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

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Wilson DF, Vinogradov SA. Mitochondrial cytochrome c oxidase: mechanism of action and role in regulating oxidative phosphorylation. J Appl Physiol 117: 1431–1439, 2014. First published October 16, 2014; doi:10.1152/japplphysiol.00737.2014.—Mitochondrial oxidative phosphorylation has a central role in eukaryotic metabolism, providing the energy (ATP) required for survival. Regulation of this important pathway is, however, still not understood, largely due to limitations in the ability to measure the essential metabolites, including oxygen (pO2, oxygen pressure), ADP, and AMP. In addition, neither the mechanism of oxygen reduction by mitochondrial cytochrome c oxidase nor how its rate is controlled is understood, although this enzyme determines the rate of oxygen consumption and thereby the rate of ATP synthesis. Cytochrome c oxidase is responsible for reduction of molecular oxygen to water using reducing equivalents donated by cytochrome c and for site 3 energy coupling in oxidative phosphorylation. A mechanism-based model of the cytochrome c oxidase reaction is presented in which transfer of reducing equivalents from the lower- to the higher-potential region of the coupling site occurs against an opposing energy barrier, Q. The steady-state rate equation is fitted to data for the dependence of mitochondrial respiratory rate on cytochrome c oxidase and for site 3 energy coupling in oxidative phosphorylation. 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 coworkers (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].

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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 coworkers (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 of cytochrome a3 and the high-potential copper atom. Formation of a bridged peroxide intermediate would facilitate breaking of the oxygen-oxygen bond and is consistent with the known chemistry of oxygen. It is also compatible with the very positive potential at which cytochrome c oxidase reduces oxygen to water, and the extensive biophysical measurements of the oxidase, including the crystal structure of the isolated enzyme [see (37)].

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).

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which the reduction occurs. Oxygen can be a powerful oxidant, but at

dynamic constraints; in particular, the oxidation-reduction potential at

reaction site. This occurs through cytochrome a and the lower poten-

tial copper portion of the oxidase, but these are near redox equilibrium
with cytochrome c (49).

\[
a_{3}^{3+} - Cu^{2+} + c^{2+} \rightleftharpoons k \times 8 \times 10^{9} \times 8 \times 10^{7} \rightarrow c^{3+} + a_{3}^{3+} - Cu^{4+} \quad (I)
\]

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 mitochondrial, 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/k1 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 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).

**Oxygen Reduction by Cytochrome c Oxidase**

![Fig. 1. A schematic (model) for the reduction of oxygen by cytochrome c oxidase.](http://jap.physiology.org/)

**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 thermody-
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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

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**Table 1. Thermodynamics of oxygen reduction**

<table>
<thead>
<tr>
<th>Half Reaction</th>
<th>Half Reduction Potentials at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. O₂ + H⁺ + e⁻ = HO₂</td>
<td>−0.33 V, −0.283 V, −0.33 V</td>
</tr>
<tr>
<td>2. O₂ + 2H⁺ + 2e⁻ = H₂O₂</td>
<td>+0.28 V, 0.267 V, 0.281 V</td>
</tr>
<tr>
<td>3. O₂ + 4H⁺ + 4e⁻ = 2H₂O</td>
<td>+0.815 V, 0.81 V, 0.815 V</td>
</tr>
<tr>
<td>4. H₂O₂ + 2H⁺ + 2e⁻ = 2H₂O</td>
<td>+1.35 V, 1.36 V, 1.35 V</td>
</tr>
<tr>
<td>5. O₂⁻ + 2H⁺ + e⁻ = H₂O₂</td>
<td>+0.93 V, 1.07 V, 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.*
The second-order rate constant for the reaction of oxygen with fully reduced cytochrome c oxidase has been reported (4, 10) to be near $2 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and estimated to be as high as $1 \times 10^7 \text{M}^{-1}\text{s}^{-1}$. Oxygen binds with high affinity ($K_0 < 10^{-7} \text{M}$), and the off constant has been estimated to be less than $10^{-3} \text{s}^{-1}$ (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 $0.57 \text{V}$ (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.

$$a^3_3^- + Cu^{1+} + O_2 + H^+ \leftrightarrow \frac{k_2 = 6 \times 10^8}{k_{2r} = 10} \rightarrow a^3_3^- + Cu^{1+} + O_2^- (2)$$

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$$a^3_3^- + Cu^{1+} + O_2 + H^+ \leftrightarrow \frac{k_3 = 2 \times 10^6}{K_3 = 2 \times 10^6 \text{M}^{-1}} \rightarrow O_2^- - a^3_3^- + Cu^{2+} (3)$$

Fit of the steady-state rate expression to the data gives a value for the equilibrium constant, $K_3$, of $2 \times 10^6 \text{M}^{-1}$ 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 equilibrium constant, $K_3$, of $2 \times 10^6 \text{M}^{-1}$.

$$O_2^- - a^3_3^- + Cu^{2+} + c^{2+} \rightarrow a^3_3^- + O_2^- + Cu^{2+} + c^{2+} (4b)$$

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

$$a^3_3^- + O_2^- + Cu^{2+} + 2c^{2+} + 2H^+ \leftrightarrow \frac{K_5 = 1 \times 10^9}{K_5 = 1 \times 10^9} \rightarrow 2c^{2+} + a^3_3^- + Cu^{2+} + 2H_2O (5)$$

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.

