Mitochondrial cytochrome c oxidase: mechanism of action and role in regulating oxidative phosphorylation

David F. Wilson and Sergei A. Vinogradov

Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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TO SUPPORT LIFE, THE THOUSANDS of different enzymes and metabolites in living cells must function as a highly organized and efficient unit. Malfunction of any part of the metabolic ensemble is life-threatening due to an accumulation of too much, too little, or just inappropriate relative amounts of critical components of the system. To make this complex ensemble work, there needs to be a control system that is programmed to set the operating point (metabolic homeostasis) and to coordinate the activities of many different reactions, making them work together to maintain the appropriate set point (40). There is only one metabolic pathway that interacts with all of the rest of cellular metabolism and yet can respond quickly (within seconds) to functionally significant perturbations in metabolism. This is oxidative phosphorylation, which provides the ATP required for molecular and ion transport, synthesis of proteins and nucleic acids, molecular synthesis, and structural assembly required for cell survival. In eukaryotic cells, mitochondrial oxidative phosphorylation is responsible for synthesis of most of the required ATP (>95%), and the rate of ATP synthesis is determined by the rate of oxygen consumption. The oxygen used for ATP synthesis is consumed exclusively by mitochondrial cytochrome c oxidase. Therefore, understanding the mechanism of oxygen reduction by cytochrome c oxidase and how it is regulated is essential to understanding metabolic homeostasis.

Mitochondrial cytochrome c oxidase is uniquely positioned to act as the control unit that sets and maintains metabolic homeostasis [see above and (40, 45)]. It is the third energy-coupling site of oxidative phosphorylation (site 3) and is responsible for reducing all of the oxygen used for ATP synthesis. The enzyme contains two cytochromes, a and a3 (18), and two copper atoms [see for example (2, 9, 10, 13, 14, 21–24, 32, 35, 37)]. At pH 7.2, cytochrome a and one copper atom have half-reduction potentials of 0.210 V and 0.225 V (21–24), similar to the 0.235 V for cytochrome c, whereas cytochrome a3 and the other copper atom have higher half-reduction potentials of 0.375 V and 0.350 V, respectively (21–24). The active site for oxygen binding and reduction is generally considered to be cytochrome a3. Lindsay and co-workers (21–23), however, showed that when potentiometric titrations were carried out in the presence of CO, an oxygen analog, the CO compound was an n = 2 electron acceptor (i.e., both cytochrome a3 and the high-potential copper atom had to be reduced for CO to bind with high affinity). They proposed that the site at which the oxygen was reduced was between the two metal atoms and that reduction proceeded through an intermediate with peroxide bridged between the iron and copper atoms of the reaction site has a central role in coupling mitochondrial respiration to the [ATP]/[ADP][Pi].

This paper presents a mechanism (model) for oxygen reduction by cytochrome c oxidase and critically evaluates the chemical and thermodynamic requirements of each step of oxygen reduction. The kinetic equations for this mechanism have been solved assuming the intermediates in oxygen reduction are in a steady state. The resulting steady-state rate expression has been fit to the measured relationships of the rate of oxygen reduction to the level of reduction of cytochrome c (the source of the reducing equivalents), oxygen pressure (pO2), and energy state as measured in suspensions of isolated mitochondria (44, 45).
dynamic constraints; in particular, the oxidation-reduction potential at reduction potential of reaction site. This occurs through cytochrome a and the lower potential.

Starting with the fully oxidized oxygen reaction site, the first step is transfer of a reducing equivalent (electron) from cytochrome c to the reaction site. This occurs through cytochrome a and the lower potential copper portion of the oxidase, but these are near redox equilibrium with cytochrome c (49).

\[
a_{3}^{3+} + Cu^{2+} + c^{2+} \overset{k_{1}}{\overset{k_{1r}}{\rightleftharpoons}} c_{3}^{3+} + a_{3}^{3+} - Cu^{4+} \quad (1)
\]

The half-reduction potential at pH 7.0 for cytochrome c is 0.235 V, and that of the high-potential copper is 0.35 V (21–24). These half-reduction potentials are both pH-independent. In uncoupled mitochondria, the equilibrium constant for this reaction is near 100. The values for the rate constants used in fitting the data are constrained, therefore, to give a ratio of k1/k1r near 100, and are consistent with the reported intrachain electron transfer rates [near 1,000 s−1; see for example (4, 10)].

