) of reduced and oxidized Cytc (\( k_{+2} \) and \( k_{+3} \), respectively) are the same and likewise for the off rate constant \( (k_{off}) \) of reduced and oxidized Cytc (\( k_{-2} \) and \( k_{-3} \), respectively). This implies that the binding constants \( (K_d=k_{off}/k_{on}) \) of reduced and oxidized Cytc are the same. Using this assumption, Van Buuren et al. [10] estimated that \( k_{+2} = k_{+3} = 40\times 10^6 \text{ M}^{-1}\text{s}^{-1}, \)
\[ k_{-2} = k_{-3} = 1200 \text{ s}^{-1} \]
and \( k_b = 300 \text{ s}^{-1} \) giving a \( k_{cat} \) of 240 s\(^{-1}\) and a \( K_d \) of 30μM.

In order to explore the effect of changing the relative \( K_d \) of oxidized and reduced Cytc, which affects the midpoint potential of bound Cytc (see supplementary information), we made the \( k_{on} \) for oxidized and reduced Cytc the same but adjusted the \( k_{off} \) for oxidized and reduced Cytc according to:

\[ k_{-2} = 1200e^{-\Delta E_{m,b}/2RT} \]
\[ k_{-3} = 1200e^{\Delta E_{m,b}/2RT} \]

where \( \Delta E_{m,b} \) is the difference in midpoint potential between bound Cytc and free Cytc and the factor 1200 is the off rate determined by Van Buuren et al. Fig 2a compares the predicted turnover of the Minnaert model with the experimental data as the midpoint potential of bound Cytc is changed according to eqn. 5. The model qualitatively matches the experimental data well when using 10μM of total Cytc, as per the experimental data, and the Van Buuren et al. values for the binding rate constants and \( k_b, \) (fig 2a) but the noise on the turnover data precludes using it to assess the midpoint

![Figure 2](image.png)

Figure 2. Comparison of experimental turnover data with the predicted by the Minnaert and the one-center model as the midpoint potential of either bound Cytc (\( E_{m,b} \)) and the donating center (\( E_{m,l} \)) is varied. All models use 10μM of Cytc as per the experimental data and a \( k_{-2} \) and \( k_{-3} \) of 40×10^6 M\(^{-1}\)s\(^{-1}\). a) Minnaert model with \( k_b \) of 300 s\(^{-1}\) as the midpoint potential of bound Cytc (\( E_{m} \)) is varied using eqn. 2. b) One-center model with \( k_b \) of 86 s\(^{-1}\), keeping \( E_{m,b} \) constant at 260mV and varying the midpoint potential of the donating center (\( E_{m,l} \)), c) as b) but keeping \( E_{m,l} \) constant at 260mV and varying \( E_{m,b} \). The insert shows the turnover as \( E_{m,b} \) is varied when \( E_{m,l} \) is held at 260mV with 10μM of reduced Cytc.
potentials of the different centers. Instead, the condition that the enzyme must follow Conrad-Smith kinetics (linearity between turnover and Cytc oxidation state at fixed Cytc concentration) was used to determine their correct midpoint potentials. As expected, the model only precisely reproduces Smith-Conrad kinetics when the midpoint potential of the bound Cytc is equal to the free Cytc (260mV [6]), with the relationship between Cytc and turnover becoming non-linear when the midpoint potentials are not equal (i.e. $k_{-2} \neq k_{-3}$). Furthermore, increasing the midpoint potential of bound Cytc increases the $k_{\text{off}}$ of oxidized Cytc and so increases turnover, and vice versa.

