The Mechanism of Superoxide Production by the Antimycin-inhibited Mitochondrial Q-cycle*

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Superoxide production from antimycin-inhibited complex III in isolated mitochondria first increased to a maximum then decreased as substrate supply was modulated in three different ways. In each case, superoxide production had a similar bell-shaped relationship to the reduction state.

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Q-cycle Superoxide

ROS production must be related to the lower reduction state of the Q pool when succinate oxidation was restricted, and they proposed that electron flow from cytochrome b$_{566}$ to reduce Q may be the mechanism by which semiquinone and superoxide are increased. Recently, Sarewicz et al. (17) further examined this hypothesis in Rhodobacter capsulatus experimentally and using a kinetic model. They found evidence to support an important role of this pathway.

In this study, we investigate, model, and describe the mechanism underlying this phenomenon in isolated mitochondria. We show that it is consistent with a conventional Q-cycle mechanism for complex III, but only if the rate constant for superoxide dismutase is assumed to be very small when antimycin is present (and even smaller when cytochrome b$_{566}$ is reduced), and the semiquinone is assumed to be stabilized when cytochrome b$_{562}$ is reduced.

EXPERIMENTAL PROCEDURES

Mitochondria—Mitochondria were isolated from hind limb skeletal muscle of female Wistar rats aged 5–8 weeks by differential centrifugation (18, 19).

Superoxide Production by Isolated Mitochondria—Superoxide production was measured indirectly as H$_2$O$_2$ production after dismutation by endogenous and exogenous superoxide dismutases. H$_2$O$_2$ production rates were determined by measurement of Amplex UltraRed fluorescence (Invitrogen) in a Shimadzu RF5301-PC spectrofluorometer (Kyoto, Japan) at the wavelength couple excitation = 530 nm and emission = 560 nm. Horseradish peroxidase was added to catalyze the reaction of extramitochondrial H$_2$O$_2$ with Amplex UltraRed to form the fluorescent product resorufin. Mitochondria (0.25 mg/ml) were incubated at 37 °C in standard assay medium containing 50 mM KCl, 5 mM HEPES, 1 mM EGTA (pH 7.2 at 20 °C), and 0.3% (w/v) bovine serum albumin. All assays contained 50 μM Amplex UltraRed, 5 units of horseradish peroxidase/ml, and 12 units of superoxide dismutase/ml. Exogenous superoxide dismutase was added to maximize conversion to H$_2$O$_2$ of extramitochondrial superoxide produced by complex III (20). Fluorescence emission data were collected and translated to H$_2$O$_2$ amounts using H$_2$O$_2$ standard curves obtained under identical conditions (20). Linear rates of change were measured for 30–60 s for each substrate condition. Superoxide production from the Q$_m$ site was defined by sensitivity to the Q$_m$ site inhibitor, stigmatellin; all data in this study refer to rates that were uninhibitable by stigmatellin, with all stigmatellin-insensitive H$_2$O$_2$ production subtracted before further calculations. All data were corrected for H$_2$O$_2$ consumption by matrix glutathione peroxidase as described previously (21). The best current estimate of the topology of superoxide production by complex III is that 63% is directed to the matrix (21), based on the empirical observation of 50% matrix-directed superoxide production (22) and correcting for matrix glutathione peroxidase activity. We therefore corrected the empirical rates of stigmatellin-sensitive H$_2$O$_2$ production using equation 1 (21),

\[ \text{corrected rate of } H_2O_2 \text{ production} = \frac{\text{observed rate}}{1 + (1.43 \times 0.5 \times \text{observed rate})/(0.55 + 0.5 \times \text{observed rate})} \]

All chemicals were purchased from Sigma unless otherwise noted.

ATP-generated Protonmotive Force—Mitochondrial membrane potential in the presence of respiratory chain inhibitors, nigericin, and ATP is equal to the protonmotive force and was determined using an electrode sensitive to methyltriphosphonium as described previously (23). Skeletal muscle mitochondria were incubated under the same conditions as for H$_2$O$_2$ production measurements at 37 °C in standard buffer with nigericin. ATP hydrolysis routinely generated a protonmotive force of 130–150 mV, similar to values described previously (24).

Reduction States of Cytochromes b$_{566}$ and b$_{562}$—Experiments were performed in parallel with measurements of H$_2$O$_2$. Mitochondria were suspended at 1 mg/ml in standard assay medium, and absorbance change was measured in an Olis DW-2 dual wavelength spectrophotometer (Bogart, GA). The b$_{566}$ signal was followed at the wavelength pair 566–575 nm at 37 °C with stirring (25, 26). Reduction of b$_{566}$ was assumed to be 0% with no added substrate and 100% with saturating substrates plus antimycin A. The contribution of cytochrome b$_{562}$ to the b$_{566}$ signal was corrected by subtracting 50% of the signal at 561–569 nm (26). Where experimental data were normalized to moles of superoxide produced per mol of bc$_1$ complex, calculations used the cytochrome b$_{566}$ extinction coefficient $\varepsilon = 25$ liters·mmol$^{-1}$·cm$^{-1}$ (27). The cytochrome b$_{562}$ signal was followed at 561–569 nm (26). For the modeling (see below) the redox state of b$_{566}$ ($b_{566}^{\text{red}}$) was calculated as a function of $b_{566}^{\text{red}}$ redox state ($b_{566}^{\text{red}}$ in %) by assuming thermodynamic equilibrium between the two b hemes (Equation 2),

\[ E_m^{b_{566}} + \frac{RT}{F} \ln \frac{100 - b_{566}^{\text{red}}}{b_{566}^{\text{red}}} = E_m^{b_{566}} + \frac{RT}{F} \ln \frac{100 - b_{566}^{\text{red}}}{b_{566}^{\text{red}}} \]

(Eq. 2)

A function was generated by solution of Equation 2 for the reduction state of b$_{562}$ shown in Equation 3,

\[ b_{566}^{\text{red}} = 100\left(1 + \frac{100}{b_{566}^{\text{red}}} - 1\right) \exp\left(-\frac{E_m^{\text{red}}}{RT}\right) \]

(Eq. 3)

where $E_m^{\text{red}} = E_m^{b_{566}} - E_m^{b_{562}}$ is the molar gas constant (8.314 J·K$^{-1}$·mol$^{-1}$), $T$ is the temperature (310 K), and $F$ is the Faraday constant (96485 C·mol$^{-1}$).

