Direct and Indirect Roles of Cytochrome b in the Mediation of Superoxide Generation and NO Catabolism by Mitochondrial Succinate-Cytochrome c Reductase

Mitochondrial superoxide (O$_2^-$) production is an important mediator of oxidative cellular injury. Succinate-cytochrome c reductase (SCR) of the electron transport chain has been implicated as an essential part of the mediation of O$_2^-$ generation and an alternative target of production because of its stability, when an inhibitory effect on O$_2^-$ generation by SCR was measured with the EPR spin-trapping technique using DEPMPO (5-diethoxylphosphoryl-5-methyl-1-pyrroline N-oxide) as the spin trap. In the presence of succinate, O$_2^-$ generation from SCR was detected as the spin adduct DEPMPO/OOH. Inhibitors of the Q$_i$ site only marginally reduced (20–30%) this O$_2^-$ production, suggesting a secondary role of O$_2^-$ in the mediation of O$_2^-$ generation. Addition of cyanide significantly decreased (~70%) O$_2^-$ production, indicating the involvement of the heme component. UV-visible spectral analysis revealed that oxidation of ferrocytochrome c was accompanied by cytochrome c$_1$ reduction, and the reaction was mediated by the formation of an O$_2^-$ intermediate, indicating a direct role for cytochrome b in O$_2^-$ generation. In the presence of NO, DEPMPO/OOH production was progressively diminished, implying that NO interacted with SCR or trapped the O$_2^-$. The consumption of NO by SCR was investigated by electrochemical detection using an NO electrode. In the presence of succinate, SCR-mediated NO consumption was observed and inhibited by the addition of superoxide dismutase, suggesting the involvement of O$_2^-$. Under the conditions of argon saturation, the NO consumption rate was not enhanced by succinate, suggesting a direct role for O$_2^-$ in the mediation of NO consumption. In the presence of succinate, oxidation of the ferrocytochrome b moiety of SCR was accelerated by the addition of NO, and was inhibited by argon saturation, indicating an indirect role for cytochrome b in the mediation of NO consumption.

Mitochondria are the major cellular source of oxygen-free radical production (1, 2). They are also an important target for the endothelium-derived relaxant factor, nitric oxide (NO) (3, 4). The generation of oxygen-free radical(s) and the effect of NO on mitochondria are particularly relevant under physiological conditions of low oxygen tension such as state IV respiration or certain pathological conditions such as inflammation and ischemia-reperfusion injury (5–7).

Under these conditions, a decrease in the rate of mitochondrial phosphorylation can increase the production of oxygen-free radicals, in the form of superoxide (O$_2^-$), from the early stages of the electron transport chain. Two segments of the electron transport chain have been implicated in O$_2^-$ generation. One, on the NADH dehydrogenase of Complex I, operates via electron leakage from the reduced flavin mononucleotide (8–11). The other, on Complex III, mediates O$_2^-$ generation through the Q cycle mechanism (Fig. 1), in which electron leakage presumably results from the autoxidation of ubisemiquinone (12) and reduced cytochrome b$_1$ (13).

In the Q cycle mechanism, there are two ubisemiquinones formed in different parts of the cycle. An unstable ubisemiquinone (Q$_{u1}$) formed near the cytoplasmic site (Fig. 1) is considered to be the major source of O$_2^-$ therefore, inhibitors such as stigmatellin and myxothiazol that block the formation of Q$_{u1}$ would be expected to decrease overall O$_2^-$ generation. The other ubisemiquinone (Q$_{u2}$), formed near the matrix site, is stable and EPR-detectable (Fig. 1). Although generally Q$_{u2}$ is not thought to be a source of O$_2^-$ production because of its stability, when an inhibitor such as antimycin A is used to block the Q$_i$ site a reverse electron flow will be induced, thus enhancing the formation of Q$_{u2}$ and increasing O$_2^-$ generation (Fig. 1). Furthermore, electron leakage for O$_2^-$ production mediated by low potential cytochrome b$_1$ (or b$_{sem}$) was postulated as shown in the Q cycle pathway (Fig. 1) (13, 14), whereas the importance of this autoxidation mediated by the ferrocytochrome b moiety in overall O$_2^-$ generation remains unclear.

NO produced from endothelial cells has the capacity to modulate mitochondrial functions in regulation of metabolism, respiration, and mitochondrial biogenesis (3, 4, 15, 16). It is well known that within the electron transport chain NO serves as a physiological regulator of cellular respiration in vivo, primarily via reversible binding to the binuclear center of cytochrome c oxidase. Complex II and Complex III are also important targets of NO in respiratory regulation (17, 18). However, an independent pathway for NO utilization in mitochondria has been implicated as well: the Q cycle mechanism (19, 20) (Fig. 1). Specifically, (a) QH$_2$ has been suggested as the primary target of NO, as NO can oxidize QH$_2$ in vitro, resulting in ubisemiquinone and nitroxyl anion (NO$^-$); and (b) ubisemiquinone at the Q$_i$ site provides a source of electron leakage, forming O$_2^-$, which is in turn trapped by NO to generate NOONO$^-$ (Fig. 1).

The mechanism of NO consumption, as mediated by the Q cycle of Complex III, is not well understood, especially with regard to interaction with O$_2^-$. An ideal model for investigation of this mechanism is the succinate-cytochrome c reductase (SCR) supercomplex purified from the bovine
SCR-mediated Superoxide Generation and NO Catabolism

FIGURE 1. Superoxide generation and NO catabolism mediated by the Q cycle mechanism in the QCR. This scheme is adapted from Ref. 23. Gray areas symbolize the reactions involved in O$_2^-$ production and peroxynitrite formation. P, O, and C represent positive, outside or oxidation, and cytoplasmic side, respectively, N, I, and M stands for negative, inside or reduction, and matrix side, respectively. Q$_H$, ubiquinol; Q$_2$, ubiquinone; Q, ubiquinone; O$_2^-$, superoxide anion; RISP, Rieske iron-sulfur protein.

heart, comprising succinate-ubiquinone reductase (SQR or Complex II) and the cytochrome $bc_1$ complex (QCR or Complex III) (21, 22).