The Minnaert model assumes that the bound reduced Cytc is oxidized in an irreversible process. In reality, electrons are passed to Cuₐ and heme $a$ with forward and reverse rate constants that are large with respect to the net electron flux. This process was first modeled by extending the Minnaert model to include one center which accepts electrons from bound Cytc, with reversible rate constants, and donates them to the BNC, with an irreversible rate constant of $k_b$ (fig 1b). The general solution for the one-center model is considerably more complex than the Minnaert model and given by:

$$v_e = \frac{k_+ k_- k_b k_{\text{on}} [e^{2+}]}{k_e A + k_+ k_b B + CA/D}$$

$$A = k_+ k_2 [e^{2+}] + k_2 k_3 [e^{3+}] + k_{-2} k_{-3}$$

$$B = k_2 [e^{2+}] + k_3 [e^{3+}] + k_{-3}$$

$$C = (k_{-3} + k_b) k_2 [e^{2+}] + (k_{-2} + k_b) k_{-2} k_{-3}$$

$$D = A + k_b B + (k_{-2} + k_b) k_{-b}$$

where $k_+$ and $k_-$ are the forward and reverse rate constants for electron transfer between bound and reduced Cytc and the redox center. Similar to before, the midpoint potential of this center was varied by setting $k_{+e}$, $k_{-e}$ to:

$$k_{+e} = 10^5 e^{F(AE_{m1} - AE_{m2})/2RT}$$

$$k_{-e} = 10^5 e^{-F(AE_{m1} - AE_{m2})/2RT}$$

where $AE_{m1}$ is the difference of the midpoint potential of the center and free Cytc, and the factor $10^5$ is the experimentally determined minimum rate for electron transfer from bound Cytc to Cuₐ [8]. The inclusion of $AE_{m2}$ ensures that the midpoint potential of the center is independent of the midpoint potential of bound Cytc (see supplementary information). This model used the same Cytc binding constants as the Minnaert model but, when using the same value for $k_b$, the turnover was much greater. This occurred because, although the donating center has the same oxidation state as the bound Cytc, only a fraction of the binding sites are occupied by Cytc and so the product of rate constant and concentration is smaller in the Minnaert model than in the one-center model. For this reason, the $k_b$ used in the one-center model was decreased to 86 s⁻¹, so that the turnover numbers of the two models matched for a given Cytc oxidation state. The dependency of this model on the midpoint potential of the donating center was then explored. For that, the binding rate constants for reduced and oxidized Cytc were set the same values as in the Minnaert model (midpoint potential of bound Cytc is equal to free Cytc) and then the midpoint potential of the donating center was changed by varying the electron transfer rate constants according to eqn. 7 (fig 2b). As visible, the model reproduced Smith-Conrad kinetics when the donating center had the same midpoint potential as free Cytc but showed a non-linear relationship between turnover and Cytc when it differed (fig 2b).

To determine if the one-center model still required an equal $k_{\text{off}}$ for both reduced and oxidized Cytc, further simulations were carried out. We used the same $k_{\text{on}}$ for reduced and oxidized Cytc, fixed the midpoint potential of the donating center at 260mV and varied the midpoint potential of bound Cytc
according to eqn. 5 and 7. Note this required changing the $k_{off}$ of both reduced and oxidized Cyt c (eqn. 5) and the rate constants for electron transfer from bound Cyt c to the center (eqn. 7). These simulations showed that Conrad-Smith kinetics were maintained to within experimental accuracy regardless of the midpoint potential of bound Cyt c, as long as the donating center had the same midpoint potential as free Cyt c (fig. 2c).

As found with the Minnaert model, the turnover would be expected to be higher when the midpoint potential of bound Cyt c is higher and the $k_{off}$ for oxidized Cyt c is greater. To examine this effect in more detail, simulations were carried out with 10 μM of reduced Cyt c and the turnover plotted as a function of the difference in midpoint potential between bound and free Cyt c (insert of fig. 2c). Surprisingly, the turnover was maximal when the midpoint potential of bound Cyt c was ≈ 35 to 40mV below that of free Cyt c, even though the $k_{off}$ of oxidized Cyt c was smaller.

The condition that the donating center has the same midpoint potential as free Cyt c can be expressed as $k_{-3}k_{+e} = k_{-j}k_{-e}$ when the $k_{on}$ for both oxidized and reduced Cyt c are the same. In addition, the term $k_bA$ in the denominator of eqn. 6 is small compared to the other terms ($k_b$ is much smaller than $k_{+e}$ or $k_{-e}$) and so this term can be ignored. With these two conditions, eqn. 6 can be written as:

$$v = \frac{k_{-3}k_{+e}k_bk_{+3}[c^{2+}]}{\left(k_{-2}[c^{2+}]+k_{-1}[c^{3+}]\right)\left(k_{+3}k_b+(k_{-2}+k_b)k_{-e}A/D\right)}$$