Nonlinear regression on the b heme reduction data (see Fig. 4) was performed using Equation 3, taking $E_m^{\text{red}}$ as a regression parameter $E_m^{\text{red}} = -60 \pm 3$ mV in de-energized mitochondria and 2.4 ± 5 mV in the presence of ATP. A similar redistribution of electrons between the b hemes in the presence of ATP was observed previously (28).

Modeling of Q$_m$ Site Kinetics and Superoxide Production—A kinetic model of the Q$_m$ site (Fig. 5) was built in CellDesigner™ 4.01 (29), then translated into the Systems Biology Markup Language, and exported to Mathematica 5.2 (Wolfram Research, Champaign, IL). It was solved by numerical differen-
tional equation using the SBMLNDSolve function in MathSBML 2.9.0 for a set of 15 QH$_2$/Q ratios. Superoxide production rates and % reduction of cytochrome b$_{566}$ were calculated in steady state (at $t = 10$ s). A total Q pool concentration of 7 mM in the lipid phase was assumed (30, 31).

To fit the model to the experimental data, the values of rate constants were optimized within defined boundaries. Ranges of rate constants for complex III from *Rhodobacter sphaeroides* were taken from Ref. 32, with wider ranges of values allowed based on values for bovine submitochondrial particles (35) described in Table 1. The fitting algorithm was based on the iterative solution of the model using SBMLNDSolve with varying parameters. A weighted, multitem least squares error function (Equation 4) that estimated the goodness of fit and penalized deviations from empirical thermodynamic relationships was calculated for each solution and minimized using the “NMinimize” numerical optimization function of Mathematica. The optimization was performed using the “DifferentialEvolution” method with default parameters. To calculate the error function, modeled rates were interpolated, yielding superoxide production rate as a function of b$_{566}^{\text{red}}$.

The first term of the error function estimated the goodness of fit of the interpolated modeled rates (y) to the experimental data (x) at corresponding cytochrome b$_{566}$ redox states (i) (Equation 4).

$$\text{error} = \sum_{i} \left( \frac{x_i - y_i}{x_i} \right)^2 + 20(x_{100} - y_{100})^2 + 0.08(b_{566}^{\text{red}}|_{QH_2} = 100\% - 100\%)^2 + 5(m - 0.55)^2 + 0.4\left(c - \frac{2mF}{RT}(0.066 - 0.0725)\right)$$

(Eq. 4)

To further constrain the optimization, additional terms were included in the error function. First, the superoxide production rate at fully reduced b$_{566}$ ($x_{100}$) was given additional weight, because it was well determined in the experimental data. This term was multiplied by the number of experimental observations (20 data points) to give it equivalent weight to the sum of squares. Second, at 100% Q-reduction, the model was expected to provide close to 100% reduced b$_{566}$. Third, although the model is kinetic, the results were expected to obey the known thermodynamics in steady state; therefore the known empirical relationship between Q/QH$_2$ and b$_{566}^{\text{total}}$ redox pairs (25) was assessed. To accommodate this, the average b heme redox state was calculated using the modeled b$_{562}^{\text{red}}$ from Equation 3. Linear regression was performed by taking ln(Q/QH$_2$) as an independent variable and ln(b$_{566}^{\text{total}}$/b$_{566}^{\text{red}}$) as dependent. The slope (m) of the fit is the reciprocal of the number electrons passed between QH$_2$ and b$_{566}$ and its deviations from the observed value of 0.55 (25) were weighted into the error sum. The ordinate intercept (c) is $F/RT$ times the difference of midpoint potentials of QH$_2$ ($E'_{\text{QH2}} = 66$ mV at pH 7) and the average b ($E'_{\text{b}} = 72.5$ mV at pH 7) (25). Squared errors between the expected and modeled c values were added to the error function. Weights were empirically set to obtain a robust fitting. Alternative weightings were also tested, resulting in similar fits (data not shown). The Differential Evolution optimization algorithm initializes optimization with a randomly seeded population of rate constant sets. The “convergent evolution” of this population through a random process to the global minimum of the error function provides the best fit. Therefore, optimization was repeated many times with different seeds to ensure that a global optimum was found.

Between seven and nine rate constants were optimized within specified ranges (see Fig. 5 and reactions and ranges in Table 1). We assumed that the redox state of b$_{566}$ did not affect the binding of Q and QH$_2$ ($k_1 = k_2 = k_3 = k_4$). $k_2 = k_4$. For simplicity, $k_a$ was set at $k_a/3$ (10). Reactions given no backward rate constant were considered to be physiologically irreversible because of negligible product accumulation: reactions 5 and 7 in Table 1 where no reduced Fe-S protein accumulates, and reaction 9 where superoxide is irreversibly removed by the assay system. The backward rate of reaction 8 in Table 1 was considered to be negligible.