Physiologically, the SCR supercomplex mediates electron transfer from succinate to cytochrome $c$ during mitochondrial respiration. The redox centers of SQR contain flavin adenine nucleotide (FAD), three iron-sulfur clusters (S-1, S-2, and S-3), cytochrome $b_{560}$ and ubiquinone (Q). The redox centers of QCR consist of ubiquinol (QH$_2$), cytochromes $b_1$, $b_2$ (high potential $b$ or $b_{562}$), and $c_1$, and the Rieske iron-sulfur cluster (RISP). Succinate serves as an electron donor for SQR to reduce FAD, after which an electron is transferred from the reduced FAD to the iron-sulfur clusters and cytochrome $b_{560}$, and Q is reduced to QH$_2$. The electron transfer from QH$_2$ to cytochrome $c$ is catalyzed by QCR and follows the Q cycle mechanism (Fig. 1). The use of succinate as an electron donor for SCR has the advantage of avoiding artificial O$_2^-$ generation derived from ubiquinol autoxidation (23).

In this work we have used purified SCR as a model system to address the fundamental question of how the Q cycle mediates O$_2^-$ generation and NO consumption. We have: 1) used EPR to obtain direct evidence of SCR-mediated O$_2^-$ generation and NO consumption; 2) used electrochemical detection with an NO electrode to obtain direct evidence of SCR-mediated NO consumption; and 3) demonstrated that NO scavenges O$_2^-$ generated from the ferrocytochrome $b$ moiety of SCR by forming OONO$^-$.

MATERIALS AND METHODS

Reagents—Ammonium sulfate, antimycin A, diethylenetriaminepentaacetic acid, horse heart cytochrome $c$ (highest grade available in commercial, and prepared without using trichloroacetic acid), Zn,Cu-superoxide dismutase (SOD), succinic acid, and sodium cholate were purchased from Sigma and used as received. Ascorbic acid, potassium cyanide, and sodium dithionite were purchased from Aldrich. Myxothiazol, stigmatellin, and thenoyl trifluoroacetone (TTFA) were from Fluka BioChemika (St. Louis, MO). The 5-diethoxylphosphoryl-5-othiazol, stigmatellin, and thenoyl trifluoroacetone (TTFA) were from ALEXIS Biochemicals (San Diego, CA).

Preparations of Mitochondrial Succinate-Cytochrome $c$ Reductase (SCR)—Bovine heart mitochondrial SCR was prepared and assayed according to the published method (21). The purified SCR contained 4–4.2 nmol of heme $b$ per mg of protein and exhibited an activity of $\sim$1.5 $\mu$mol of cytochrome $c$ reduced/min/mg of protein. Purified SCR was stored in 50 mM sodium/potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA.

Analytical Methods—Optical spectra were measured on a Shimadzu 2401 UV-visible recording spectrophotometer. The enzyme concentration (based on the heme $b$) of SCR was calculated from the differential spectrum between dithionite reduction and ferricyanide oxidation, using an extinction coefficient of 28.5 M$^{-1}$ cm$^{-1}$ for the absorbance difference of A$_{562}$–A$_{576}$ nm. The enzyme activity of SCR was assayed by measuring cytochrome $c$ reduction. An appropriate amount of SCR was added to an assay mixture (1 ml) containing 50 mM phosphate buffer, pH 7.4, 0.3 mM EDTA, 19.8 mM succinate, and 50 $\mu$M ferricytochrome $c$. The SCR activity was determined by measuring the increase in absorbance at 550 nm.

Electron Paramagnetic Resonance Experiments—EPR measurements were performed using the EPR Core Facilities at the Davis Heart and Lung Research Institute. Experiments were carried out on a Bruker EMX spectrometer operating at 9.86 GHz with 100 kHz modulation frequency at room temperature. The reaction mixture was transferred to a 50-$\mu$l capillary, which was then positioned in the HS cavity (Bruker Instrument, Billerica, MA). The sample was scanned using the following parameters: center field, 3510 G; sweep width, 140 G; power, 20 milliwatt; receiver gain, 10$^2$; modulation amplitude, 1 G; time of conversion, 81.92 ms; time constant, 327.68 ms. The spectral simulations were performed using the WinSim program developed by Duling (24), National Institutes of Health, NIEHS.

Nitric Oxide Consumption Rate by SCR—NO consumption rate was measured electrochemically at 37 $^\circ$C in an electrochemical vial using a Free Radical Analyzer (World Precision Institute, Sarasota, FL) and Clark NO electrode (World Precision Institute, Sarasota, FL). For measuring the NO consumption rate under anaerobic conditions, a slow flow of argon gas was maintained in the space above the solution. The electrochemical detector continuously recorded the current collected by the electrode, which is proportional to the NO concentration in the solution. The sensor was calibrated with known concentrations of NO, using NO-equilibrated solutions as described in the literature (25, 26). The quantitation of the SCR-mediated NO consumption rate with succinate or without succinate was based on: (a) the initial rate of the kinetic curve from NO decay in PBS, or (b) the decrease of peak current from the kinetic curve of NO decay in PBS (27).

RESULTS

DEPMPO Spin Trapping of O$_2^-$ Generated from SCR in the Presence of Succinate—To obtain direct evidence for O$_2^-$ production mediated by SCR, we employed the EPR spin-trapping technique to measure O$_2^-$ generation.
SCR-mediated Superoxide Generation and NO Catabolism

unambiguously and directly. Of the available spin traps, DEPMPO is ideal for quantitating O$_2^-$ production by SCR based on the following advantages: (a) DEPMPO is 40-fold more sensitive than the cytochrome c assay for the detection of O$_2^-$ (28), (b) DEPMPO traps O$_2^-$ with an efficiency of 60–70% (28), and (c) the O$_2^-$ adduct of DEPMPO/’OOH is more stable than that of DMPO/’OOH (29).