$$A = k_{-3}k_{+2}[c^{2+}]+k_{-2}k_{+3}[c^{3+}]+k_{-3}k_{-3}$$

$$D = A+k_b\left(k_{+2}[c^{2+}]+k_{+3}[c^{3+}]\right)+(k_{-2}+k_{-3}+k_b)k_{+b}$$

$$E = k_{-3}k_{+e}k_b+(k_{+2}+k_b)k_{-e}A/D$$

which approximates Smith-Conrad kinetics when $k_{+2}=k_{+3}$ because the term $A/D$ is a very weak function of Cyt c oxidation state. From this we conclude that, to generate Smith-Conrad kinetics, (1) the $k_{on}$ for oxidized and reduced Cyt c must be the same, (2) the $k_{off}$ need not be the same and (3) the center which donates to oxygen must have the same midpoint potential as free Cyt c.

A two-center model was then used to explore the disequilibrium between free Cyt c and downstream redox centers. This model has two centers (C1 and C2, fig. 1c) in rapid equilibrium with bound Cyt c before a slow step to the BNC (fig. 1c). For these simulations, both the midpoint potential of bound Cyt c and that of C2, which donates electrons to the BNC via a slow irreversible step, was fixed at 260mV to ensure Smith-Conrad kinetics. Then, the midpoint potential of C1 was varied by changing the rate constants according to eqn. 7, and the midpoint potential of C2 was fixed by varying the forward and reverse rate constant for electron transfer from centers C1 to C2 according to:

$$k'_{+e} = 10^5 e^{\Delta F_{c1}/2RT}$$

$$k'_{-e} = 10^5 e^{\Delta F_{c1}/2RT}$$

where the term $10^5$ reflects the rapid electron equilibration between CuA and heme $a$ [11]. The turnover of this model displayed Smith-Conrad kinetics and varied very little with the midpoint potential of C1 (fig. 3a). The redox potentials of the free Cyt c and of the two redox centers were calculated from their oxidation state (using an n=1 Nernst function) and their midpoint potentials. Then the $\Delta G$ for electron transfer from free Cyt c to C1, and from C1 to C2, was determined from the differences in the above calculated redox potentials according to:

$$\Delta G = -(E^a_b - E^d_b)$$

where $E^a_b$ and $E^d_b$ are the redox potentials of the accepting and donating centers, respectively. Fig 3b shows that, while there was a considerable disequilibrium between free Cyt c and C1, which
increased with electron flux, both C1 and C2 remained in close equilibrium with a ΔG smaller than −2 mV as the midpoint potential of bound Cyt c varied. These results are expected since, at a concentration of 10 μM, the effective first order rate constant for Cyt c binding is only 400 s⁻¹, which is comparable to the 64 s⁻¹ (4 times the CytOx turnover number) of the electron flux, whereas the rate constants for electron transfer between the centers are almost 3 orders of magnitude greater.

Fig. 3c compares the oxidation state of C1 when it has different midpoint potentials to the experimentally measured oxidation state of the heme a center, Cuₐ center and the 655nm band, which has been used as a surrogate of oxidized Cuₐ [6]. As visible, the data suggests that these centers have midpoint potentials of ≈280, ≈250 and ≈260 mV, respectively. Also, there is very good correspondence between the model and the data except when Cyt c is more than 80% reduced, where the model substantially underestimates the reduction of the 655nm band and slightly underestimates the reduction of heme a. This data strongly suggests that the non Nernst response of Cuₐ and heme a centers could result from the disequilibrium between free Cyt c and Cuₐ rather than from redox anti-cooperativity.

The redox chemistry at the BNC is considerably more complex than assumed in the previous models (see [12] for a review). The BNC cycles through the states P, F, O, E and R with each transition requiring an electron from heme a and a substrate proton from the matrix. The state P has Cuₐ oxidized, heme a₃ in the ferryl (Fe⁴⁺) form and a nearby tyrosine as a free radical. The first electron reduces the tyrosine to generate state F and the second electron reduces heme a₃ to the ferric (Fe³⁺) form to generate the state O. State O can relax into a slow form in which further electron transfer to the BNC is very slow and not thought to be part of the catalytic cycle [13]. Alternatively, the third electron reduces Cuₐ to form state E and the fourth electron reduces heme a₃ to form state R. Molecular oxygen binds to state R to form state A whereupon the oxygen undergoes a concerted and irreversible 4-electron reduction to regenerate state P.