Using the same model (Fig. 5), the fitting algorithm was built to increasing complexity in four trials by increasing the number of fit parameters and including dependence of the rate of reaction 6 on the redox state of cytochrome b$_{562}$ (Table 1). In trial 1, a set of 13 optimization runs on different seeds of the random generator were performed with seven parameters varied as follows: $k_1, k_{-1}, k_2, k_{-2}, k_6, k_{-6}$, and $k_a$ with fixed $k_5 = k_b = 1650$ s$^{-1}$. In trial 2, $k_9$ and $k_{-9}$ were allowed to take a wider range of values (Table 1), and the optimization was performed 11 times and typically resulted in convergence within 200 iterations in a 20-h run time on a standard PC at 2.2 GHz (Intel(R) Core(TM)2 Duo processor). Trial 3 was the same as trial 2, but in addition, $k_5$ and $k_{-5}$ were assumed to be dependent on the redox state of b$_{562}$; $k_9$ and $k_{-9}$ were calculated as an average of rate constants in the presence of reduced and oxidized b$_{562}$, weighted by the b$_{562}$ reduction state (calculated by Equation 3 from b$_{566}^{\text{red}}$). Trial 4 used the same constraints on rate constants as trial 3, but the model was solved for both energized and de-energized mitochondria. The same rate constants, but a different b$_{562}$ to b$_{566}$ redox state function (Equation 3), were used, and the error function was calculated as the sum of Equation 4 calculated for the two conditions. Because this added extra constraints, the last two terms in Equation 4 were omitted for trial 4, resulting in a wider range of fitted values. Each run for trials 3 and 4 typically converged within 400 iterations in 22 and 82 h, respectively. The presence or absence of inflections and peaks in the superoxide production rate curves was determined by numerical differentiation and root search to ensure objectivity.

**RESULTS**

**Substrate Limitation Increases Superoxide Production from Complex III in Isolated Mitochondria**—Previous work (15, 16) showed that addition of malonate, a competitive inhibitor of succinate dehydrogenase, causes a substantial increase in ROS production from antimycin-inhibited isolated complex III and submitochondrial particles in the presence of a high concentration of succinate. Fig. 1a recapitulates this result in isolated skeletal muscle mitochondria (where malonate inhibits both succinate dehydrogenase and succinate transport). At saturat-
ing succinate concentration (5 mM), in the absence of malonate and the presence of rotenone to inhibit complex I and antimycin A to inhibit electron flow through the Q site of complex III, the stigmatellin-sensitive H$_2$O$_2$ production rate was about 2 nmol·min$^{-1}$·mg protein$^{-1}$. The sensitivity to stigmatellin defined superoxide generated in the Qo site of complex III, with the reasonable assumption that no other superoxide-generating site changed redox state when stigmatellin was added under these conditions. As malonate was progressively added, the rate of H$_2$O$_2$ production more than doubled, with a peak rate of 5 nmol·min$^{-1}$·mg protein$^{-1}$, before decreasing again at higher malonate concentrations as the supply of electrons from succinate was attenuated.

To examine previous suggestions that this effect was caused by oxidation of the Q pool, and was not specific to malonate or complex II, we tested whether it could be reproduced in other ways. Fig. 1b shows that the result could be replicated by progressive titration with succinate; H$_2$O$_2$ production increased with succinate concentration, reached a peak at about 1 mM succinate, and then decreased at higher succinate concentrations. Fig. 1c shows that the same was true when complex II was bypassed, and electrons entered the Q pool from complex I instead, during titration with glutamate plus malate in the absence of rotenone.

These results show that saturating substrate concentrations do not lead to maximal superoxide production from the Qo site of complex III; instead, the results are consistent with superoxide generation being maximal at an intermediate Q reduction state.

**Superoxide Production Rate Is a Function of Cytochrome b$_{566}$ Redox State**—Cytochrome b$_{566}$ receives the second electron during quinol oxidation at the Qo site. In the presence of antimycin, excess substrate, and oxygen to keep cytochrome c$_1$ oxidized, b$_{566}$ will become maximally reduced because the normal egress of electrons from the system through site Qi is inhibited. Fig. 1 (d–f) shows the redox behavior of cytochrome b$_{566}$ during the substrate titrations in Fig. 1 (a–c). In each case, as the available substrate increased, b$_{566}$ became more reduced. When the rates of H$_2$O$_2$ production were plotted against the reduction state of cytochrome b$_{566}$ (Fig. 2), each substrate combination gave a bell-shaped curve. The three curves were very similar, suggesting that there was a single relationship between H$_2$O$_2$ production and the reduction state of cytochrome b$_{566}$ under these conditions; in each
The suppression of superoxide production at intermediate substrate supply required ATP hydrolysis, because it was prevented by addition of oligomycin to inhibit the F₁Fₒ-ATPase (data not shown).

The Distribution of Electrons between Cytochrome b₅₆₆ and Cytochrome b₅₆₂ Is Altered by ATP Hydrolysis—ATP hydrolysis generates an electrical potential across the mitochondrial inner membrane, which is expected to alter the distribution of electrons between cytochrome b₅₆₆ (on the cytosolic side of the mitochondrial inner membrane) and cytochrome b₅₆₂ (on the matrix side) (34). Because of its more positive midpoint potential, b₅₆₂ is the preferred electron acceptor when there is no membrane potential (28). However, in the presence of membrane potential, the apparent midpoint potentials become similar, leading to a more equal distribution of electrons. These predictions were checked under the conditions of Figs. 2 and 3. Fig. 4a shows that in the presence of rotenone and antimycin, but the absence of a membrane potential, cytochrome b₅₆₂ initially remained almost fully reduced, but cytochrome b₅₆₆ became oxidized as malonate concentration was increased, consistent with the more positive midpoint potential of b₅₆₂. However, Fig. 4b shows that in the presence of rotenone, antimycin, and ATP, the electron distribution between b₅₆₆ and b₅₆₂ was more equal, and both became oxidized as malonate increased, indicating that in the presence of a membrane potential b₅₆₂ was no longer preferentially reduced.

Thus, the imposition of a membrane potential markedly changed the relationship between superoxide production and b₅₆₆ reduction (Figs. 2 and 3). It also markedly changed the relationship between the redox states of cytochromes b₅₆₂ and b₅₆₆ (Fig. 4). These results suggest that the imposition of a membrane potential affects superoxide production rate from the Qₒ site of complex III by changing the redox state of cytochrome b₅₆₂. This suggestion is examined more fully below.

Is the Observed Maximum in Superoxide Production Rate Predicted by the Q-cycle?—To test whether maximum superoxide production at submaximum substrate supply and submaximum cytochrome b₅₆₆ reduction (Fig. 2) can be quantitatively explained by the Q-cycle mechanism of complex III, we generated a kinetic model of the Qₒ site of the antimycin-inhibited Q-cycle (Fig. 5).