When purified SCR (2 μM, based on the heme b concentration) was incubated with DEPMPO (20 mM) in PBS and the reaction was initiated by the addition of succinate (18 μM), a multi-line EPR spectrum was produced that was characteristic of DEPMPO/’OOH (Fig. 2A, solid line) based on the hyperfine coupling constants (isomer 1: a$^N$ = 13.14 G, a$^H$ = 11.04 G, a$^{3}$ = 0.96 G, a$^{3}$ = 49.96 G (80% relative concentration); isomer 2: a$^N$ = 13.18 G, a$^H$ = 12.59 G, a$^{3}$ = 3.46 G, a$^{3}$ = 48.2 G (20% relative concentration) (11, 29)) obtained from the computer simulation (Fig. 2A, dashed line). Trapping of SCR-mediated O$_2^-$ by DEPMPO was thus highly specific and suitable for quantitative analysis.

That the DEPMPO/’OOH adduct arose from the trapping of O$_2^-$ was confirmed by the addition of Zn,Cu-containing superoxide dismutase (SOD, 100 units/ml) to the reaction system (Fig. 2B); upon its addition the adduct formation was completely prevented. In the absence of SCR, no DEPMPO/’OOH was detected (Fig. 2C), indicating the enzymatic dependence of the DEPMPO adduct formation. When native SCR was replaced with heat-denatured (70 °C for 5 min) SCR, the formation of DEPMPO/’OOH was inhibited (Fig. 2D), indicating that the electron transfer activity in the enzyme is required for O$_2^-$ generation. The detection of the superoxide adduct of DEPMPO was strongly inhibited when succinate was omitted from the system (Fig. 2E), supporting a direct role for succinate as the electron donor for SCR-mediated O$_2^-$ production.

In the presence of an electron acceptor, ferricytochrome c (cytochrome c, 6 μM), the production of O$_2^-$ by succinate-energized SCR was nearly completely inhibited (Fig. 2F), suggesting two possibilities: (a) electron leakage from SCR was efficiently prevented by a stoichiometric amount of cytochrome c under the conditions of enzyme turnover, i.e. in the presence of both succinate and cytochrome c; and (b) any O$_2^-$ produced by SCR was presumably quenched by cytochrome c reduction.

The enzymatic activity of SQR in SCR is sensitive to TTFA, which inhibits electron transfer from SQR to QCR through binding to the S3 center and the cytochrome b$_{560}$ binding protein in SQR (30). Pretreatment of SCR with TTFA (10 mM) inhibited ~87% of the electron transfer activity from succinate to cytochrome c but only 65% of the O$_2^-$ generation (Fig. 2G), whereas complete inhibition of electron transfer activity and O$_2^-$ generation can be achieved by higher dosage of TTFA (50 mM); implying that QCR controlled most of the SCR-mediated O$_2^-$ generation.

**Electron Leakage to Oxygen by SCR under the Conditions of Enzyme Turnover**—To measure the electron leakage from SCR to oxygen under the conditions of enzyme turnover, Zn,Cu-SOD (0.5 units/μl) was added to the reaction mixture containing 36 pmol of SCR and 30 μM cytochrome c in PBS. The reduction of cytochrome c was subsequently induced by the addition of various amounts of succinate (30 μM to 1.2 mM). Electron leakage to oxygen under these turnover conditions was calculated as the amount of SOD-inhibitable cytochrome c reduction. The observed electron leakage was dependent upon the ratio of succinate/cytochrome c. There was no detectable O$_2^-$ generation under the ratio of 15 or below. Above a ratio of 15, the observed electron leakage increased as the succinate/cytochrome c increased, reaching a plateau at a maximum of 14% of the total cytochrome c reduction when the ratio was greater than 30 (data not shown).

**Involvement of the Q Cycle Mechanism in SCR-mediated O$_2^-$ Generation**—Antимycin A is a well known inhibitor of QCR; it blocks the transfer of the second electron of QH$_2$ from cytochrome b$_{560}$ to Q, and inhibits the formation of Q$_{10}^-$ (see Fig. 1). In the presence of antymycin A, electrons would accumulate in the high-potential heme b (b$_{560}$) of QCR, and electron leakage to molecular oxygen should be accelerated due to enhanced formation of Q$_{10}^-$, a potential source of O$_2^-$.

Two other inhibitors of QCR, myxothiazol and stigmatellin, block electron transfer at the Q$_b$ site. Myxothiazol is bound in the Q$_b$ pocket slightly toward low-potential heme b$_{560}$, which causes the release the RISP into a mobile conformation (31, 32). It inhibits the first electron transfer from QH$_2$ to RISP (Fig. 1). Whereas stigmatellin is bound at a different subsite in the Q$_b$ pocket, which immobilizes the RISP in the b conformation (31, 32). It interrupts the transfer of the first electron from the Q$_b$ site to cytochrome c$_1$ (Fig. 1). Both inhibitors and QH$_2$ are mutually competitive for binding, so either would prevent QH$_2$ occupying the site, and therefore would prevent the formation of Q$_{10}^-$ (33, 34), presumably inhibiting O$_2^-$ generation.

The above three inhibitors inhibited more than 99.9% of the electron transfer activity of SCR at concentrations the same as the enzyme. In spin trapping with DEPMPO in the absence of cytochrome c, their effects on O$_2^-$ generation by SCR varied. When SCR (2 μM) was

---

**FIGURE 2. EPR spin-trapping of O$_2^-$ generated from SCR in the presence of DEPMPO.** A, the computer simulation (dashed line) superimposed on the experimental spectrum (solid line) obtained using SCR (2 μM), DEPMPO (20 mM), diethylenetriaminepentaacetic acid (1 mM), and succinate (suc) (18 μM) in PBS. The experimental spectrum was recorded after signal averaging 3 scans at room temperature. B, the same as A, except that SOD (0.1 units/μl) was added to the mixture before the reaction was initiated by succinate. C, the same as A, except that the enzyme was omitted from the system. D, the same as A, except that the enzyme was heated at 70 °C for 5 min prior to EPR measurement. E, the same as A, the substrate succinate was omitted from the system. F, the same as A, but the system contained cytochrome c (6 μM). G, the same as A, except that SCR was pretreated with TTFA (10 mM) prior to succinate initiation.
pretreated with antimycin A (10 μM), O$_2^-$ generation by SCR was enhanced by up to 20%, whereas both stigmatellin and myxothiazol inhibited succinate-initiated O$_2^-$ production by up to 20–30% (Fig. 3A). These results are consistent with the idea that the ubisemiquinone at the Q$_b$ site (Q$_b^*$) contributes an appreciable fraction of the total O$_2^-$ generation.