**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>$k_1$</td>
<td>$8 \times 10^9 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_{1r}$</td>
<td>$8 \times 10^7 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$6 \times 10^8 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_{2r}$</td>
<td>$1 \times 10^7 \text{s}^{-1}$</td>
</tr>
<tr>
<td>$K_3$</td>
<td>$2 \times 10^4 \text{M}^{-1}$</td>
</tr>
<tr>
<td>$k_a$</td>
<td>$1 \times 10^{55}$</td>
</tr>
<tr>
<td>$k_{4a}$</td>
<td>$2.5 \times 10^4 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_{4b}$</td>
<td>$8 \times 10^7 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
</tbody>
</table>

either the difference in the redox potential of the respiratory chain components ($\Delta E$) or the [ATP][ADP][P] (where P represents inorganic phosphate). For net oxygen reduction (respiration) to occur, $\Delta E$ must be equal to or greater than the energy that is coupled to ATP synthesis.

**Steady-state kinetic equations for the cytochrome c oxidase model.** The rates of the intermediate reactions have been shown to be fast compared with the net rate of oxygen reduction, so the levels of the intermediates may be considered to be in a steady state. The fit of the model to the data is consistent with interconversion of intermediates III and IV (reaction 3) and V to I (reaction 5) being in near equilibrium. The two-electron reduction of intermediates III and IV to V (reactions 4A and 4B) is irreversible, and therefore rate limiting, under all conditions. The assumption that the levels of the intermediates are in a steady state is reasonable because in most unstimulated cells the cytochrome c turnover number (TN) is less than $10^{-1} \text{s}^{-1}$ whereas 1 electron transfer between redox components has been reported to have first-order rate constants (TNs) of at least $1,000 \text{s}^{-1}$ for cytochrome c (4, 10); 2 the maximal rate of oxygen reduction (high energy state and fully reduced cytochrome c) is for a cytochrome c TN of about $200 \text{s}^{-1}$; and 3) the reactions are fully reversible, and transfer of reducing equivalents from the low- to high-potential side of each site can synthesize ATP and, conversely, hydrolysis of ATP can drive reducing equivalents from the high to low potential side of each site.

For near-equilibrium reactions, the energy loss is negligible and the energy conserved in the intermediate reactions is independent of the internal mechanism. This means energy coupling can be accurately represented by the appropriate energy barrier without specifying the mechanism. We have chosen to represent energy coupling by assuming that the electron transfers result in a charge separation that generates a voltage difference, Q (equal to but opposite in sign to $\Delta E$). This formally couples the equilibrium and rate constants involved in transferring electrons from the lower potential to the higher potential to Q, and charge transfer occurs only by driving the voltage difference, Q. The steady-state rate expression for the model and its derivation are shown in APPENDIX A. The voltage difference, Q, can be converted to an equivalent free energy of ATP synthesis by multiplying the volts by 46.133 kcal/volt (193.2 kJ/volt). For conversion to the energy state, the equilibrium constants for ATP synthesis have been taken from Lawson and coworkers (19, see also 11) for the appropriate pH and Mg$^{2+}$ concentration.

**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 (fread) 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 Mg²⁺, 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, \( y = a(f_{red})^b \), where y is the respiratory rate as the TN for cytochrome c, \( f_{red} \) 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 experimental 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 experimental data.
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 ($\text{red}$) 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 ($\text{TN}$), and therefore is not comparable to experimental measurements of the $P_{50}$ for respiration.

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 $\text{[ATP]/[ADP][Pi]}$ 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 $\text{[ATP]/[ADP][Pi]}$ (1 mM Mg$^{2+}$, pH 7.35) fell from $4.91 \times 10^4$ M$^{-1}$ to $1.01 \times 10^3$ 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...
oxidase is nearly all in the form of V (Fig. 5). As the value of Q increases from 0.200 to 0.300 V, the fraction of the oxidase in the form of intermediates increases. The levels of the intermediates are shown as their fraction of the total oxidase, summing to 1.0. As the value of Q increases, the levels of intermediates III and V increase 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_cysteine is less than 0.2 and Q is near 0.300 V, the oxidase is nearly all in the form of V (Fig. 5).

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_cysteine for cytochrome c is 0.2. The levels of the intermediates are calculated for the conditions given for Fig. 6. The levels of intermediates I through V 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. As the value of Q increases, the levels of intermediates III and V increase 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_cysteine 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 a₃ 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 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 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 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 preparations without intact energy coupling.

