Mitochondrial Complex II Can Generate Reactive Oxygen Species at High Rates in Both the Forward and Reverse Reactions*

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Background: Complex II is not considered a significant contributor to mitochondrial ROS production. Results: Complex II generates ROS in both the forward reaction, from succinate, and the reverse reaction, from the reduced ubiquinone pool. Conclusion: Occupancy and reduction state of the flavin dictate its ROS producing behavior. Significance: Based on the maximum rates observed, complex II may be a contributor to physiological ROS production.

Respiratory complex II oxidizes succinate to fumarate as part of the Krebs cycle and reduces ubiquinone in the electron transport chain. Previous experimental evidence suggested that complex II is not a significant contributor to the production of reactive oxygen species (ROS) in isolated mitochondria or intact cells unless mutated. However, we find that when complex I and complex III are inhibited and succinate concentration is low, complex II in rat skeletal muscle mitochondria can generate superoxide or H\textsubscript{2}O\textsubscript{2} at high rates. These rates approach or exceed the maximum rates achieved by complex I or complex III. Complex II generates these ROS in both the forward reaction, with electrons supplied by succinate, and the reverse reaction, with electrons supplied from the reduced ubiquinone pool. ROS production in the reverse reaction is prevented by inhibition of complex II at either the ubiquinone-binding site (by atpenin A\textsubscript{5}) or the flavin (by malonate), whereas ROS production in the forward reaction is prevented by malonate but not by atpenin A\textsubscript{5}, showing that the ROS from complex II arises only from the flavin site (site I\textsubscript{f}). We propose a mechanism for ROS production by complex II that relies upon the occupancy of the substrate oxidation site and the reduction state of the enzyme. We suggest that complex II may be an important contributor to physiological and pathological ROS production.

Several potential sites of superoxide and H\textsubscript{2}O\textsubscript{2} production have been identified in the Krebs cycle and the electron transport chain of mammalian mitochondria. They include the dihydrolipoate moieties of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (1–3), glycerol-3-phosphate dehydrogenase (GPDH),\textsuperscript{4} the electron transferring flavoprotein:Q oxidoreductase (ETFQOR) of fatty acid β-oxidation, the flavin site of complex I (site I\textsubscript{f}); the ubiquinone-reducing site in complex I (site I\textsubscript{Q}); and the ubiquinol-oxidizing site in complex III (site III\textsubscript{Q\textsubscript{o}}) (4–8). Of these, site I\textsubscript{f} in complex I and site III\textsubscript{Q\textsubscript{o}} in complex III (9, 10, 12,13) have been investigated in greatest detail because of their ability to generate superoxide at high rates when they are fully or partially reduced. Sites III\textsubscript{Q\textsubscript{o}}, I\textsubscript{Q}, and I\textsubscript{f} have high maximum rates, and sites I\textsubscript{f} and III\textsubscript{Q\textsubscript{o}} dominate native superoxide production during oxidation of NAD-linked substrates in vitro.\textsuperscript{5} Wild-type complex II makes little contribution to superoxide or hydrogen peroxide production by isolated mammalian mitochondria under normal incubation conditions (8, 14). However, mutations in the enzyme can lead it to generate reactive oxygen species (ROS) at high rates and cause pathology (15).

Mitochondrial complex II (succinate:quinone oxidoreductase; succinate dehydrogenase; SDH) oxidizes succinate to fumarate in the Krebs cycle and reduces ubiquinone (Q) in the respiratory chain. Complex II is ubiquitous in diverse aerobic species. It consists of four subunits: SDHA, the flavoprotein subunit, which contains FAD bound covalently in the active site; SDHB, the iron-sulfur protein subunit, which contains a chain of three iron-sulfur clusters, [2Fe-2S], [4Fe-4S], and [3Fe-4S]; and SDHC and SDHD, the two transmembrane cytochrome b heme subunits, which contain a heme moiety (16). The reaction mechanism of complex II has been explored in depth (17–20). During succinate oxidation, two electrons are transferred to the flavin at site II\textsubscript{Q}, then passed singly through the Fe-S clusters to reduce ubiquinone to ubiquinol at site II\textsubscript{Q\textsubscript{o}}. It is not a requirement for electrons to pass through the b heme because the 7.1 Å edge-to-edge distance between the [3Fe-4S] center and Q facilitates electron tunneling (16). Also, the very...
low midpoint potential of the heme (−185 mV) (21) implies that its steady-state reduction level will be negligible.

In *Escherichia coli*, fumarate reductase is a structural homologue of succinate dehydrogenase that catalyzes the reverse reaction under anaerobic conditions. *E. coli* and *Ascaris suum* fumarate reductases generate superoxide and hydrogen peroxide at high rates when run in reverse as succinate dehydrogenases in the presence of oxygen (22, 23). Succinate dehydrogenases from *E. coli* and *Saccharomyces cerevisiae* also generate superoxide, although at lower rates (24–27). The specific site of superoxide production has been proposed to be the flavin at site II₂, based on the autoxidation properties of flavins (28) and evidence from the *E. coli* studies (24, 29). However, site II₁₂ was proposed in the yeast studies (25, 27). The recent study on *A. suum* suggested that both sites were responsible for significant ROS production (23).