In the presence of cytochrome c at one-third the level of the succinate concentration (6 μM), DEPMPO/OOH was not detected (Fig. 3B). However, O$_2^-$ generation was significantly stimulated when SCR was preincubated with any of the above three inhibitors (Fig. 3B). This stimulation could be due to blocking of the electron transfer pathway by the inhibitors, leading to electron leakage to molecular oxygen. Antimycin A was observed to induce marginally higher stimulation of O$_2^-$, which was decreased to 4 μM. Only the spectra obtained from the 2nd, 4th, 6th, and 8th scans were shown. C, same as A, except that DEPMPO (100 μM) and SOD (0.1 units/μl) were included in the reaction mixture prior to the addition of succinate. Only the spectra obtained from the 8th scan were compared. D, the experiment was carried out under argon saturation.

The observed spectral interchange between heme b and heme c$_1$, which was partially inhibited by DEPMPO (Fig. 4C, dashed line) and completely inhibited by SOD (Fig. 4C, dotted line). Under conditions of argon saturation, spectral interchange between heme b and heme c$_1$ was not observed (Fig. 4D). These data imply that oxygen mediates the intra-molecular electron transfer of succinate-re-
duced SCR via the formation of an $O_2^\cdot$ intermediate from the autooxidation of ferrocytochrome $b$ (Equations 1 and 2).

\[
\text{Ferrocytochrome } b (\text{Fe}^{2+}) + O_2 \rightarrow \text{ferricytochrome } b (\text{Fe}^{3+}) + O_2^\cdot \quad (\text{Eq. 1})
\]

\[
\text{Ferricytochrome } c_1 (\text{Fe}^{3+}) + O_2^\cdot \rightarrow \text{ferrocytochrome } c_1 (\text{Fe}^{2+}) + O_2 \quad (\text{Eq. 2})
\]

It is known that $O_2^\cdot$ can be quenched by ferricytochrome $c$ via one-electron reduction (rate constant, $k \approx 10^6 \text{ M}^{-1} \text{s}^{-1}$) (35). The difference in $E_{m,\gamma}$ between cytochrome $c$ and cytochrome $c_1$ is small ($<10 \text{ mV}$), and both share an identical heme configuration (c-type heme). Therefore, cytochrome $c_1$ should be as efficient as cytochrome $c$ at oxidizing $O_2^\cdot$. It is worth noting that similar intramolecular electron transfer mediated by $O_2^\cdot$ as an intermediate was also observed in the case of native SCR, antimycin A-treated SCR, and myxothiazol-treated SCR (data not shown). Therefore, these data provide strong evidence that reduced cytochrome $b$ is a source of $O_2^\cdot$. This concept was further supported by measurement of the NO consumption rate by stigmatellin-inhibited SCR (see below).

**Interaction of NO with SCR Prevents $O_2^\cdot$ Generation**—Poderoso et al. (19, 36) have proposed that the Q cycle mechanism represents an independent pathway in mitochondria that competes with cytochrome $c$ oxidase in NO utilization. Therefore, the Q cycle has been implicated as an important target for NO-mediated respiratory regulation. This cytochrome $c$ oxidase-independent pathway is proposed to regulate $O_2^\cdot$ generation by forming peroxynitrite (OONO$^-$) (19). It is important to pinpoint two fundamental questions here: (a) how does NO affect SCR-mediated $O_2^\cdot$ generation, and (b) how does the Q cycle pathway mediate NO consumption by SCR?

To address the first question, a saturated NO solution (2 mM) was used to titrate the $O_2^\cdot$ generation system containing SCR (2 mM), DEPMPO (20 mM), and succinate (18 mM). The EPR spectra of the reaction mixture, obtained from a signal average of 5 scans, showed that the addition of stoichiometric amounts of NO progressively diminished the DEPMPO*/OOH adduct (Fig. 5). The result suggested that NO can directly or indirectly decrease SCR-mediated $O_2^\cdot$ production, presumably NO directly scavenges $O_2^\cdot$ via the formation of OONO$^-$.

**SCR-mediated NO Consumption as Measured by Electrochemical Detection**—Electrochemical detection using the NO electrode is a powerful and sensitive approach to measuring the rate of NO consumption and production. This technique has been widely employed to study the interaction of NO with the mitochondrial electron transport chain (27, 37–40), as NO traps $O_2^\cdot$ efficiently to form OONO$^-$ ($k = 10^9–10^{10} \text{ M}^{-1} \text{s}^{-1}$, Equation 3) (41). We therefore chose the NO electrode as a sensitive alternative way to probe the mechanism of $O_2^\cdot$ generation by SCR.

\[
\text{NO} + O_2^\cdot \rightarrow \text{OONO}^- \quad (\text{Eq. 3})
\]

We had previously observed that the NO consumption rate in PBS was not significantly increased by the presence of fully oxidized SCR, presumably because of the weak interaction of its heme centers (heme $b$ and heme $c_j$) with NO (27). Here, we observed that the rate of NO consumption by SCR (1 $\mu$M) was greatly enhanced initially by the addition of succinate (1 $\mu$M) (Fig. 6, A and B (trace a)), but decreased gradually upon repeated NO exposures (Fig. 6, A and B, traces b–d) as the succinate-energized SCR reacted with NO.