The metal atoms in cytochrome a3 and the high-potential copper are in close proximity and their half-reduction potentials are similar. This allows rapid exchange of electrons and equilibration of the reducing equivalent between the iron and copper atoms. Reaction 1 suggests that copper is first reduced but, due to the rapid equilibration (reaction 3), this would be the iron of cytochrome a3. As soon as one of the metal atoms has been reduced, oxygen can enter the site and bind to the reduced metal atom (21–23).

**MATERIALS AND METHODS**

**Mitochondrial Data**

Measurements of the dependence of respiration by suspensions of isolated mitochondria are taken from published papers [see (44, 45)]. Graphics were prepared using Origin 7 (OriginLab, Northampton, MA) and computer fitting was performed with MatLab (MathWorks, Natick, MA).

Oxygen reduction by cytochrome c oxidase has strict thermodynamic constraints; in particular, the oxidation-reduction potential at which the reduction occurs. Oxygen can be a powerful oxidant, but at pH 7.0 acceptance of the first electron to form HO2 has a half-reduction potential of −0.33 V (Table 1), near that of the NAD couple (−0.32 V). Even two-electron reduction of oxygen to hydrogen peroxide has a half-reduction potential of 0.3 V, similar to that of cytochrome c. Oxidative phosphorylation, however, couples the oxidation of each NADH, with a potential of −0.26 V in liver (49) by oxygen (0.81 V) to synthesis of three ATP. Synthesis of ATP under cellular conditions (liver) requires about −14 kcal/mol (48), the energy available when two reducing equivalents are transferred down a redox potential difference of about 0.3 V. Thus in liver, the synthesis of three ATP per NADH oxidized accounts for 0.90 V of the 1.07 V redox potential difference between the NAD couple and oxygen. This means the site at which oxygen is reduced to water needs to have a potential near 0.6 V. Site 3, cytochrome c oxidase, is responsible for synthesis of one ATP for each two electrons transferred from cytochrome c to oxygen (17, 20). Cytochrome c is typically about 20% reduced in normoxic liver cells, for a redox potential of 0.27 V. To provide the 0.30 V needed to make ATP, the positive side (the oxygen reduction site) of site 3 needs to be at a redox potential near 0.57 V, consistent with the values calculated using all three sites. A summary of the thermodynamics for each step of oxygen reduction by cytochrome c oxidase is presented in Table 1.

**Oxygen Reduction by Cytochrome c Oxidase**

The mechanism of oxygen reduction by cytochrome c oxidase shown in Fig. 1 is consistent with current knowledge of the chemistry of oxygen and the properties of cytochrome c oxidase. The rate and equilibrium constants used to fit the data are summarized in Table 2. Starting with the fully oxidized oxygen reaction site, the first step is transfer of a reducing equivalent (electron) from cytochrome c to the reaction site. This occurs through cytochrome a and the lower potential copper portion of the oxidase, but these are near redox equilibrium with cytochrome c (49).

\[
a_{3}^{3+} + Cu^{2+} + c^{2+} \underset{k_{1}}{\overset{k_{1r}}{\rightleftharpoons}} c_{3}^{3+} + a_{3}^{3+} - Cu^{4+} \quad (1)
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The metal atoms in cytochrome a3 and the high-potential copper are in close proximity and their half-reduction potentials are similar. This allows rapid exchange of electrons and equilibration of the reducing equivalent between the iron and copper atoms. Reaction 1 suggests that copper is first reduced but, due to the rapid equilibration (reaction 3), this could be the iron of cytochrome a3. As soon as one of the metal atoms has been reduced, oxygen can enter the site and bind to the reduced metal atom (21–23).