The Qₒ site contains a Q-binding site and cytochrome b₅₆₂ (and the Rieske Fe-S center, which was assumed for simplicity to be rapidly reoxidized by cytochrome c₁ whenever it became reduced). Because there are four states of the Q-binding site (empty, QH₂-bound, Q-bound, and semiquinone (SQ)-bound) and two states of cytochrome b₅₆₆ (reduced, shown in yellow on the left, and oxidized, shown in blue on the right in Fig. 5), there are eight possible states. These states are shown in Fig. 5 as the vertices of a cube, connected by the reactions that interconvert them. These reactions are detailed in Table 1, where reactions 1–4 describe quinone binding, reactions 5–8 describe internal electron movements, and reaction 9 describes the reaction of semiquinone with O₂ to form superoxide. The semiquinone can be formed by the single electron oxidation of the quinol, in Q-cycle fashion (reactions 5 and 7 in Table 1), or it can be formed by reverse electron flow from reduced cytochrome b₅₆₆ to an oxidized Q (reaction 6 in reverse). Normal Q-cycle reac-

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The Dependence of Superoxide Production on Cytochrome b₅₆₆ Reduction Is Altered by Protonmotive Force—In the presence of antimycin, oxidation of succinate (or of glutamate plus malate) is inhibited, and protons are not pumped across the mitochondrial inner membrane. Under these conditions, hydrolysis of added ATP by the F₁Fₒ-ATPase can be used to generate a protonmotive force (measured to be 130–150 mV under our conditions). Fig. 3 shows that addition of 2 mM ATP caused a dramatic change in the relationship between superoxide production and cytochrome b₅₆₆ reduction; the maximum rate of superoxide production was strongly decreased, and the peak at intermediate reduction of b₅₆₆ was no longer apparent.

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**FIGURE 2. Relationship between superoxide production rate from the Qₒ site of complex III and the reduction state of cytochrome b₅₆₆.** Hydrogen peroxide production rates with each substrate from Fig. 1, a–c, are plotted against the appropriate percent reduction of cytochrome b₅₆₆ from Fig. 1, d–f. Data are means ± S.E. (n = 4–5).

**FIGURE 3. Relationship between superoxide production rate from the Qₒ site of complex III and the reduction state of cytochrome b₅₆₆.** Filled symbols, superoxide production rate and cytochrome b₅₆₆ reduction were measured as in Fig. 1, a and d, respectively, except that 2 mM ATP was present, and the total concentration of succinate plus malonate was kept constant at 5 mM while altering their ratio. Data are means ± S.E. (n = 4). Open symbols, data for malonate titration of succinate as substrate, taken from Fig. 2, for comparison.
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FIGURE 4. Relationships between the steady-state reduction of cytochromes b_{562} and b_{566} in the absence and presence of ATP. Cytochrome b_{566} was measured at 566–575 nm with correction for cytochrome b_{562} by subtracting 50% of the signal at 561–569 nm. Cytochrome b_{562} was measured at 561–569 nm. a, ATP absent, conditions as Fig. 1 (or it leaves the Q_{o} site then reacts with oxygen to form superoxide, not shown). The second electron is donated to cytochrome b_{566} in reaction 6 in Table 1, forming Q (top left). In the absence of antimycin, cytochrome b_{566} is reoxidized by cytochrome b_{562}; Q is released by reaction 2, and the Q-cycle proceeds. In the presence of antimycin, cytochrome b_{566} cannot be reoxidized through the low potential chain once cytochrome b_{562} is reduced, so Q is released by reaction 4 in Table 1. The next QH_{2} to enter binds to the reduced complex (reaction 3 in Table 1) and generates a semiquinone by reaction 7. This semiquinone can be reduced to QH_{2} by cytochrome b_{566} in reaction 8 (Table 1), or it can be reoxidized by forming superoxide in reaction 9. However, if Q enters instead of QH_{2} (reaction 4 in Table 1), it can reoxidize cytochrome b_{566} in reaction 6, forming semiquinone.

Trial 1—In trial 1, we attempted to fit the observed data in Fig. 2 (absence of ATP) using plausible ranges of rate constants from the literature (Table 1). We used the constants from R. sphaeroides (32), because they are well determined, but relaxed the allowed ranges to include those found using bovine heart submitochondrial particles (35), because these may be more relevant for the mammalian (rat) mitochondria used here. The values of k_{5} and k_{7} were fixed at 1650 s⁻¹, which is the approximate rate constant for this step (32), and k_{10} was allowed to vary widely because its value is unknown. To model different conditions of substrate supply, we fixed QH_{2}/Q at a range of different values and calculated the superoxide production rate and the reduction state of cytochrome b_{566} from the steady-state solution of the model. In this trial, as we varied QH_{2}/Q we found no acceptable fits to the observed bell-shaped curve depicting H_{2}O_{2} as a function of b_{566} reduction. Specifically, fits lacked the peak at 70–80% reduction of b_{566} and the concave inflection at lower b_{566} reduction and had poor least squares values (Table 2). The best of these poor fits (lowest value of the error function given by Equation 4) is shown in Fig. 6a. Thus, a
straightforward model of the Q-cycle, with strict adherence to published Q-cycle rate constants, failed to fit the observed superoxide production data.

**Trial 2**—In trial 2, we relaxed the constraints on the rate constants for semiquinone formation by reactions 5 and 7 in Table 1, because in the presence of antimycin A, the rates of Q cycle reactions have been observed to slow considerably (36–38). In particular, the rate of quinol oxidation (reactions 5 and 7 in Table) was suggested to slow as much as 500-fold in the presence of antimycin A (13). We allowed $k_5$ and $k_7$ to vary independently between 0.001 and 1650 s$^{-1}$. With this change, the best fits in trial 2 produced a curve, but, as shown in Table 2 and Fig. 6b, failed to recapitulate the inflection point and peak found in the experimental data in Fig. 2 and did not produce an acceptable fit. Thus, a straightforward model of the Q-cycle, with the constraints on the rate constants of quinol oxidation relaxed to accommodate particular experimental observations from the literature, still failed to fit the observed data. This was the case even though the mechanism proposed by Droese and Brandt (16), reduction of Q in the Qo site by cytochrome $b_{566}$, was a fully functioning part of the model.