In a parallel experiment, NO consumption by SCR (1 $\mu$M heme $b$) was induced by 80-s post-addition of succinate (1 $\mu$M) during the period of NO decay. As indicated in Fig. 6C, the NO decay in PBS was enhanced by succinate, with a decay rate linearly proportional to the amount of added succinate (Fig. 6C, inset). This allowed us to quantitate the SCR-mediated NO consumption rate based on the kinetic curve of NO decay in PBS. The initial rate of consumption was estimated to be 9.6 pmol of NO/s/mmol of $b$ (n = 3, for 1 $\mu$M succinate). The detected NO consumption by SCR was completely inhibited by pretreatment of the enzyme with heat denaturation (70 °C for 5 min, data not shown), suggesting that electron transfer activity is required for SCR to catalyze NO consumption.

**Involvement of OONO$^-$ Formation in the SCR-mediated NO Consumption**—As demonstrated previously, $O_2^\cdot$ was derived from the electron leakage from the reduced cytochrome $b$ moiety of SCR (Fig. 4A). Therefore, it is logical to predict that the electron leakage from the reduced heme $b$ could be tightly coupled with NO consumption, resulting in the formation of OONO$^-$.

To address this issue, we added Zn, Cu-SOD (from 0.05–0.5 units/µl) to the reaction mixture prior to succinate-induced NO consumption. As indicated in Fig. 7, the rate of SCR-mediated NO consumption was progressively exponentially slowed as the concentration of SOD increased. The maximal inhibition of the SCR-mediated NO consumption rate by SOD was ~77% at a SOD concentration of ~0.4 units/µl (Fig. 7, inset), indicating that $O_2^\cdot$ generation by succinate-energized SCR largely controls the NO consumption.
FIGURE 6. Polarographic measurements of SCR-mediated NO consumption induced by succinate using an NO sensor. A, a reaction mixture containing purified SCR (1 μM heme b) in PBS was incubated at 37 °C prior to succinate (suc) (1 μM) initiation. A solution containing NO (2 μM) was sequentially injected into the enzyme solution as indicated by the arrows. B, the trace in A was re-plotted, and the NO decay profiles for each injection were superimposed. C, same reaction mixture as A, except that different dosages (indicated by gray arrows) of succinate were added to the enzyme solution during the period of NO decay. Inset, various concentrations of succinate used were plotted against the corresponding NO consumption rate calculated based on the initial rate of the kinetic curve from NO decay in PBS.
rate by forming OONO⁻. A further increase of SOD dosage to 1 unit/μl did not change the maximal inhibition of the SCR-mediated NO consumption rate (data not shown), suggesting that NO can outcompete SOD in scavenging the produced O₂⁻.

Involvement of the Q Cycle Pathway in the SCR-mediated NO Consumption Induced by Succinate—As demonstrated above, the presence of cytochrome c efficiently prevented O₂⁻ generation by succinate-energized SCR as detected by EPR spin-trapping (Fig. 2E). A similar result was also observed in the system for detecting SCR-mediated NO consumption. As indicated in Fig. 8A (trace a) and Table 1, the presence of ferrocytochrome c (1 μM) in the enzyme solution efficiently inhibited (~95%, Table 1) the NO consumption induced by succinate induction.

Any O₂⁻ generated through the Q cycle pathway would be expected to cause NO consumption because of the extremely fast rate constant (Equation 3) of its reaction with NO. To test the direct involvement of the Q cycle pathway in the mediation of NO consumption rate, we treated SCR with antimycin A (1 μM) prior to polarographic measurement in the same reaction system containing cytochrome c (1 μM). As indicated in Fig. 8A (trace c) and Table 1, the replacement of native SCR with antimycin A-SCR significantly boosted the NO consumption rate induced by the addition of succinate (1 μM), implying that O₂⁻ contributed to the NO consumption rate via O₂⁻ production.

A similar enhancement in NO consumption rate was also observed when the SCR was pretreated with the Q₁ site inhibitors, stigmatellin (Table 1) or myxothiazol (Table 1), suggesting that a direct or indirect role for Q₁ is not absolutely required for NO consumption by succinate-reduced SCR. The addition of SOD (0.5 units/μl) inhibited most NO consumption (70–80% in Table 1) by SCR pretreated by any of the above three inhibitors, supporting a direct role for O₂⁻ derived from the Q cycle pathway in the mediation of NO consumption. It should be noted that neither Q₁ site nor Q₀ site inhibitor alone was observed to interact with NO to affect the measured NO consumption rate by SCR (data not shown).

Other redox centers of QCR that could potentially contribute to the SCR-mediated NO consumption include ferrocytochrome b, reduced RISP, and ferrocytochrome c₁. To clarify the roles of RISP and ferrocytochrome c₁, we initiated SCR-mediated NO consumption with ascorbate (5 μM and 10 μM) because it can selectively reduce RISP and heme c₁ but not heme b. As indicated in Fig. 8B and Table 1, post-addition of ascorbate during the period of NO decay did not significantly enhance the NO consumption rate, thus eliminating the possible involvement of reduced RISP and reduced heme c₁ in the SCR-mediated NO consumption. This data also implied that no electron leakage suitable for O₂⁻ generation occurred at the sites of the reduced RISP and heme c₁.

Indirect Role of Ferrocytochrome b in SCR-mediated NO Consumption—To evaluate whether ferrocytochrome b interacts directly with NO to cause NO consumption, we pretreated SCR with the heme blocker cyanide (1 mM); this pretreatment decreased the succinate-induced NO consumption rate by ~62.5% (n = 3, Fig. 8C and Table 1), implying that a heme component, presumably heme b, of SCR was directly or indirectly involved in the NO consumption rate by succinate-energized SCR. Heme c₁ was eliminated as a mediator by the failure to detect SCR-mediated NO consumption initiated by ascorbate (Fig. 8A and Table 1, see above).