**Table 1. Thermodynamics of oxygen reduction**

<table>
<thead>
<tr>
<th>Oxygen Reduction in Aqueous Solution</th>
<th>Half Reduction Potentials at pH 7.0</th>
<th>Ref. (1)</th>
<th>Ref. (51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. O2 + H+ + e− = HO2</td>
<td>−0.33 V</td>
<td>−0.283 V</td>
<td>−0.33 V</td>
</tr>
<tr>
<td>2. O2 + 2H+ + 2e− = H2O2</td>
<td>+0.28 V</td>
<td>0.267 V</td>
<td>0.281 V</td>
</tr>
<tr>
<td>3. O2 + 4H+ + 4e− = 2H2O</td>
<td>+0.815 V</td>
<td>0.81 V</td>
<td>0.815 V</td>
</tr>
<tr>
<td>4. H2O2 + 2H+ + 2e− = 2H2O</td>
<td>+1.35 V</td>
<td>1.36 V</td>
<td>1.35 V</td>
</tr>
<tr>
<td>5. O2− + 2H+ + e− = H2O2</td>
<td>+0.93 V</td>
<td>1.07 V</td>
<td>0.89 V</td>
</tr>
</tbody>
</table>

*Estimated using the indicated Ka for oxygen, but it could be more positive than this. The off-rate constant for oxygen dissociation is determined to be only <10 s−1 by the fitting procedure. †All the values are for pH 7.0 except these, which are the values without adjusting to pH 7.0.

**Fig. 1. A schematic (model) for the reduction of oxygen by cytochrome c oxidase.**
Table 2. Values of the kinetic parameters used to fit the steady-state rate expression to the experimental data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value Used for Fitting</th>
</tr>
</thead>
<tbody>
<tr>
<td>k1</td>
<td>8 x 10^8 M^-1 s^-1</td>
</tr>
<tr>
<td>k1r</td>
<td>8 x 10^7 M^-1 s^-1</td>
</tr>
<tr>
<td>k2</td>
<td>6 x 10^8 M^-1 s^-1</td>
</tr>
<tr>
<td>k2r</td>
<td>1 x 10^9 s^-1</td>
</tr>
<tr>
<td>K3</td>
<td>2 x 10^6 M^-1</td>
</tr>
<tr>
<td>K5</td>
<td>1 x 10^5</td>
</tr>
<tr>
<td>k4a</td>
<td>2.5 x 10^8 M^-1 s^-1</td>
</tr>
<tr>
<td>k4b</td>
<td>8 x 10^8 M^-1 s^-1</td>
</tr>
</tbody>
</table>

\[ a^{1+}_3 - Cu^{1+} + O_2 \leftrightarrow \frac{k2}{k2r} \times 10^8 \rightarrow a^{1+}_3 - Cu^{1+} - O_2 \]  (2)

The second-order rate constant for the reaction of oxygen with fully reduced cytochrome c oxidase has been reported (4, 10) to be near 2 x 10^8 M^-1 s^-1 and estimated to be as high as 1 x 10^10 M^-1 s^-1. Oxygen binds with high affinity (Kd < 10^-7 M), and the off constant has been estimated to be less than 10 s^-1 (48). Oxygen dissociation is sufficiently slow relative to enzyme turnover that the oxygen has little opportunity to dissociate. Importantly, binding of oxygen stabilizes the reduced form of the metal atom and shifts the redox potential for half-reduction to near 0.57 V (see below and reaction 6 in Table 1).

The half-reduction potential for cytochrome a3 is pH-dependent [see (23)], whereas that of the high-potential copper is not, and a proton is taken up when cytochrome a3 is reduced.

\[ a^{1+}_3 - Cu^{1+} - O_2 + H^+ \leftrightarrow K3 \times 2 \times 10^6 M^-1 \rightarrow O_2 - a^{2+}_3 - Cu^{2+} \]  (3)

Fit of the steady-state rate expression to the data gives a value for the equilibrium constant, K3, of 2 x 10^6 M^-1 or 0.2 at pH 7.0. The oxygen remains as bound oxygen until a second electron (reducing equivalent) arrives in the active site. The half-reduction potential for the second electron transferred to oxygen is 1.27 V, so it initiates a highly exergonic two-electron reduction of the oxygen to bound peroxide (reaction 4). Fit to the steady-state rate expression indicates this reaction occurs at somewhat different rates whether the oxygen is bound to a or Cu.

\[ a^{1+}_3 - Cu^{1+} - O_2 + c^2+ \rightarrow K4a \times 25 \times 10^5 \rightarrow c^{3+} + a^{1+}_3 - O^{2-} - Cu^{2+} \]  (4a)

\[ O_2 - a^{2+}_3 - Cu^{2+} + c^2+ \rightarrow K4b \times 8 \times 10^7 \rightarrow c^{3+} + a^{1+}_3 - O^{2-} - Cu^{2+} \]  (4b)