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 P₅₀ for the respiratory rate is dependent on the experimental conditions used, particularly the behavior of the energy 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

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 dependency is expressed not by a decrease in the rate of respiration, but by changes in leak and/or rate of ATP utilization, and these are not dependent on oxygen pressure until the levels of ATP fall substantially. The rate of respiration is much more dependent on energy state and f_red than on pO_2 per se.

**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.

\[
\begin{align*}
\frac{dl}{dt} &= k_1 \cdot c + I - k_1 \cdot c + II + k_{q1} \cdot c + III - k_q \cdot c + IV \\
\frac{dl}{dt} &= k_2 \cdot O + II - k_2 \cdot III - k_1 \cdot c + I + k_{q1} \cdot c + II \\
\frac{dl}{dt} &= + k_{2r} \cdot III - k_2 \cdot O + II - (k_{q4} \cdot c + III + k_qb \cdot c + IV) \\
\frac{dV}{dt} &= k_1 \cdot cr + I - k_1 \cdot cr + II - k_2 \cdot cr + III - k_qb \cdot cr + IV
\end{align*}
\]

**K3** = \[\frac{IV}{III + H}\]  
**IV** = K3 + H + III  
**K5** = \|[1 + Q]/(V + H^2)| \cdot (co/cr)^2  
From equation A2:

\[
\begin{align*}
k_2 \cdot O + II &= k_2r \cdot III + k_4a \cdot cr + III + k_qb \cdot cr + IV \\
II &= (k_2r + k_4a \cdot cr + + k_qb \cdot cr + K3 + H)/k_2 \cdot O + III \\
II &= A + III
\end{align*}
\]

From equation A1:

\[
\begin{align*}
k_1 \cdot c + I &= k_2 \cdot O \cdot A + III - k_2r \cdot III + k_{1r} \cdot c + A + III \\
I &= [(k_2 \cdot O + A - k_{2r} + k_{1r} \cdot c + A) / k_1 \cdot c] + III \\
I &= B + III
\end{align*}
\]

From equation A5:

\[
V = ([O]/(K_{5} + H^2) \cdot (co/cr)^2 + B + III \\
V &= C + III 
\]

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

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

or

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

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

\[
v = (ko \cdot c + K3 + H + k_{4b} \cdot cr + III + k4b/cr) \cdot (cyt c turnover number)
\]

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^{9.0295} for n = 1 and 10^{9.0295} 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.

\[
k_1 = 8 \times 10^9 \\
k_{1r} = 8 \times 10^7 \\
k_2 = 6 \times 10^8 \\
k_{2r} = 1 \times 10^4 \\
k_3 = 2 \times 10^6 \\
k_5 = 1 \times 10^25 \\
k_4a = 2.5 \times 10^8 \\
k_4b = 8 \times 10^7 \\
ak_3t = 1 \times 10^4 - 6; \text{ % cytochrome a3 concentration} \\
cr = 2 \times 10^{-4} - 6; \text{ % cytochrome c concentration (2 \times cytochrome a3)} \\
x = (1:100)^{1/2}; \text{ % used to generate 100 levels for cytochrome c reduction}
\]

\[
f_q = 1:10; \text{ % used to generate 10 levels for the energy state in volts} \\
cr = x^{1.6} \times 10^{-8}; \text{ % cr is reduced cytochrome c - increased in 100 steps}
\]

\[
\begin{align*}
co &= ct - \text{cr; \ % co is oxidized cytochrome c} \\
W &= 7.35; \ % W = pH of the medium \\
H &= 10^{-W}; W; \ % H = hydrogen ion concentration \\
Q &= 0.200 + q; 0.01; \ % sets 10 levels of energy state in volts \\
O &= 2 \times 10^{-4} - 4; \ % O is the oxygen concentration \\
G &= Q \cdot 46.183; \ % Gibb’s free energy in kcal for two-electron transfer
\end{align*}
\]

\[S = Q^{0.059}; \ % coupling value for energy conservation \\
z = 10^{0.5S}; \\
kf1 = k_1z^{0.5}; \ % couples k1 to energy state \\
k_{1r} = k_{1r}z^{0.5}; \ % couples k1r to energy state \\
A = (k_{2r} + k_4a \cdot cr + k_{4b} \cdot cr \cdot K3 \cdot H)/k_2 \cdot O; \ % variable A in the steady-state expression \\
B = (k_2 \cdot O \cdot A + k_{1r} \cdot co \cdot A + k_{2r})/(k_{1r} \cdot cr); \ % variable B in the steady-state rate expression \\
C = K5 \cdot 1 \cdot (1/H)z^{2.2} \cdot (co/cr)^{2} \cdot z^{2} \cdot B; \ % variable C in the steady-state rate expression
\]
REFERENCES