Next, we measured the SCR-mediated NO consumption rate under anaerobic conditions. Whether succinate was present in the SCR system or not, the decay rate of NO was significantly slower under conditions of argon saturation (Fig. 9, dashed line) than under the conditions of air saturation (Fig. 9, solid line), demonstrating that oxygen plays an essential role and that NO did not interact directly with ferrocytochrome b. A similar situation was observed for stigmatellin-treated SCR (data not shown).
**SCR-mediated Superoxide Generation and NO Catabolism**

UV-visible absorption spectra recorded at the Soret α and β bands of SCR provide additional evidence that clarifies the indirect role of cytochrome b in SCR-mediated NO consumption induced by succinate. These spectra demonstrate that the oxidation of ferrocytochrome b is closely related to SCR-mediated NO consumption in the air-saturated solution (Fig. 10A). In the presence of NO, the oxidation of reduced heme b in succinate-reduced SCR was significantly accelerated (Fig. 10A, dashed line and trace b), presumably because of indirect interaction with heme b via scavenging O$_2^-$. To determine the effect of oxygen, the experiment was carried out under conditions of argon saturation (Fig. 10B). The spectral intensity of the succinate-reduced heme b was not significantly changed in the presence of NO, supporting the indirect role of ferrocytochrome b in the mediation of the NO consumption rate.

**DISCUSSION**

**Direct Role of Ferrocytochrome b Moiety in the SCR-mediated O$_2^-$ Generation**—With a combination of EPR and UV-visible spectroscopies, it was concluded that the Q cycle pathway unambiguously controls O$_2^-$ generation mediated by succinate-energized SCR. EPR spin-trapping with DEPMPO revealed that the ubisemiquinone radical at the Q$_o$ site only contributes 20–30% of the O$_2^-$ generation by succinate-energized SCR (Fig. 3A). Under enzyme turnover conditions in the presence of cytochrome c, the Q$_o$ site inhibitors stimulated O$_2^-$ generation, presumably because most electron leakage occurred at the site of ferrocytochrome b oxidation. The same situation was observed when SCR was pretreated with the Q$_o$ site inhibitor (Fig. 10, C and D) or the Q site inhibitor (data not shown).

UV-visible absorption spectra (Figs. 4 and 10) obtained from a system of succinate/stigmatellin-SCR, supporting the direct role of ferrocytochrome b in the mediation of O$_2^-$ formation.

DEPMPO can specifically and efficiently trap the O$_2^-$ radicals produced to yield a stable adduct detected by EPR. However, it was observed that even

**TABLE 1**

SCR-mediated NO consumption in the presence of succinate and the effect of cytochrome c, QCR inhibitors (antimycin A, myxothiazol, and stigmatellin), ascorbate, Zn,Cu-SOD, and KCN

| Experiment | NO consumption % |
|------------|------------------|
| SCR        | 0                |
| SCR + succinate | 100          |
| SCR + SOD + succinate | 23.0          |
| SCR + cytochrome c + succinate | 5.0          |
| SCR + antimycin A + cytochrome c + succinate | 83.3          |
| SCR + antimycin A + cytochrome c + succinate + SOD | 16.5         |
| SCR + myxothiazol + cytochrome c + succinate | 61.5          |
| SCR + myxothiazol + cytochrome c + succinate + SOD | 17.2         |
| SCR + stigmatellin + cytochrome c + succinate | 68.6          |
| SCR + stigmatellin + cytochrome c + SOD + succinate | 19.9         |
| SCR + cytochrome c | 3.0          |
| SCR + KCN + succinate | 37.5          |
| SOD + succinate | 0            |

**FIGURE 8.** The effect of cytochrome c, antimycin A, ascorbate, and KCN on the SCR-mediated NO consumption rate as measured by an NO sensor. A, a reaction mixture containing purified SCR or antimycin-SCR (1 μM heme b) with cytochrome c (1 μM) or without cytochrome c in PBS was incubated at 37 °C. The profiles of sensor current were recorded after the injection of NO solution (2 μM), and NO consumption was initiated by succinate (Suc) (1 μM) as indicated by the arrows. B, same as A, but cytochrome c was omitted from the reaction mixture, and succinate (1 μM) or ascorbate (5 and 10 μM, as indicated by X) was used to initiate NO consumption. C, same as A, but KCN (1 mM) was added to the enzyme solution prior to the measurement of NO consumption.

**FIGURE 9.** Polarographic measurements of SCR-mediated NO consumption under the conditions of air saturation (solid lines) and argon saturation (dashed lines). The reaction mixture containing purified SCR (1 μM heme b) in PBS was incubated at 37 °C. After the addition of succinate (1 μM), an NO-containing solution (2 μM) was immediately injected into the enzyme solution as indicated by the arrows. The experiments under anaerobic conditions were carried out as described under “Materials and Methods.” It is worth noting that autoxidation of succinate-reduced heme b was marginally higher under air saturation than under argon saturation (Fig. 10, A versus B, dashed lines), implying that oxygen is involved in the mediation of ferrocytochrome b oxidation. The same situation was observed when SCR was pretreated with the Q$_o$ site inhibitor (Fig. 10, C and D) or the Q site inhibitor (data not shown).
SCR-mediated Superoxide Generation and NO Catabolism

A high concentration of DEPMPO was not able to efficiently prevent cytochrome c$_1$ reduction by O$_{2}^\cdot$ (Fig. 4C), presumably because of its slower reaction rate constant ($k \sim 60–90$ M$^{-1}$ s$^{-1}$) for trapping O$_{2}^\cdot$ (42). As the rate constant for dismutation by SOD is as high as $10^{6–10^{9}}$ M$^{-1}$ s$^{-1}$ (43), SOD can prevent cytochrome c$_1$ reduction more efficiently than DEPMPO by dismutating O$_{2}^\cdot$.