The peroxide is very tightly bound, with the thermodynamic calculations giving a dissociation constant at pH 7 of near 10^-10 M. The bound peroxide is further reduced to water in a two-electron transfer reaction:

\[ a^{2+}_3 - O^{2-} - Cu^{2+} + 2c^{2+} + 2H^+ \leftrightarrow K5 \times 1 \times 10^9 \rightarrow 2c^{3+} + a^{1+}_3 - Cu^{2+} + 2H_2O \]  (5)

Two H^+ ions are taken up in this reaction, as indicated by the pH dependence of the data. These two hydrogen ions plus the one ion taken up in reaction 3 account for three of the four hydrogen ions required for formation of the two water molecules. The point in the mechanism at which the fourth hydrogen ion enters remains to be determined.

**Comparison of Simulated Behavior of the Steady-State Kinetic Equation Derived for the Proposed Model with Experimental Observations**

Relationship of cytochrome c reduction to respiratory rate in isolated mitochondria. The steady-state rate equation has been programmed for MatLab (APPENDIX B). Figure 2 shows the predicted dependence of the respiratory rate on the fraction of cytochrome c reduced (fCyt) as a function of Q, the energy state expressed in volts (n = 2), at pH 7.95. As the value of Q is increased from a very low value of 0.040 V to 0.320 V, there is little effect of increasing Q until it is above about 0.160 V. Above 0.160 V, the respiratory rate at each level of reduction of cytochrome c is progressively suppressed with increasing Q. The straight-line relationship predicted for Q less than 0.160 V is consistent with that observed experimentally for mitochondrial suspensions treated with uncoupler (Figs. 3 and 4). We therefore simulated uncoupler-treated mitochondria as having a Q value of 0.160 V.

Fit of the simulated behavior with experimental measurements of the relationship of cytochrome c reduction and respiratory rate at pH 7.35 is shown in Fig. 2. As noted above, uncoupler-treated mitochondria are well fitted using a value of 0.160 V for Q. For well-coupled mitochondria with or without the addition of ATP, a good fit is observed when Q is near 0.30 V, with the best fit for different
mitochondrial preparations varying from 0.29 to 0.30 V. For mitochondrial suspensions treated with an [ATP]/[ADP][Pi] of 300 M\(^{-1}\), fit was obtained with a Q value of 0.260 V (at pH 7.35). The free energy of hydrolysis of ATP calculated for 300 M\(^{-1}\) (0 Mg\(^{2+}\), pH 7.35) is \(-11.9\) kcal/mol and for \(n = 2\); this is equivalent to a voltage difference of 0.257 V (11, 19).

Relationship of cytochrome c reduction to respiratory rate measured in isolated mitochondria at pH values from 6.5 to 8.35. The dependence of the rate of oxygen consumption on the level of reduction of cytochrome c at pH values of 6.5, 7.35, and 8.35 (45) is shown in Fig. 4. The simulated behavior of the model fits, within experimental error, the measurements at each pH both for coupled mitochondria (Q = 0.300 V) and uncoupled mitochondria (Q = 0.160 V). At more alkaline pH, such as pH 8.3, the respiratory rates when cytochrome c is less than 50% reduced are so low that contributions due to auto-oxidation of the ascorbate and endogenous substrates become significant. In addition, at these low respiratory rates the energy state (Q) would be expected to be lower than for higher respiratory rates. Both of these factors could contribute to the poorer fit of the model to the data when cytochrome c turnover is very low.

In our companion paper (45) the dependence on pH was summarized by fitting the data to an empirical equation, \(y = a(f_{\text{red}})^b\), where \(y\) is the respiratory rate as the TN for cytochrome c, \(f_{\text{red}}\) is the fraction of cytochrome c reduced, and a and b are parameters obtained by fit to the data. This allowed visualization of the variability among experiments and showed that as the pH increased from 6.5 to 8.35 the value of a decreased, whereas the value of b increased. The behavior predicted by the steady-state rate expression has been calculated and then fitted to the same empirical equation. Figure 5, A and B, compares the predicted dependencies with those measured experimentally. The predicted values are consistent with the experimental values and show similar dependencies on pH, indicating, as in Figures 3 and 4, the steady-state rate expression provides a good fit to the experimental data throughout the pH range over which measurements were made.