The midpoint potentials of the P/F and F/O couples are very high (≈800 mV) [14-15] making these transitions essentially irreversible. Heme a and the BNC are sufficiently close that electron transfers occur on the nanosecond timescale [16]. However, the reduction of the BNC from P to F and from F to O are much slower than the pure electron transfers because these transitions also involve proton pumping and proton uptake to the BNC which determine the equilibrium. Nonetheless, these transitions are rapid with respect to turnover and state P is reduced to state F and O with time constants of 0.2 and 3 ms after oxygen reduction when both Cuₐ and heme a are initially reduced [17]. Likewise the binding and reduction of oxygen is also very rapid with rate constants of 1.38×10⁸ M⁻¹ s⁻¹ (2.76×10⁹ at 200 μM of oxygen) and 0.32×10⁵ s⁻¹, respectively [18].
turnover, heme $a_3$ is observed to be highly oxidized. This can easily be explained if either the O/E or E/R transition is slow compared to oxygen binding and reduction such that heme $a_3$ is oxidized much faster than it is reduced. When reduced CytC and oxidized CytOx are mixed in a stop flow experiment, there is an initial burst phase in which Cu$_A$ and heme $a$ are reduced that is then followed by a slow phase of CytC oxidation with a rate 51 s$^{-1}$ that does not have spectral signal, consistent with the reduction of Cu$_B$ [19]. This would suggest that the O/E transition is slow.

Using these observations, a model was developed in which the BNC cycled through states P to R on transfer of an electron from heme $a$, and then spontaneously to state A before regenerating P (fig. 1d). The model used the same rate constants for CytC binding as the Minnaert model and the same rate constants for electron transfer from bound CytC to Cu$_A$ and heme $a$ with the latter centers having a midpoint potential of 280 and 245 mV, respectively. The forward rate constants for the P/F and F/O transitions were set to 5.0×10$^6$ and 3.33×10$^2$ s$^{-1}$, respectively, consistent with the experimental results [17], and the reverse rates constants were essentially zero due to the large $\Delta G^0$ of the electron transfer. The reverse rate constant of the E/R transition had no effect on turnover because the rapid binding and reduction of oxygen reduces the probability of finding the enzyme in the R state to near zero. The forward rate constant and midpoint potential of the O/E transition and the forward rate constant of the E/R transition was then tuned by trial and error. The best correspondence between experimental and modeled data was found when Cu$_B$ had a midpoint potential of 260 mV and the forward rate constants for the O/E and E/R transitions were 200 and 30 s$^{-1}$, respectively (fig. 4). With these parameters...

![Figure 4](image)

**Figure 4.** Comparison of experimental data with that predicted by the BNC model. a) Experimental (circles) and modelled (solid line) turnover data. The dotted line is a regression to the linear component of the modelled turnover. b) Experimental oxidation states of Cu$_A$, heme $a$ and the 655nm band (circles) compared to modelled oxidation state of Cu$_A$, heme $a$ and Cu$_B$ (lines). c) Modelled population of states P, F, O and E during turnover.