**Indirect Role of Ferrocytochrome b Moiety in the NO Consumption**—SCR-mediated O$_{2}^\cdot$ production was clearly affected by NO as indicated by the studies of EPR spin-trapping and polarographic measurements. We have observed that ~20–25% of SCR-mediated NO consumption was not inhibited by SOD (Fig. 7 and Table 1). One plausible reason is that the rate constant for NO trapping of O$_{2}^\cdot$ is higher than that for SOD-catalyzed O$_{2}^\cdot$ dismutation ($10^{9–10^{10}}$ M$^{-1}$ s$^{-1}$ versus $10^{6–10^{8}}$ M$^{-1}$ s$^{-1}$). Another possible explanation is that a portion of NO may oxidize ferrocytochrome b directly, yielding NO$^\cdot$ (nitrosyl ion) as seen in the case of yeast reduced iso-cytochrome c (44) and ubiquinol (20). However, this second possibility can be eliminated based on the anaerobic results (Figs. 9 and 10). Under argon saturation, electrochemical detection showed no interaction between NO and succinate-reduced SCR or succinate-reduced stigmatellin-treated SCR. As indicated by UV-visible spectroscopy, NO-mediated heme b autoxidation was entirely dependent on the presence of O$_{2}^\cdot$, demonstrating that the NO consumption rate was directly mediated by O$_{2}^\cdot$ via the formation of OONO$^\cdot$, and indirectly mediated by the oxidation of ferrocytochrome b (Fig. 10).

The significant enhancement of the SCR-mediated NO consumption rate by the Q$_o$ site inhibitors provided additional evidence to support a direct role of ferrocytochrome b in O$_{2}^\cdot$ generation (Table 1 and Fig. 10C), because there was no O$_{2}^\cdot$ production derived from Q$_o^\cdot$.

A slightly higher level of NO consumption was detected when SCR was treated with antimycin A (Table 1). This increase was attributed to the increase in generated O$_{2}^\cdot$ derived from Q$_o^\cdot$, leading to marginally enhanced NO consumption by SCR. In support of this explanation, we observed that stimulation of O$_{2}^\cdot$ production by antimycin A is more profound than that by stigmatellin or myxothiazol under the conditions of enzyme turnover (Fig. 3B). These data were consistent with those obtained by EPR spin-trapping and UV-visible spectroscopic analysis, in which ferrocytochrome b controlled most of the SCR-mediated O$_{2}^\cdot$ production, causing most of the NO consumption.

In this study, cyanide has been shown to efficiently inhibit both O$_{2}^\cdot$ production and NO consumption (Figs. 3A and 8C), thus providing extra evidence to support the critical role of the heme component. Cyanide inhibition of the electron transfer activity of SCR ($K_i$ measured ~3.7 mM) is much less efficient than its inhibition of cytochrome c oxidase ($K_i$ ~ 4 µM), whereas it has been widely used as a heme blocker; presumably because of its ability to replace the 6th ligand (histidine) of heme b at the higher concentration (5 mM) used here.

**Physiological Implications**—In our current studies, the enzymatic activity of SCR did not suffer any loss from repeated exposures of SCR (1 µM) to NO (2 µM) up to 4 times in the presence of succinate (1 µM) (Fig. 6A). Therefore, OONO$^\cdot$ from SCR-mediated NO catabolism was not formed in large enough amounts to induce protein nitration under normal physiological conditions. In contrast, it can actually play the role of scavenger to remove O$_{2}^\cdot$ produced in mitochondria (19), supplementing the scavenging action of SOD. Physiologically, this may represent an independent pathway for NO utilization in mitochondria, which effectively competes with the binding of NO to cytochrome c oxidase; thereby releasing cytochrome c oxidase inhibition by NO and restoring oxygen uptake (19, 45). Alternatively, OONO$^\cdot$ might act as a substrate for cytochrome c oxidase and be recycled back to nitrite by the peroxynitrite reductase activity of the cytochrome c oxidase (46). The nitrite produced can be, in turn, recycled back to NO when catalyzed by cytochrome c (39), which can potentially increase the bioavailability of NO in mitochondria.

**Superoxide Generation by the Succinate Dehydrogenase of SCR**—It should not be rule out the involvement of succinate dehydrogenase moiety in the SCR-mediated O$_{2}^\cdot$ production. The generation of O$_{2}^\cdot$ by the flavin autoxidation of succinate dehydrogenase has been reported in the enzymatic systems from bovine (23) and *Escherichia coli* (47). We have conducted the measurements in the systems of SCR and isolated SQR by using EPR spin-trapping and electrochemical detection. Indeed, TTFA stimulates both SCR-mediated O$_{2}^\cdot$ production and NO consumption under the conditions of enzyme turnover. Investigation of this mechanism is currently under progress.

**CONCLUSIONS**

The present studies have demonstrated how the cytochrome b moiety of the Q cycle mechanism mediates O$_{2}^\cdot$ generation and NO consumption. The mechanism addressed here provides a useful concept for understanding the fundamental question of how mitochondria utilize a cytochrome c oxidase-independent pathway to modulate NO catabolism. Clearly, the major function of this event is to scavenge O$_{2}^\cdot$ whose concentration can be increased during state IV respiration or under certain pathophysiological conditions of disease. A secondary role may be to modulate respiration via indirect interaction with the Q cycle.

---

$^3$ Y. R. Chen and J. L. Zweier, unpublished results.
and/or reduced heme \( b \) (19, 45). Recognition of this cytochrome \( c \) oxidase-independent pathway for NO catabolism is important in understanding the fundamental mechanisms by which oxidants and nitric oxide modulate mitochondrial function in a variety of diseases associated with inflammation and oxidant toxicity.