**Trial 3**—The inflection point in the observed superoxide production rate suggests a pseudo second order process. Therefore, in trial 3, we took into account the possibility that a variable related to the reduction state of cytochrome $b_{562}$ also affected certain rate constants and therefore the rate of superoxide production in this system. As noted above, the redox state of cytochrome $b_{562}$ was a candidate for this related variable. The presence of antimycin A in the Qi site does not slow entry of the Q-cycle Superoxide Production data.

**Table 1**

| Reactions | Rate constant range trial 1 | Rate constant range trial 2 | Rate constant range trials 3 and 4 |
|-----------|-----------------------------|-----------------------------|----------------------------------|
| 1 $QH_2 + b_{ox}$ $\rightarrow$ $K_1$ $QH_2b_{ox}$ | $k_1=(3-600) \times 10^6$ $k_1=(4-10) \times 10^3$ | $k_1=(3-600) \times 10^6$ $k_1=(4-10) \times 10^3$ | $k_1=(3-600) \times 10^6$ $k_1=(4-10) \times 10^3$ |
| 2 $Q + b_{ox}$ $\rightarrow$ $K_2$ $Qb_{ox}$ | $k_2=(3-6) \times 10^6$ $k_2=(2-4) \times 10^5$ | $k_2=(3-6) \times 10^6$ $k_2=(2-4) \times 10^5$ | $k_2=(3-6) \times 10^6$ $k_2=(2-4) \times 10^5$ |
| 3 $QH_2 + b_{red}$ $\rightarrow$ $K_3$ $QH_2b_{red}$ | $k_3=(3-6) \times 10^6$ $k_3=(4-8) \times 10^3$ | $k_3=(3-6) \times 10^6$ $k_3=(4-8) \times 10^3$ | $k_3=(3-6) \times 10^6$ $k_3=(4-8) \times 10^3$ |
| 4 $Q + b_{red}$ $\rightarrow$ $K_4$ $Qb_{red}$ | $k_4=(3-6) \times 10^6$ $k_4=(2-4) \times 10^5$ | $k_4=(3-6) \times 10^6$ $k_4=(2-4) \times 10^5$ | $k_4=(3-6) \times 10^6$ $k_4=(2-4) \times 10^5$ |
| 5 $QH_2b_{ox} + Fe-S_{ox}$ $\rightarrow$ $K_5$ $SQb_{ox} + Fe-S_{red}$ | $k_5=1650$ $k_5=n/a$ | $k_5=0.001-1650$ $k_5=n/a$ | $k_5=0.001-1650$ $k_5=n/a$ |

* See under “Experimental Procedures.”

**Value not known therefore arbitrarily constrained as indicated.**
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TABLE 2
Criteria used to determine the best fits in each trial
The experimental data in Fig. 2 showed both an inflection point (below 50% reduction of cytochrome b566) and a peak (between 70 and 80% reduction of b566). Therefore, the best model fits were selected based on four criteria as follows: 1) presence of an inflection point; 2) presence of a peak; 3) best least squares fit of the line to the raw data in Fig. 2 with no extra weighting, calculated using only the first term in Equation 4; 4) best least squares fit of the line to the raw data in Fig. 2 with extra weighting as described under “Experimental Procedures,” calculated using Equation 4. The presence or absence of inflections and peaks in the superoxide production rate curves was determined by numerical differentiation and root search to ensure objectivity.

| Trial     | Inflection | Peak | Unweighted least squares error (truncated Equation 4) | Weighted least squares error (full Equation 4) |
|-----------|------------|------|-----------------------------------------------------|-----------------------------------------------|
| 1         | No         | No   | 8.9                                                 | 9.2                                           |
| 2         | No         | No   | 8.9                                                 | 9.2                                           |
| 3         | Yes        | Yes  | 0.95                                                | 1.7                                           |
| 3 + ATP   | Yes        | Yes  | 2.9                                                 | 3.4                                           |
| 4         | Yes        | Yes  | 0.93                                                | 3.4                                           |

![FIGURE 6. Model-generated fits to the data in Fig. 2.](image-url)

The experimental data (open symbols) were converted to moles of superoxide per s per mol of bc1 complex. The antimycin-inhibited Q-cycle model (Fig. 5) was solved in steady state by numerical optimization of the rate constants within the ranges specified in Table 1, in four separate trials with different constraints, to give the best fit to the data and the known thermodynamic relationships between Q and the b hemes. The black squares show the best fit for each trial; superoxide production rates were reevaluated with finer steps of QH2/Q ratios using the best fit rate constants. The criteria for goodness of fit are shown in Table 2. a, Trial 1. Rate constants were held to strict Q-cycle ranges. b, Trial 2. As trial 1, but rate constants for semiquinone formation (k5 and k7) were allowed to vary within a wide range. c, Trial 3. As trial 2 but rate constants for reaction 6 were allowed to depend on the reduction state of cytochrome b562 (see Table 1). d, rate constants from trial 3 used to fit the data in Fig. 3 with ATP present. In this case, the model used the b562 reduction profile shown in Fig. 4b instead of that in Fig. 4a to calculate reaction 6 rate constants. e, Trial 4. As trial 3, but the model simultaneously fit the data sets from Figs. 2 and 3 using the relevant b reduction profiles from Fig. 4, a and b, to generate a single set of rate constants that defined both data sets.