**Table 1 Reactions, rate constants and standard free energies of the BNC model.**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>BNC</th>
<th>$k_f$</th>
<th>$k_r$</th>
<th>$\Delta G^0$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB CytC$^{2+}$</td>
<td>40.0×10$^6$ [C$^{2+}$]</td>
<td>1.2×10$^3$</td>
<td>-</td>
<td>-278</td>
</tr>
<tr>
<td>PB CytC$^{3+}$</td>
<td>40.0×10$^6$ [C$^{3+}$]</td>
<td>1.2×10$^3$</td>
<td>-</td>
<td>-278</td>
</tr>
<tr>
<td>ET CytC $\rightarrow$ Cu$_A$</td>
<td>100×10$^3$</td>
<td>175×10$^3$</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>ET Cu$_A$ $\rightarrow$ heme $a$</td>
<td>100×10$^3$</td>
<td>27.0×10$^3$</td>
<td>-35</td>
<td></td>
</tr>
<tr>
<td>ET heme $a$ $\rightarrow$ BNC (P $\rightarrow$ F)</td>
<td>5×10$^3$</td>
<td>1.74×10$^5$</td>
<td>-520</td>
<td></td>
</tr>
<tr>
<td>ET heme $a$ $\rightarrow$ BNC (F $\rightarrow$ O)</td>
<td>3.33×10$^2$</td>
<td>1.16×10$^6$</td>
<td>-520</td>
<td></td>
</tr>
<tr>
<td>ET heme $a$ $\rightarrow$ BNC (O $\rightarrow$ E)</td>
<td>200</td>
<td>423</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>ET heme $a$ $\rightarrow$ BNC (E $\rightarrow$ R)</td>
<td>30</td>
<td>63.4$^\dagger$</td>
<td>20$^\dagger$</td>
<td></td>
</tr>
<tr>
<td>MB O$_2$ $\rightarrow$ BNC (R $\rightarrow$ A)</td>
<td>1.38×10$^8$[O$_2$]</td>
<td>38.6×10$^3$</td>
<td>-220</td>
<td></td>
</tr>
<tr>
<td>OR (A $\rightarrow$ P)</td>
<td>6.67×10$^3$</td>
<td>0.01</td>
<td>-360</td>
<td></td>
</tr>
</tbody>
</table>

Key: PB: protein binding, ET: electron transfer, MB: molecular binding, OR: oxygen reduction. $^\dagger$ The turnover of the model has negligible dependency on these values because the R state population is near zero. The square brackets denote concentration in Molar.
(summarized in table 1), the model produced Conrad-Smith kinetics when Cytc was more than \( \approx 40\% \) reduced (fig 4a), and there was a good correspondence between the modeled and experimental oxidation states of CuA and heme \( a \) and between CuB and the 655nm band (fig 4b). Furthermore, the model predicted that CytOx population of the P and F states were below 10\% (fig. 4c) and negligible reduction of heme \( a_3 \), both consistent with experimental observations [6].

**Discussion**

In this paper we have taken the Minnaert model and added additional complexity to arrive at a very simple but more realistic model of CytOx turnover that can account for the non Nernst response of CuA and heme \( a \) and gives a good approximation to Smith-Conrad kinetics. The model predicts that the effective midpoint potential of CuA and heme \( a \) are \( \approx 245 \) and \( \approx 280 \text{mV} \), respectively, the former in good agreement with redox titrations (250mV) [7] and the latter in good agreement with calculations from time resolved studies at short timescales (270mV) [11, 20] but markedly different from 340mV recorded for heme \( a \) when heme \( a_3 \) is oxidized in long-timescale redox titrations [21].

This work finds that the non Nernst response of CuA and heme \( a \) is most likely due to the disequilibrium between free Cytc and CuA, as result of the relatively slow kinetics of Cytc binding and release compared to turnover, rather than redox anti-cooperativity. While the Minnaert model requires the \( k_{off} \) to be the same for both reduced and oxidized Cytc to reproduce Smith-Conrad kinetics, the more realistic models find this is not necessary. Our model including the BNC predicts low populations of CytOx in the P, F and R states during turnover, as found experimentally [6]. Overall, this work paints a picture in which the transfer of electrons from the pool of free Cytc to CuA is relatively slow (\( \tau \approx 2.5 \text{ms} \)), due to the slow binding and release of Cytc, the equilibration of electrons between bound Cytc, CuA and heme \( a \) is fast (\( \tau \approx 8 \text{μs} \)) whereas the transfer of electrons to oxygen is, again, slow. The electron transfer to the BNC is fastest in states F and P (\( \tau \approx 0.2 \) and 3ms, respectively), relatively slow transfer in the state O (\( \tau \approx 5 \text{ms} \)) and slowest in state E (\( \tau \approx 30 \text{ms} \)), when electron transfer reduces heme \( a_3 \).