Succinate dehydrogenase purified from bovine heart has also been reported to generate superoxide under some conditions (30). Furthermore, mutations in the flavoprotein subunit of complex II lead to the progressive neurodegenerative disease Leigh syndrome (15). Germ line mutations in the subunits SDHB, SDHC, and SDHD have been observed in patients with hereditary paragangliomas, a rare type of tumor that arises from neuroendocrine tissue in the head and neck (31). Mutations in the SDHC subunit of embryonic fibroblasts result in increased ROS production and enhanced tumorigenesis (32). Similarly, mutations in SDHB, SDHC, and SDHD have been shown to increase ROS production and lead to the stabilization of hypoxia-inducible factor 1α (HIF-1α) and to subsequent cell proliferation (33). Two mechanisms have been proposed for this tumor formation. First, succinate may act as a signaling molecule when it accumulates following loss-of-function mutations in succinate dehydrogenase; and second, ROS generated by mutated succinate dehydrogenase may cause oxidative stress or act as a signal. These mechanisms may not be mutually exclusive (31).

In the present study, we show that the flavin site of mammalian complex II (site II₂) can make superoxide or hydrogen peroxide at high rates in isolated mitochondria. The maximal rates are similar to those of the classic sites in complexes I and III. Based on the conditions required to generate ROS from this site, we suggest that complex II may be a significant source of ROS *in vivo*.

**EXPERIMENTAL PROCEDURES**

*Animals and Mitochondria*—Mitochondria were isolated from hind limb skeletal muscle of female Wistar rats aged 5–8 weeks, by differential centrifugation (34, 35). The animal protocol was approved by the Buck Institute Animal Care and Use Committee, in accordance with IACUC standards.

*Superoxide and H₂O₂ Production by Isolated Mitochondria*—Superoxide and hydrogen peroxide production rates were measured (without discriminating between them) as H₂O₂ production rate in the presence of endogenous and exogenous superoxide dismutase. H₂O₂ production rates were determined using Amplex UltraRed (Invitrogen, Carlsbad, CA) in a Cary Varian spectrofluorometer at the wavelength couple Ex = 560 nm, Em = 590 nm. Horseradish peroxidase was added to catalyze reaction of extramitochondrial H₂O₂ with Amplex UltraRed to form the fluorescent product resorufin. Mitochondria (0.3 mg protein/ml) were incubated at 37 °C in standard assay medium containing 120 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 horseradish peroxidase/ml, and 12 units superoxide dismutase/ml. Fluorescence emission data were calibrated using H₂O₂ standard curves obtained under identical conditions (14). Linear rates of change were measured for 30–60 s for each condition. All values are the empirical rates of H₂O₂ production (in nmol·min⁻¹·mg mitochondrial protein⁻¹), except for the data in Fig. 6, which were corrected for H₂O₂ consumption by matrix peroxidases as described previously using Equation 1 (13).

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\text{CDNB corrected rate} = \text{measured H}_2\text{O}_2 \text{rate} + \frac{\text{measured H}_2\text{O}_2 \text{rate} \times 1.43}{\text{measured H}_2\text{O}_2 \text{rate} + 0.55} \quad (\text{Eq. 1})
\]

Superoxide released to the intermembrane space side of the inner membrane was measured directly by the acetylated cytochrome c assay (36).

*Succinate Dehydrogenase Activation State*—Mitochondria (1 mg protein/ml) were incubated for 5 min with inhibitors and different succinate concentrations under conditions identical to those above, in standard assay medium (120 mM KCl, 5 mM HEPES, 1 mM EGTA) at 37 °C. To assay the activation state of succinate dehydrogenase in each condition, the mitochondria were lysed in assay medium maintained at 15 °C containing 0.1% (v/v) Triton X-100. This follows the protocol in Ref. 17 and is based on observations that below 20 °C, succinate added in the activity assay cannot compete off inhibitory oxaloacetate, and change the activation state of succinate dehydrogenase. Therefore, the activation state observed in this activity assay directly reflects the activation state during the 37 °C incubation, and during the parallel ROS assays. Succinate dehydrogenase activity was determined spectrophotometrically at 15 °C in an Olis dual beam spectrophotometer in split beam mode (λ = 600 nm, slits = 5 nm) as the rate of phenazine methosulfate (PMS) (1 mM) linked reduction of dichlorophenolindophenol (DCPIP) (50 μM) instigated by addition of 10 mM succinate. The reference cuvette contained identical components except that instead of succinate, 10 mM malonate was added. 1 mM sodium cyanide was present in all assays. Data were converted to molar units using the DCPIP extinction coefficient 21 mM⁻¹·cm⁻¹ (37).