first pair of electrons from QH2 into the Qo site but only subsequent pairs once cytochromes c1 and b562 are reduced (13). This important observation suggests that the reduction state of cytochrome b562 (and not simply the presence of antimycin) may influence rate constants in the Qo site. Therefore, trial 3 not only allowed the rate constants k5 and k7 to vary independently of each other as in trial 2, but also allowed the rate constants of reaction 6 in Table 1 to vary as a function of the reduction state of cytochrome b562. To do this, one set of reaction 6 rate constants was used when b562 was reduced, and a different set when b562 was oxidized (Table 1). Reaction 6 in Table 1 was chosen partly because it is the most obvious way to allow the redox state of cytochrome b562 to affect semiquinone concentration and superoxide production, and partly because there is some experimental evidence suggesting that it is crucial in superoxide formation (17). Notably, similar trials implementing an effect of b562 redox state on k5 and k7 did not produce acceptable fits (data not shown).
Every run that converged in trial 3 (25 of 34 attempts) produced acceptable fits to the data in Fig. 2 (Table 2), with only small ranges for most of the rate constants (Table 3). The fit from Table 3 with the smallest error is shown in Fig. 6e. All fits shared two significant characteristics as follows: slowed rate constants for quinol oxidation, particularly when $b_{566}$ was reduced, and stabilization of the $Q_s$ site semiquinone when $b_{562}$ was reduced.

In every run, the rate constant for quinol oxidation to form the semiquinone when $b_{566}$ was oxidized ($k_5$) was between 1.4 and 3.2 s$^{-1}$, 500–1200 times slower than the rate constant of 1650 s$^{-1}$ during Q-cycle operation. When $b_{566}$ was reduced, values of $k_5$ were between 0.36 and 0.52 s$^{-1}$, 4.2 times slower (range was 3–6) than when $b_{566}$ was oxidized. Thus, successful fits required the rate constant for quinol oxidation to be about 1000 times slower in our experiments (with antimycin present and cytochrome $b_{562}$ mostly reduced) than when the Q-cycle runs (with antimycin absent and $b_{562}$ mostly oxidized), and about 4000 times slower when cytochrome $b_{566}$ was also reduced.

Because we allowed the rate constants for the transfer of an electron between $b_{566}$ and the semiquinone in the $Q_s$ site ($k_6$ and $k_9$) to vary independently, trial 3 was able to test whether the redox state of $b_{562}$ affected the equilibrium constant of this reaction ($K_{eq6} = (Q_b_{566,red})/(SQ_b_{566,ox}) = k_6/k_9$), i.e. the preference of the electron for $SQ_b_{566}$ compared with reduced $b_{566}$ in the $Q_s$ site. Without exception, all trial 3 fits changed the equilibrium constant of semiquinone formation when $b_{562}$ was reduced. Values of the equilibrium constant were always greater than 1 when $b_{562}$ was oxidized (median value = 245) and always less than 1 when $b_{562}$ was reduced (median value = 0.023), strongly suggesting that reduction of $b_{562}$ stabilizes the semiquinone bound to oxidized $b_{566}$ compared with quinone bound to reduced $b_{562}$.

Thus, the Q-cycle explains the observed data well, but only when the redox states of the cytochrome $b$ hemes in the enzyme are allowed to exert considerable control over the rate constants of semiquinone formation and oxidation in the $Q_s$ site.

The model also predicted other unknown rate constants, such as that for superoxide production. For simplicity, this rate constant ($k_9$) was assumed to be the same regardless of the reduction state of the enzyme but was otherwise allowed to vary widely, yet all fits gave values between 2.9 and 39.5 s$^{-1}$ (Table 3). Other rate constants, such as those for $QH_2$ and $Q$ binding (reactions 1–4 in Table 1), were within the delineated range, but are not particularly illuminating as they are well described in the literature.

A powerful test of the robustness of the model was to ask if the rate constants fitted to Fig. 2 in trial 3 could predict the quite different relationship between superoxide production and the redox state of $b_{566}$ observed in the presence of a membrane potential, shown in Fig. 3. In this case, instead of defining the rate constants in reaction 6 (Table 1) with the $b_{562}$ reduction state described in Fig. 4a, we used the relationship between $b_{562}$ and $b_{566}$ determined in the presence of ATP (Fig. 4b). All 25 sets of rate constants generated from the data in the absence of ATP predicted a relationship approximating the experimental data shown in Fig. 3 in the presence of ATP (Table 2; trial 3 plus ATP). Fig. 6d shows the fit using the rate constants of Fig. 6a overlaid on the data from Fig. 3. Bearing in mind that the parameters were generated using a completely different data set, the fit is remarkably good. However, each set of rate constants from Table 3 predicted a small peak between the last two experimental data points that was not resolved by our experimental data.

**Trial 4**—In trial 3 above, the rate constants were optimized to fit the data measured under de-energized conditions (Fig. 2) and subsequently applied to the data measured under energized conditions (Fig. 3), giving a reasonable prediction of the energized results (Fig. 6d). In contrast, in trial 4 we optimized a single set of rate constants to provide the best fit to both sets of data simultaneously. Trial 4 gave seven runs that converged.
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(out of 12 attempts). It gave similar results to trial 3, fitting both data sets well, and (as expected) giving an even better fit to the energized data (Table 2 and Fig. 6e), but there was greater variability between runs. The values of the rate constants for the best fit and their ranges for the different fits are given in Table 3, and give similar conclusions to trial 3.

Thus, the Q-cycle can explain the experimental data in both de-energized (Fig. 2) and energized conditions (Fig. 3), whether the model is fitted to one data set or to both, as long as the model uses one set of rate constants of semiquinone formation and oxidation in the Qo site when cytochrome b_{562} is reduced and another when b_{562} is oxidized.

DISCUSSION

We examined the kinetic mechanism of superoxide production by complex III. In mitochondria given succinate and antimycin, superoxide production increased when malonate was added and then decreased as malonate concentration was increased further (Fig. 1a). This effect was not specific to malonate or to succinate dehydrogenase, as it was recapitulated by low concentrations of succinate (Fig. 1b) or glutamate plus malate (Fig. 1c). Thus, limitation of substrate in three different ways maximized superoxide production from complex III, presumably by allowing partial oxidation of the Q pool, the first intermediate that is common to all three conditions. These observations suggest that the Q-pool redox state affects superoxide production from complex III (through its effects on reduction of cytochromes b_{566} and b_{562}).