The simplest way to determine if the non-Nernst response between Cytc, CuA, heme \( a \) and the 655nm band is due to redox anti-cooperativity or redox disequilibrium would be to repeat the studies with varying concentrations of Cytc. If there is close equilibrium between free Cytc and CuA and the non-Nernst response is due to redox anti-cooperativity then the response should not change with Cytc concentration. In contrast, if the response is due to disequilibrium between Cytc and CuA then the disequilibrium should become greater at low Cytc concentrations.

The 655nm absorption band is thought to originate from a charge transfer band from oxidized heme \( a_3 \) and a bound ligand that is only present when CuB is oxidized [22]. Therefore, it is expected to appear when the BNC is in the state O but not the state E and, as such, it has been used previously by others as a surrogate of oxidized CuB [6]. The band titrates with a midpoint potential of \( \approx 400 \text{mV} \) in redox titrations [22] and would be expected to have a midpoint potential \( >460 \text{mV} \) from the time resolved O to E transition [11]. Here the band has a good correlation with CuB when it has a midpoint potential of 260mV. Surprisingly, this value is needed by the donating center in the simplest models to produce Smith-Conrad kinetics even though electrons are donated to the BNC by heme \( a \) and not CuB. It is known that the state O is metastable [13] and can relax into a slow form which cannot pump protons and in which CuB has a low midpoint potential reminiscent of the form found here [23]. However, the low apparent midpoint potential of CuB cannot be attributed to the relaxation of state O under the conditions modeled here because the lifetime of O is at least 30 seconds [13] whereas the enzyme is turning over on a much shorter timescale. The reason why CuB has such an apparent low midpoint potential is not known but raising the midpoint potential in the model led to larger deviations from Smith-Conrad kinetics and a substantial mismatch between the 655nm band and the oxidation state of CuB.

Based on the Dutton ruler [24], the intrinsic electron transfer rate from heme \( a \) to the BNC is expected to be on the nanosecond timescale due to their proximity [16], whereas the model uses rate
constants on the millisecond timescale. This disparity is thought to occur because electron transfer is coupled to protonation and the proton uptake is slow. In state O, prior to proton uptake, the midpoint potential of heme $a$ is $\approx 270\text{mV}$ and that of the BNC is very low ($<140\text{mV}$) so that the equilibrium prevents electron transfer [11]. Uptake of the pump proton ($\tau \approx 0.15\text{ms}$) raises the midpoint potential of heme $a$ and the BNC via an electrostatic interaction to values approximating those seen in steady state redox transitions ($\approx 340\text{mV}$), altering the equilibrium so that the electron can be shared between heme $a$ and the BNC. Subsequently, the substrate proton is taken up ($\tau \approx 0.8\text{ms}$) and reacts with the oxygen intermediates at the BNC raising the midpoint potential of Cu$_B$ to $>460\text{mV}$ [11]. Technical limitations prevent such detailed studies on the E to R transition, but it could be expected that the pump proton raises the midpoint potential of heme $a$ and $a_3$ similarly to the O to E transition, because it occurs through similar electrostatic interactions. In contrast the substrate proton undergoes different chemistry at the BNC and so will have a different effect on the midpoint potential of heme $a_3$ compared to Cu$_B$. The F to P and P to O transitions will be different again because heme $a_3$ is in the ferryl state instead of the ferric state and electron transfer has been found to occur before uptake of the pump proton [25]. However, the slow electron transfer to the state O seen here ($\tau \approx 5\text{ms}$) is still slow compared to the uptake of the pump proton ($\tau \approx 0.15\text{ms}$) and the substrate proton ($\tau \approx 0.8\text{ms}$) in the time resolved studies. A similar discrepancy has been observed for the release of a proton during back flow studies on the R to E transition and has been attributed to different proton kinetics in the soluble enzyme modeled here compared to the time resolved studies carried out with the enzyme inserted into a membrane [26]. During catalytic turnover, the probability of finding the enzyme in states O and E is $\approx 90\%$ (fig. 4c). In these states, heme $a$ is expected to have midpoint potentials of $\approx 270\text{mV}$, prior to pump proton uptake, and $\approx 340\text{mV}$ after proton uptake. The observed heme $a$ midpoint potential of $\approx 280\text{mV}$ would suggest that the rate limiting step is the pump proton uptake rather than the substrate proton.