REFERENCES

1. Raha, S., and Robinson, B. H. (2000) *Trends Biochem. Sci.* 25, 502–508
2. Turrens, J. F. (2003) *J. Physiol.* 552, 335–344
3. Moncada, S., and Erusalimsky, J. D. (2002) *Nat. Rev. Mol. Cell. Biol.* 3, 214–220
4. Ramachandran, A., Levonen, A. L., Brookes, P. S., Ceaser, E., Shiva, S., Barone, M. C., and Darley-Usmar, V. (2002) *Free Radic. Biol. Med.* 33, 1465–1474
5. Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1406–1407
6. Zweier, J. L., Kuppusamy, P., Williams, R., Rayburn, B. K., Smith, D., Weisfeldt, M. L., and Flaherty, J. T. (1990) *J. Biol. Chem.* 264, 18890–18895
7. Ambrosio, G., Zweier, J. L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P. P., Tritte, L., Cirillo, P., Condorelli, M., Chiarriello, M., Williams, R., Rayburn, B. K., Smith, D., Weisfeldt, M. L., and Flaherty, J. T. (1993) *J. Biol. Chem.* 268, 18532–18541
8. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. (1977) *Arch. Biochem. Biophys.* 180, 248–257
9. Turrens, J. F., and Boveris, A. (1990) *Biochem. J.* 191, 421–427
10. Kudin, A. P., Rimpolie-Buta, N. Y., Vielhaber, S., Elger, C. E., and Kunz, W. S. (2004) *J. Biol. Chem.* 279, 4127–4135
11. Chen, Y. R., Chen, C. L., Zhang, L., Green-Church, K. B., and Zweier, J. L. (2005) *Circulation* 111, 2966–2972
12. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) *J. Biol. Chem.* 260, 37339–37348
13. Nohl, H., and Jordan, W. (1986) *Biochem. Biophys. Res. Commun.* 138, 533–539
14. Gong, X., Yu, L., Xia, D., and Yu, C. A. (2005) *J. Biol. Chem.* 280, 9251–9257
15. Nisioli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., Bracale, R., Valerio, A., Francozoli, M., Moncada, S., and Carruba, M. O. (2003) *Science* 299, 896–899
16. Zhao, X., He, G., Chen, Y. R., Pandian, R. P., Kuppusamy, P., and Zweier, J. L. (2005) *Circulation* 111, 2966–2972
17. Geng, Y., Hansson, G. K., and Holme, E. (1992) *Circ. Res.* 71, 1268–1276
18. Welter, R., Yu, L., and Yu, C. A. (1996) *Arch. Biochem. Biophys.* 331, 9–14
19. Poderoso, J. J., Lisdero, C., Scolpo, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) *J. Biol. Chem.* 274, 37709–37716
20. Cadenas, E., Poderoso, J. J., Antunes, F., and Boveris, A. (2001) *Free Radic. Res.* 33, 747–756
21. Yu, L., and Yu, C. A. (1982) *J. Biol. Chem.* 257, 2016–2021
22. Gwak, S. H., Yu, L., and Yu, C. A. (1986) *Biochemistry* 25, 7675–7682
23. Zhang, L., Yu, L., and Yu, C. A. (1998) *J. Biol. Chem.* 273, 33972–33976
24. Duling, D. R. (1994) *J. Magn. Reson. B* 104, 105–110
25. Liu, X., Miller, M. J., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A., and Lancaster, J. R. (1998) *J. Biol. Chem.* 273, 18709–18713
26. Lee, C. I., Liu, X., and Zweier, J. L. (2000) *J. Biol. Chem.* 275, 9369–9376
27. Chen, Y. R., Chen, C. L., Liu, X., He, G., and Zweier, J. L. (2005) *Arch. Biochem. Biophys.* 439, 200–210
28. Rosbaud, V., Sankarapandi, S., Kuppusamy, P., Tordo, P., and Zweier, J. L. (1997) *Anat. Biochem.* 247, 404–411
29. Frejaville, C., Karoui, H., Tuccio, B., Le Moigne, F., Cukasi, M., Pietri, S., Lauricella, R., and Tordo, P. (1995) *J. Med. Chem.* 38, 258–265
30. Sun, F., Huo, X., Zhai, Y., Wang, A., Xu, J., Su, D., Bartlam, M., and Rao, Z. (2005) *Cell* 121, 1043–1057
31. Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, I. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998) *Nature* 392, 677–684
32. Esser, L., Quinno, B., Li, Y. F., Zhang, M., Elberry, M., Yu, L., Yu, C. A., and Xia, D. (2004) *J. Mol. Biol.* 341, 281–302
33. Trumpower, B. L. (1990) *J. Biol. Chem.* 265, 11409–11412
34. Crofts, A. R., Barquera, B., Gennis, R. B., Kuras, R., Gueguérou-Kuras, M., and Berry, E. A. (1999) *Biochemistry* 38, 15807–15826
35. Butler, J., Koppenol, W. H., and Margoliash, E. (1982) *J. Biol. Chem.* 257, 10747–10750
36. Poderoso, J. J., Carreras, M. C., Schopfer, F., Lisdero, C. L., Riobo, N. A., Giulivi, C., Boveris, A. D., Boveris, A., and Cadenas, E. (1999) *Free Radic. Biol. Med.* 26, 925–935
37. Shiva, S., Brookes, P. S., Patel, R. P., Anderson, P. G., and Darley-Usmar, V. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 7212–7217
38. Liu, X., Cheng, C., Zorko, N., Cronin, S., Chen, Y. R., and Zweier, J. L. (2004) *Am. J. Physiol.* 287, H2421–H2426
39. Chen, Y. R., Chen, C. L., Liu, X., Li, H., Zweier, J. L., and Mason, R. P. (2004) *Free Radic. Biol. Med.* 37, 1591–1603
40. Chen, Y. R., Chen, C. L., Liu, W., and Zweier, J. L. (2004) *Antioxid. Redox Signal.* 6, 195–199
41. Villamena, F. A., and Zweier, J. L. (2004) *Antioxid. Redox Signal.* 6, 619–629
42. Sun, J., and Trumpower, B. L. (2003) *Arch. Biochem. Biophys.* 419, 198–206
43. Sharpe, M. A., and Cooper, C. E. (1998) *Biochem. J.* 332, 9–19
44. Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996) *Arch. Biochem. Biophys.* 328, 85–92
45. Pearce, L. L., Pitt, B. R., and Peterson, J. (1999) *J. Biol. Chem.* 274, 35763–35767
46. Messner, K. R., and Imlay, J. A. (2002) *J. Biol. Chem.* 277, 42563–42571