The observation that malonate addition caused oxidation of cytochrome b_{566} (Fig. 1d), even though the thermodynamic driving force for Q reduction was unaltered, implies that this system was kinetically limited, with inflow of electrons maintaining a steady-state reduction of intermediates against a background rate of electron flow out of the Q site (to O_2 to form superoxide, and by any other reactions that bypass inhibition by antimycin A). The substrate-limiting conditions applied in these experiments should then allow a quasi-equilibrium to be achieved among the intermediates between electron entry and exit, i.e. between the redox states of bound Q and cytochrome b_{566}. Therefore, the redox status of cytochrome b_{566} acts as a reporter of the concentration of semiquinone in site Q_{o} (as long as cytochrome b_{562} redox state is constant). Because superoxide production rate should be a function of semiquinone concentration in the Q_{o} site, cytochrome b_{566} redox state is also a reporter of superoxide production rate from this site. With the same constraint, cytochrome b_{566} redox state also reports the redox status of the bulk Q pool. Indeed, in redox titrations, the b_{566}^{ox}/b_{566}^{red} couple has been shown to be a function of the redox status of the QH_{2}/Q couple (25).

In the absence of membrane potential, superoxide production was a function of the reduction state of the b_{566} heme in the superoxide-generating Q_{o} site (Fig. 2), with the highest rates at 70–80% reduction of b_{566} independently of the substrates used. However, the relationship was different in the presence of a membrane potential (Fig. 3). In the absence of a membrane potential, b_{562} was preferentially reduced (Fig. 4a), but in the presence of a membrane potential electrons were distributed more equally between the two b hemes (Fig. 4b). We propose that the altered relationship between superoxide production and b_{566} results from changes to the stability constant of the semiquinone, which is controlled directly by b_{562} redox state and indirectly by membrane potential. Indeed, membrane potential was shown to play a defining role in superoxide production by the reconstituted bc_{1} complex (39).

Decrease in the Rate Constant for Quinol Oxidation Caused by Antimycin and b_{562} Reduction—The fits in trials 3 and 4 indicate that the rate constants for quinol oxidation decrease considerably in the presence of antimycin. When b_{566} is oxidized, the rate constant (k_{566}^{ox}) is ~1000 times smaller than during normal Q-cycle operation. When b_{566} is reduced, k_{566} is ~4000 times smaller than normal, and ~4-fold slower than when b_{566} is oxidized (Table 3). There is considerable precedent for this behavior. Q_{o} site catalysis is strongly decreased by the presence of antimycin A in the Q_{o} site (40–42), and it has been proposed that this functions to decrease bypass reactions that short-circuit the Q-cycle (13). However, antimycin does not slow the entry of the first pair of electrons from FH_{2} into the complex; it only slows subsequent pairs once cytochromes c_{1} and b_{562} are reduced (13). Therefore, we propose that the changes in rate constants for quinol oxidation and semiquinone oxidation are not (entirely) due to the presence of antimycin in site Q_{o}, which would be physiologically irrelevant, but could also be caused indirectly by the reduction of the b cytochromes.

The electron transfer capacity of the Rieske Fe-S protein is mediated by macromovements of its soluble head domain, which swings on a "hinge" between the b position (near cytochrome b) and the c_{1} position (near cytochrome c_{1}) during each electron transfer event (43, 44). The macro movements and equilibrium position of the mobile head domain are modified by the presence of inhibitors in either the Q_{o} or Q_{i} site (43). Mutations to cytochrome b residues result in dramatic alterations in rates of electron transfer through the Fe-S protein (45). This suggests that the properties of cytochromes b_{562} and b_{566} are communicated to the Fe-S protein. This communication could be achieved through large scale protein conformation changes, or it is conceivable that the reduction of b_{566} affects its interaction with nearby amino acid residues, particularly the glutamate within the conserved PEWY region of the protein, and thereby alters the rate constants of the Q_{o} site-Fe-S protein interaction (13).

Stability of the Q_{o} Site Semiquinone—Trials 3 and 4 allowed semiquinone oxidation (reaction 6 in Table 1) to use two sets (k_{562}^{red}, k_{562}^{ox}, k_{566}^{red}, k_{566}^{ox}) of rate constants depending the redox state of cytochrome b_{562}. The model predicts that k_{566} alters relative to k_{-6} when b_{562} becomes reduced, and so alters the equilibrium constant (K_{eq} = (Q.b_{566}.red)/(SQ.b_{566}.ox) = k_{566}^{ox}/k_{566}^{red}) of reaction 6 in Table 1 and the stability of "semiquinone.b_{566}.ox" relative to "quinone.b_{566}.red". To maintain the thermodynamics of the QH_{2}/Q couple, this implies that k_{-6}/k_{566} also changed, but this was not apparent in the model because we assumed that reaction 5 in Table 1 was irreversible under our conditions. K_{eq} was large when b_{562} was oxidized (semiquinone destabilized) and small when b_{562} was reduced (semiquinone stabilized).

There are many models of Q_{o} site catalysis and little consensus on the stability of the semiquinone. Some models suggest it is stabilized (46, 47) and others that it is destabilized to limit its
concentration and reaction with oxygen (48, 49). Our model predicts that the fully reduced low potential chain will maximally stabilize the semiquinone. Therefore, under the more physiologically relevant conditions of an ATP-generated membrane potential (Fig. 3), where cytochrome \( b_{562} \) is less reduced at intermediate substrate levels, we observe the highest rate of superoxide production near to the maximal reduction of the complex, as semiquinone is less stabilized at intermediate substrate levels. Conversely, the data in Fig. 2 can also be described by semiquinone stabilization. The peak rate of superoxide production observed when \( b_{562} \) becomes fully reduced but \( b_{566} \) is only 60–80% reduced (Figs. 2 and 4) is explained by the increased semiquinone stability coupled to increased rate of semiquinone formation (reaction 5 in Table 1) under these conditions.