Transferring electrons from free Cytc into CytOx with a small $\Delta G$ is a challenge. Electron transfer between Cytc, Cu$_A$ and heme $a$ is much faster than the off rate for Cytc, therefore the electron will equilibrate between these three centers in the time that Cytc remains bound. Assuming midpoint potentials of 260, 250 and 280mV for Cytc, Cu$_A$ and heme $a$, respectively, the equilibrium will distribute one electron over the three redox centers at a proportion of 26:18:56\%, respectively. In this case, Cytc will leave the docking site reduced in 26\% of docking events and only 74\% of the events will result in electron transfer. If the three centers do not reach equilibrium before release of Cytc then even fewer binding events will result in electron transfer. The situation is worse for the second electron because the equilibrium is 66:50:84 for two electrons over the three redox centers and so only 34\% of dockings events will lead to electron transfer. This means that the burst phase of Cu$_A$ and heme $a$ reduction in time resolved studies [19-20, 27-28] must involve multiple dockings, and net electron transfer occurs with decreasing efficiency until there is equilibration of the redox potentials of the free Cytc, Cu$_A$ and heme $a$ [27]. The electron transfer efficiency of a docking can be increased if oxidized Cytc binds tighter to CytOx lowering the midpoint potential of the bound Cytc. For instance, lowering the midpoint potential by 30mV would increase the electron transfer efficiency to 90\% and 61\% for the first and second electron, respectively. Equal affinity for reduced and oxidized Cytc is a condition for the Minnaert model to generate Smith-Conrad kinetics, but we show this condition is relaxed in more realistic models. The $k_{on}$ of reduced and oxidized Cytc must be the same to generate Smith-Conrad kinetics (the term $k_{+2}[e^{2+}]+k_{+3}[e^{3+}]$ appearing in the rate equation must be independent of oxidation state), but the $k_{off}$ and $K_d$ can be different, as found when the $K_d$ was measured directly [29]. When the midpoint potential of bound Cytc is lower than free Cytc, the increase in electron transfer efficiency comes at the expense of a decreased $k_{off}$ of oxidized Cytc and, although maximum turnover occurred in the one-center model when the bound Cytc had a midpoint potential 40mV lower than free Cytc (inset of fig 2c), the increase in turnover was marginal (1\%). Nevertheless, this work shows that $k_{off}$ cannot be assumed to be the same for oxidized and reduced Cytc in the burst phase of time resolved models.
The Cytc concentration in the intermembrane space of mitochondria in living cells (≈700μM [30]) is much higher than that used here (10μM) and, consequently, the apparent first order \( k_{on} \) (\( k'_{i2} \) and \( k'_{i3} \) of eqn. 2) will be much higher than modeled here (28000s\(^{-1}\) compared to 400s\(^{-1}\)). This will bring the Cu\(_A\) center closer to equilibrium with the free Cytc for a given turnover number under these conditions and the \( k_{off} \) will become the limiting factor in maintaining equilibrium between bound and free Cytc. The \( k_{off} \) is equal to the product of \( k_{on} \) and the \( K_d \) so that the \( K_d \) becomes an important parameter when understanding the function in-vivo. The Minnaert model provides a simple framework to calculate \( K_d \) from experimental data because it predicts that the \( K_d \) is equal to the \( K_M \) [31]. However, this relationship is not maintained in the more realistic models where the \( K_M \) (3.48μM, 2.85μM and 3.56μM for the one-center, two-center and BNC model, respectively) was found to be an order of magnitude smaller than the \( K_d \) of the model (30μM). The \( K_d \) has also been estimated from time resolved studies where either reduced Cytc and CytOx are rapidly mixed in a stopped-flow apparatus [20] or the free Cytc is rapidly photo-reduced in a pre-mixed solution [19, 28]. The stopped-flow study found that the binding rates were strongly dependent on the ionic strength, suggesting that electrostatic interactions funnel the positively charged Cytc towards the negatively charged binding site on CytOx [32], but the \( K_d \) was independent of ionic strength consistent with binding occurring mainly through hydrophobic interactions [31]. The \( K_d \) was found to be 1-2μM in the stopped-flow study and ≈10-13μM in the photo-reduction studies. All the studies required the use of a model to calculate \( k_{on} \) and \( K_d \) from the experimental data. The stopped-flow measurements were calculated with a model in which Cytc binding was slow but electron transfer was very fast, consistent with later direct measurements of electron transfer rates from bound Cytc to Cu\(_A\) and heme \( a \) [8]. In contrast, the photo-reduction studies used a model in which Cytc binding was very fast but electron transfer from bound Cytc was very slow, placing a question mark on the accuracy of the calculated \( K_d \). Where the \( K_d \) was measured directly from the bound and free concentrations of Cytc [29], it was found to be even lower at 0.35 and 0.13μM for reduced and oxidized Cytc, respectively. Thus the different methodologies have produced values for \( K_d \) varying over two orders of magnitude from 0.13μM to 30μM with the higher values being calculated using questionable models. If the \( K_d \) is as low as 1μM, or even lower, then the \( k_{on} \) must be an order of magnitude greater than used here to support a \( k_{cat} \) of 25 O\(_2\)/s.