The high degree of control of the rate of oxygen reduction by cytochrome c oxidase is readily observed. At pH 7.35 and 25% reduction of cytochrome c, for example, the TN is about 1 s\(^{-1}\) for the coupled mitochondria, 12 s\(^{-1}\) for an [ATP]/[ADP][Pi] of 300 M\(^{-1}\), and 60 s\(^{-1}\) for uncoupled mitochondria. The combined dependences on energy state and cytochrome c reduction provides sufficient control to explain the observed increases of 50-fold and more in respiratory rate as skeletal muscles go from rest to maximal work.

Fig. 2. Dependence of respiratory rate on the fraction of reduced cytochrome c as a function of energy state as predicted by the model. The steady-state rate expression was solved for values of voltage difference, Q, from 0.320 V to 0.040 V, decreasing the value of Q in increments of 0.020 V. For mitochondrial preparations varying from 0.29 to 0.30 V, so each is labeled with the value of Q used in the calculation. For Q less than 0.200 V the dependencies are nearly linear and tightly spaced, so only that for 0.040 V is labeled.

Fig. 3. Dependence of the rate of oxygen reduction on the fraction of cytochrome c reduced \((f_{\text{red}})\): experimental data and fit by the model. The experimental data are taken from Wilson et al. (45) and are for rat liver mitochondria at pH 7.35. The reduction of cytochrome c was titrated with N,N,N’-tetramethyl-p-phenylenediamine (TMPD; 6 mM ascorbate) and the oxygen pressure was held above 60 torr. The turnover number for cytochrome c is plotted against the fraction of cytochrome c reduced for mitochondria coupled (●), treated with an [ATP]/[ADP][Pi] of 300 M\(^{-1}\) (3.3 mM Pi, 1 mM ATP, and 1 mM ATP) (▲), and after adding the uncoupler p-trifluoromethyl phenylhydrazone of carbonyl cyanide (FCCP) (○). The data corresponding to fits by the steady-state rate expression are shown with smaller, nearly continuous symbols, and are for Q values of 0.300, 0.260, and 0.160 V, respectively.

Fig. 4. The pH dependence of the relationship of cytochrome c reduced \((f_{\text{red}})\): experimental data and fit by the model. The experimental data for coupled rat liver mitochondria in media at pH 6.5 (●), 7.35 (▲), and 8.35 (○) and at the same pH values but after treatment with FCCP were taken from Figs. 2 and 3 of Wilson et al. (45). Fit by the model is shown as the smaller, nearly continuous symbols. The Q values used in the fit were 0.300 V for the coupled data at each pH and 0.160 V for the FCCP data.

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Behavior of cytochrome c reduction and respiratory rate as mitochondria deplete oxygen from the surrounding medium. The simulated oxygen dependence for constant reduction of cytochrome c ($f_{\text{red}} = 0.2$) and different, but constant values of $Q$, is shown in Fig. 6. The oxygen pressure for a 50% decrease in respiratory rate ($P_{50}$) is a function of the energy state ($Q$) decreasing from 20 torr when $Q$ is 0.300 V to near 4 torr when $Q$ is decreased to 0.200 V. This decrease in $P_{50}$ (with decrease in $Q$) is accompanied by a large increase in respiratory rate (cytochrome c TN), and therefore is not comparable to experimental measurements of the $P_{50}$ for respiration. To approximate the conditions most typically used to experimentally measure the $P_{50}$ for respiration, a hypothetical experiment was carried out in which mitochondria at 110 torr (14.7 kPa) oxygen pressure (cytochrome c TN = 1.6 sec$^{-1}$) and cytochrome c reduction ($f_{\text{red}} = 0.3$) were progressively depleted of oxygen down to 5 torr (0.67 kPa) while holding the level of reduction of cytochrome c and the respiratory rate constant. Five torr was chosen as the lower limit because, assuming a hyperbolic dependence of rate on oxygen pressure, no change in rate down to 5 torr would correspond to a $P_{50}$ of less than 1 torr. For this hypothetical experiment the value of $Q$ decreased from 0.300 V to 0.280 V. The calculated change in $[\text{ATP}]/[\text{ADP}][\text{Pi}]$ and concentrations of ADP and AMP are shown in Fig. 7. The levels have been normalized to their respective values at 110 torr to allow plotting in the same graph. The $[\text{ATP}]/[\text{ADP}][\text{Pi}]$ (1 mM Mg$^{2+}$, pH 7.35) fell from $4.91 \times 10^2 \text{M}^{-1}$ to $1.01 \times 10^2 \text{M}^{-1}$, ATP and Pi concentrations consistent with cellular values were assumed for 110 torr oxygen pressure (3 mM and 1.5 mM, respectively) and the adenylate kinase reaction was assumed to be near equilibrium. The concentrations of
free ADP and AMP could then be calculated to be $4.07 \times 10^{-5}$ M and $5.81 \times 10^{-7}$ M, respectively at 110 torr. During oxygen depletion the ADP and AMP concentrations increased by 4.9-fold and 24-fold, respectively, with no significant change in [ATP] and a 10% increase in [Pi].