The Q\( \text{b} \) site semiquinone is notoriously difficult to observe by electron paramagnetic resonance even under conditions that should favor its presence; this has led to proposals that it is spin-coupled and EPR silent or that the site is primarily occupied by QH\( \text{b} \) with oxidized Rieske Fe-S (50). However, under anaerobic conditions utilizing freeze-quench, the semiquinone was detectable (48). Our model agrees with these findings as follows: when \( b_{566} \) is reduced, the rate constant for semiquinone formation (\( k_s \)) is 0.3–0.4 \( \text{s}^{-1} \) (Table 3), considerably slower than the rate constant for its reaction with O\( \text{b} \) to form superoxide (\( k_o \)), at 5–17 \( \text{s}^{-1} \), so as soon as semiquinone is formed, it readily reacts with O\( \text{b} \) and is removed, and the semiquinone concentration in the steady state is very low. At zero O\( \text{b} \), reaction 9 (Table 1) stops, and the semiquinone becomes detectable by EPR (48). We predict that with subsaturating substrate, detection of semiquinone by EPR may be possible even in the presence of O\( \text{b} \).

**Explanation of the Peak in Superoxide Production at Intermediate Substrate Availability**—The bell shapes of the curves in Figs. 1 (a–c) and 2 are explained by the effect of bulk QH\( \text{b}/Q \) on the rate of production and stability of semiquinone, mediated by \( b_{566} \) and \( b_{562} \) redox states. Reduction of Q by cytochrome \( b_{566} \) (reaction \(-6\) produces semiquinone, as proposed by Ref. 16, but this alone is not sufficient to give a bell-shaped response to substrate availability (trials 1 and 2; Fig. 6, a and b). However, this reaction also tends to generate the state in which \( b_{562} \) is reduced but \( b_{566} \) is oxidized, and this state sets rate constants that are necessary for the highest semiquinone concentrations and the highest peaks of superoxide production from complex III (trials 3 and 4; Fig. 6c). When ATP is present, \( b_{562} \) never reaches much higher reduction level than \( b_{566} \) and the peak is suppressed (Fig. 6d).

Fig. 7 summarizes these effects, showing the modeled percent of complex III occupied by a superoxide-generating semiquinone in different environments as a function of \( b_{566} \) reduction. In the absence of membrane potential (Fig. 7a), at intermediate \( b_{566} \) reduction states when \( b_{562} \) is reduced, semiquinone concentration peaks then falls, accounting for the observations in Figs. 1 and 2. It is clear that the model predicts that it is the concentration of semiquinone on complex III in which cytochrome \( b_{566} \) is oxidized but \( b_{562} \) is reduced that is responsible for the bell-shaped curve in Fig. 2. In the presence of membrane potential, \( b_{562} \) reduction tracks \( b_{566} \) reduction, and the peak is diminished and pushed to the right (Fig. 7b).

**Physiological and Biochemical Implications of the Model**—Slowing QH\( \text{b} \) oxidation by reduction of cytochrome \( b_{562} \) (and further slowing by reduction of \( b_{566} \)) could be a critically important physiological mechanism in two ways. First, to limit short-circuiting of the Q-cycle, in which the second electron follows the first down the high potential chain, and proton pumping and energy conservation by the Q-cycle is lost. As electrons accumulate on the b hemes, which would tend to promote the short circuit, the entry of electrons is slowed to oppose this trend. The second mechanism that could be of physiological importance is to limit superoxide production by complex III when substrate is abundant but energy demand is low. Under these “state 4” conditions, protonmotive force is high, electrons back up into complex III, and there is a risk that the semiquinone in...
site Q, will build up and cause very high superoxide production. Despite the potential for a stabilized semiquinone under these conditions, the reaction is limited by semiquinone formation rates. Although the small $K_{066}$ makes the reduction of Q to generate semiquinone a possibility, this is not observed unless there is enough Q present in the Q$_s$ site to facilitate this reaction. Note that state 4 conditions would ensure a relatively reduced Q-pool and limit the possibility of electron backflow from b$_{566}$ to Q to generate semiquinone. In this light, the slowing of the rate constants for semiquinone formation in site Q$_o$ would ensure that the site is largely occupied by QH$_2$ and would act as a safety mechanism to prevent excessive superoxide production by complex III.

Our conclusion that reduction of cytochrome b$_{566}$ lowers the rate constant for semiquinone production and that reduction of cytochrome b$_{566}$ lowers it further could explain two confusing observations in the literature. First, it explains why the rate of superoxide production at site Q$_o$ when cytochrome b is reduced is essentially independent of [O$_2$] above about 10 $\mu$M concentration (51) and not second-order and proportional to [O$_2$] as might be expected; most of the rate limitation is in semiquinone formation and not superoxide formation (Table 3). Only when [O$_2$] is very low does control shift away from quinol oxidation and allow sensitivity to [O$_2$] to appear. Second, it may explain why superoxide production from complex III in the presence of antimycin increases under hyperoxia (10, 52) even though the reaction is already saturated with O$_2$ under normoxia (51). This could occur if oxygen at very high concentrations can mimic Q of the Q$_c$ site and oxidize cytochrome b$_{566}$, increasing the rate constant for quinol oxidation, causing increased superoxide production by the mechanism discussed above.

The results of our study imply that cytochrome b$_{566}$ and b$_{562}$ redox states can reliably predict the rate of superoxide generation by the Q$_c$ site of complex III in the presence of antimycin A. They strongly suggest that reduction of cytochromes b$_{562}$ and b$_{566}$ slows electron input into complex III from quinol oxidation at site Q$_o$ and modulates the stability of semiquinone. This could be a physiologically relevant mechanism that prevents short circuiting of the Q-cycle, strongly limits superoxide production from complex III, and makes this superoxide production independent of [O$_2$] except when [O$_2$] is very low or very high.

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