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References


Figure Captions

**Figure 1.** Graphical representations of the 4 models describing binding of Cytc to CytOX and electron transfer. Both reduced Cytc ($c^{2-}$) and oxidized Cytc ($c^{2+}$) bind reversibly in all models. In the a) Minnaert model: bound reduced Cytc is oxidized by the BNC in a single irreversible step. In the b) One-center model: the electron from reduced Cytc is accepted reversibly by a single center ($C_1$) before being irreversibly oxidized by the BNC. In the c) Two-center model, the electron passes reversibly through two centers ($C_1$ and $C_2$) before being passed irreversibly to the BNC. Finally, in the d) BNC model: the electron is passed through the CuA and heme a centers before being passed to the BNC, which cycles through the 5 reductions states (P, F, O, E and R) before oxygen binding (A) and reduction of oxygen to regenerate (P). Blue bi-arrows depict reversible Cytc binding and black bi-arrows/arrows denote reversible and irreversible electron transfers, respectively.

**Figure 2.** Comparison of experimental turnover data with the predicted by the Minnaert and the one-center model as the midpoint potential of either bound Cytc ($E_{m,b}$) and the donating center ($E_{m,1}$) is varied. All models use 10μM of Cytc as per the experimental data and a $k_{-2}$ and $k_{-3}$ of 40×10$^5$ M$^{-1}$ s$^{-1}$. a) Minnaert model with $k_b$ of 300 s$^{-1}$ as the midpoint potential of bound Cytc ($E_m$) is varied using eqn. 3. b) One-center model with $k_b$ of 86 s$^{-1}$, keeping $E_{m,b}$ constant at 260mV and varying the midpoint potential of the donating center ($E_{m,1}$). c) as b) but keeping $E_{m,1}$ constant at 260mV and varying $E_{m,b}$. The insert shows the turnover as $E_{m,b}$ is varied when $E_{m,1}$ is held at 260mV with 10μM of reduced Cytc.

**Figure 3.** Disequilibrium between free Cytc and the redox centers (C1 and C2) in a two-center model, in which the midpoint potentials of both bound Cytc and of C2 were held at 260mV. a) Comparison of experimental (circles) and modelled turnover data as the midpoint potential of the C1 is varied. b) Modelled disequilibrium between free Cytc and C1, and between C1 and C2, when...
C1 and C2 have a midpoint potential of 260mV. c) Comparison of the experimental oxidation states of CuA, heme a and the 655nm band (circles) with the modelled oxidation state of C1 when the midpoint potential of the center is varied between 230 and 290mV (lines).

**Figure 4.** Comparison of experimental data with that predicted by the BNC model. a) Experimental (circles) and modelled (solid line) turnover data. The dotted line is a regression to the linear component of the modelled turnover. b) Experimental oxidation states of CuA, heme a and the 655nm band (circles) compared to modelled oxidation state of CuA, heme a and CuB (lines). c) Modelled population of states P, F, O and E during turnover.
Graphical Abstract