Role of individual intermediates in oxygen reduction by cytochrome c oxidase in regulating the rate of the reaction. The steady-state rate expression for the model for cytochrome c oxidase allows calculation of the concentrations of each of the intermediates in the reaction. Figure 8 shows calculated levels of each intermediate (I through V) as the value of Q increased from 0.200 to 0.300 V when the pH is 7.35 and $f_{	ext{red}}$ for cytochrome c is 0.2. The levels of the intermediates are shown as their fraction of the total oxidase (sum = 1.0). As the value of Q increases, the levels of intermediates III and V decrease in proportion to the decrease in TN, consistent with their role in determining the rate of oxygen reduction. The decrease in TN is from near 29 s$^{-1}$ to 0.4 s$^{-1}$. As Q increases above 0.240 V, a rapid increase occurs in the fraction of the oxidase in the form of intermediate V, reaching 0.9 at Q = 0.300 V. The fraction of the oxidase in each intermediate species is a function of Q and the level of reduction of cytochrome c. When $f_{	ext{red}}$ is less than 0.2 and Q is near 0.300 V, the oxidase is nearly all in the form of V (Fig. 5).

DISCUSSION

In cells, oxidative phosphorylation is tightly coupled to the metabolic requirement for energy, primarily as ATP. During isolation of mitochondria, even from soft tissue such as liver, there is extensive damage to both structure and function. Structural damage, including breaking of the outer membrane, extensive swelling, disruption of the internal organization, and the presence of mitochondrial fragments has been extensively documented by electron microscopy. There are also many functional alterations, the most documented one being evident in the respiratory control ratio often used as a measure of the quality of the mitochondria. Both state 4 and state 3 (5) are experimental artifacts resulting from mitochondrial damage during isolation combined with nonphysiological assay conditions. In vivo, mitochondria can function over a wide range of rates without evidence for the existence of respiration not coupled to ATP synthesis (state 4). Muscles of insects, hummingbirds, and humans can reach metabolic rates from 50 to more than 100 times their resting rates (7, 8, 16, 30), and yet ATP is efficiently produced both when the muscle is resting (maintaining the ion gradients, synthesizing protein, etc.) and when it is at maximal work rate.

The state 4 respiration by suspensions of isolated mitochondria is due to isolation-induced leak(s) in energy coupling, but the molecular basis for the leak(s) and their effects on oxygen pressure dependence measurements is not clear. The leak is maximal when the energy level is maximal (mitochondria having adequate oxidizable substrates and oxygen but unable to make ATP) and decreases to insignificance when the energy level is low (added high concentrations of ADP and Pi or glucose plus hexokinase is used to trap the ATP as glucose-6-phosphate). Little is known about the behavior of the leak between these two extreme conditions. If we assume the leak is similar to introducing an ATPase activity (+ATP) or the addition of a small amount of uncoupler, it is not dependent on oxygen pressure. As a result, mitochondrial suspensions behave as in the hypothetical experiment in Fig. 7. The rate of respiration remains constant as the oxygen in the medium is depleted because Q changes as needed to match the rate of the leak. In cells, the situation is clearer because most of the ATP is consumed by reactions that are known to not be oxygen dependent and the energy state has to decrease as necessary to maintain the rate of ATP synthesis equal to its rate of utilization. In either case, a decrease in the rate of oxygen consumption would begin at much lower oxygen pressures (by a factor of 10 or more) than the increase in ADP and AMP.

In well-coupled mitochondria, nearly 100% of the oxidase can be in the form of the intermediate, V (Fig. 1). An energy-dependent change in the spectrum of cytochrome oxidase (43) has been reported to occur in suspensions of well-oxygenated pigeon heart mitochondria. The spectral change is consistent with the iron of cytochrome $a_3$ changing from the high-spin ferric form to the low-spin ferric form, as would be expected for transition from I to V. Intermediate V is unlikely to be observed as a significant fraction of the oxidase in preparations without intact energy coupling. The free energy of formation for V is large and negative, so it is a significant fraction of the oxidase in well-coupled mitochondria only in a high-energy state. In uncoupled mitochondria, the concentration is vanishingly small, and this would be expected for preparations of isolated cytochrome oxidase as well. It is also likely that both the formation and stability of V are dependent on precise positioning of the iron and copper (i.e., the geometry of the oxygen reduction site). Breakage of the copper-oxygen bond would likely lead to formation of more stable ferryl-oxo compounds (2, 29, 32, 35).

The oxygen dependence of respiration described by the steady-state rate expression for the model is consistent with the behavior of isolated mitochondria and intact cells (12, 31, 33, 41, 42) to well within the limits imposed by fit to the data and the experimental variability in the fitted data. The $P_{50}$ for the respiratory rate is dependent on the experimental conditions used, particularly the behavior of the enzyme state as the oxygen pressure decreases. The decrease in energy state required to maintain the respiratory rate constant to low oxygen pressures (50% decrease at <1 torr) is small. The increases in free ADP and AMP, however, are large enough (Fig. 7) to

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Fig. 8. The calculated levels of the intermediates in oxygen reduction as a function of the energy state (Q) as predicted by the steady-state rate expression for the model. The levels of intermediates I through V, as shown in Fig. 1, were calculated for the conditions given for Fig. 6. The levels of the intermediates are presented as their fraction of the total oxidase (sum of the intermediates is 1.0) as the value of Q was increased from 0.200 to 0.300 V.
cause substantial alterations in metabolism, including activation of AMP kinase signaling [see (6, 15, 25, 52)] and other signaling functions (26, 36, 38, 47). They are, however, too small to be readily measured with existing methods, particularly taking into account the difficulty in working at low oxygen pressures and the rapidity with which the values can change in response to changes in oxygen pressure. A decrease in the energy state in cell suspensions with decreasing oxygen pressure has been reported (41) using a specially designed apparatus that allowed very rapid quenching of metabolism without changes in oxygen pressure. The reported decrease is consistent with that predicted by the model.

The small decrease in energy state with decreasing oxygen pressure needed to keep the respiratory rate constant down to 5 torr provides a rational basis for the reports [see for example (3, 27, 28, 31, 33, 34)] that mitochondrial oxidative phosphorylation measured by the rate of respiration is not dependent on oxygen pressure until it is very low (< 10 torr). In these experiments the respiratory rate is being determined by the rate of leak and/or rate of ATP utilization, and these are not dependent on oxygen pressure until the levels of ATP fall substantially. As a result, the oxygen dependence is expressed not by a decrease in the rate of respiration, but by changes in the respiratory rate (and to decrease the P50 from 12 torr to 1 torr) is only 1% of the increase that can be induced by decreasing Q.

APPENDIX A

Steady-state kinetic expression for oxygen reduction by cytochrome c oxidase (Fig. 1).

Assumptions: Reactions 4A and 4B, is irreversible and the intermediates are in the steady state.

\[ \frac{dI}{dt} = k_1 \cdot cr \cdot I - k_1r \cdot co \cdot II + k_4a \cdot cr \cdot III - k_4b \cdot cr + IV \]  
\[ \frac{dII}{dt} = k_2 \cdot O \cdot II - k_2r \cdot III - k_1r \cdot II + k_1r \cdot co + II \]  
\[ \frac{dIII}{dt} = + k_2r \cdot III - k_2 \cdot O + II - (k_4a \cdot cr \cdot III + k_4b \cdot cr + IV) \]  
\[ -dIV/dt = k_1 \cdot cr \cdot I - k_1r \cdot co \cdot II - k_4a \cdot cr \cdot III - k_4b \cdot cr + IV \]  
\[ K_3 = \frac{IV}{II + III} \]  
\[ IV = K_3 \cdot H \cdot III \]  
\[ K_5 = \frac{(I + Q)/(V + H^2)}{co/cr}^2 \]  

From equation A2:

\[ k_2 \cdot O \cdot II = k_2r \cdot III + k_4a \cdot cr \cdot III + k_4b \cdot cr + IV \]  
\[ II = (k_2r + k_4a \cdot cr + k_4b \cdot cr + K_3 \cdot H)/k_2 \cdot O + III \]  
\[ II = A + III \]  

From equation A1:

\[ k_1 \cdot cr \cdot I = k_2 \cdot O + A + III - k_2r \cdot III + k_1r \cdot co + A + III \]  
\[ I = [(k_2 \cdot O + A - k_2r + k_1r \cdot co + A)/k_1 \cdot cr] \cdot III = I + B + III \]  

From equation A5:

\[ V = \frac{(O)/(K_5 \cdot H^2)}{co/cr}^2 \cdot B + III \]  
\[ V = C + III \]  

The total cytochrome a3 (a3t) is the sum of the intermediate; that is,

\[ a3t = (I + II + III + IV + V) \]  

or

\[ a3t = (1 + A + B + C + K3 \cdot H) \cdot III \]  

The rate of oxygen consumption is determined by the irreversible steps (reactions 4)

\[ v = \frac{(k_4a \cdot cr \cdot K3 \cdot H + k_4b \cdot cr) \cdot III + k_4b \cdot cr}{co/cr} \]  

\[ (cyt c turnover number) \]  

where cr is reduced cytochrome c; H is hydrogen ion; O is oxygen; co is oxidized cytochrome c; a3t is total cytochrome a3; k1, k2, k3, k4a, and k4b are forward reaction rate constants; k1r and k2r are reverse rate constants; and K3 and K5 are equilibrium constants. Q is the energy state in volts. To convert to kilocalories for each two reducing equivalents, multiply by 46.18 kcal/volt. The value of Q suitable for use in the steady-state expression (ratio of rate constants) is \(10^{0.0059}\) for \(n = 1\) and \(10^{0.0295}\) for \(n = 2\). Q is included in rate constants k1 and k1r in the MatLab program.

APPENDIX B

The program of the steady-state rate expression for MatLab calculates the respiratory rate as a function of energy state and cytochrome c reduction at an oxygen concentration of \(2 \times 10^{-4}\) M.

\[ k_1 = 8 \times 10^9 \]  
\[ k_1r = 8 \times 10^7 \]  
\[ k_2 = 6 \times 10^8 \]  
\[ k_2r = 1 \times 10^4 \]  
\[ K_3 = 1 \times 10^2 \]  
\[ K_5 = 1 \times 10^9 \]  
\[ k_4a = 2.5 \times 10^9 \]  
\[ k_4b = 8 \times 10^7 \]  
\[ a3t = 1 \times 10^{-6}; \% cytochrome a3 concentration \]  
\[ ct = 2 \times 10^8 \]  
\[ x = (1:100)^\prime; \% used to generate 100 levels for cytochrome c reduction \]

For

\[ q = 1:10; \% used to generate 10 levels for the energy state in volts \]  
\[ cr = x.*16.*10.^(-8); \% cr is reduced cytochrome c increased in 100 steps \]  
\[ co = ct - cr; \% co is oxidized cytochrome c \]  
\[ W = 7.35; \% W = pH of the medium \]  
\[ H = 10^-W; \% H = hydrogen ion concentration \]  
\[ Q = 0.200 + q.*0.01; \% sets 10 levels of energy state in volts \]  
\[ O = 2 \times 10^-4; \% O is the oxygen concentration \]  
\[ G = Q.*46.183; \% Gibb’s free energy in kcal for two-electron transfer \]  

\[ S = Q./0.059; \% coupling value for energy conservation \]  
\[ z = 10; \% S \]  
\[ kf1 = k1./z.\%0.5; \% couples k1 to energy state \]  
\[ kr1 = k1r.*0.5; \% couples kr1 to energy state \]  
\[ A = (k_2r + k_4a \cdot cr + k_4b \cdot cr)/(k_2 \cdot O) ; \% variable A in the steady-state expression \]  
\[ B = (k_2 \cdot O \cdot A + kr1.*co \cdot A - k_2r)/(kf1 \cdot cr); \% variable B in the steady-state rate expression \]  
\[ C = K_5.^-1.*(1/H^2.*(co/cr).^2.*z.^2.*B; \% variable C in the steady-state rate expression \]

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