*Respiration Assays*—Mitochondrial oxygen consumption was measured in a Clark type oxygen electrode in 120 mM KCl, 5 mM Hepes, 1 mM EGTA in the presence of either 27 mM α/β-glycerol 3-phosphate or 5 mM succinate, 4 mM rotenone, and 1 mM trifluorocarbonyl cyanide phenylhydrazone (FCCP) (33). In the glycerol 3-phosphate experiments, 200 mM free Ca²⁺ was also present to increase affinity of glycerol-3-phosphate dehydrogenase for its substrate. Atpenin A5 was tested at the concentrations described in Fig. 3C.

*Data Analysis*—Data are presented as mean ± S.E. Differences between substrate conditions were analyzed by t test,
with \( p < 0.05 \) considered significant. Where multiple means were compared ANOVA was performed with post hoc Bonferroni used to determine significance (\( p < 0.05 \)) between individual means. Normalization of the rates of \( \text{H}_2\text{O}_2 \) production to succinate dehydrogenase activity in Fig. 4 was performed on the mean ± S.E. for three experiments. The error was appropriately propagated to give the normalized standard error.

RESULTS

Succinate Oxidation Results in High Rates of \( \text{H}_2\text{O}_2 \) Production from Multiple Sites—Succinate is a useful substrate for isolated mitochondria because of its simple and rapid metabolism: it enters the mitochondrial matrix on the dicarboxylate carrier; succinate dehydrogenase oxidizes it to fumarate and passes two electrons to ubiquinone and down the electron transport chain; fumarate is converted to malate by fumarase, and malate exits on the dicarboxylate carrier in exchange for incoming succinate. The \( K_m \) values of the dicarboxylate carrier and succinate dehydrogenase for succinate are around 1 mM (18, 38), so succinate is typically added at saturating concentrations (\( \geq 5 \text{ mM} \)) to support rapid respiration.

In most mitochondria, succinate oxidation is kinetically and thermodynamically able to feed electrons rapidly into the Q pool and generate a high protonmotive force, making it a good substrate for superoxide or \( \text{H}_2\text{O}_2 \) generation. The first bar in Fig. 1a recapitulates many earlier observations that mitochondria isolated from rat skeletal muscle generate \( \text{H}_2\text{O}_2 \) at a high rate when incubated with 5 mM succinate in the absence of inhibitors of electron transport (39–41). Fig. 1b shows eight sites that might contribute to \( \text{H}_2\text{O}_2 \) production under these conditions, and the sites of action of inhibitors used in the present report to dissect out which sites run in different conditions. The second bar in Fig. 1a confirms earlier observations that addition of rotenone, an inhibitor of the Q binding site of complex I, greatly inhibits \( \text{H}_2\text{O}_2 \) formation, identifying the major source of \( \text{H}_2\text{O}_2 \) production during succinate oxidation as site \( I_{\text{Q}} \), driven by reverse electron transport from \( \text{QH}_2 \) into complex I (13, 14, 40, 41).

The remaining \( \text{H}_2\text{O}_2 \) production in the presence of rotenone is usually attributed to site \( III_{\text{Q}} \) in complex III, although a significant proportion may come from site \( I_{\text{Q}} \) following build-up of malate and NADH (42) during succinate oxidation. Addition of myxothiazol, an inhibitor at site \( III_{\text{Q}} \), will remove the contribution of site \( III_{\text{Q}} \) (and of any other site that is downstream or depends on protonmotive force). However, the third bar in Fig. 1a shows that addition of myxothiazol did not abolish the residual \( \text{H}_2\text{O}_2 \) production. As discussed elsewhere, the smallness of the change is likely due to compensatory increases at other sites as the Q pool became reduced.

In the experiments presented below we examine the source of the \( \text{H}_2\text{O}_2 \) production in this condition: the presence of succinate, rotenone and myxothiazol. Fig. 1b identifies the remaining six candidate sites under these conditions: site \( I_{\text{Q}} \) (and also the dihydrolipoate moieties of \( \alpha \)-ketoglutarate dehydrogenase and pyruvate dehydrogenase, connected through malate, malate dehydrogenase, and NADH), the flavin site (site \( II_{\text{Q}} \)) and ubiquinone-binding site (site \( II_{\text{Q}} \)) of complex II, and any other redox center that is able to accept electrons from the \( \text{QH}_2 \) pool,
ROS Production by Complex II

As expected, malonate fully prevented \( \text{H}_2\text{O}_2 \) production. To check that the apparent inhibition by malonate was not explained simply by a shift in the concentration of succinate required to give peak \( \text{H}_2\text{O}_2 \) production, we titrated \( \text{H}_2\text{O}_2 \) production with succinate at different concentrations of malonate. \( \text{H}_2\text{O}_2 \) production was inhibited at all succinate concentrations, with no major shift in the concentration required for peak rates (Fig. 3b). We conclude that malonate fully prevented \( \text{H}_2\text{O}_2 \) production by inhibiting electron supply from succinate to complex II, confirming that \( \text{H}_2\text{O}_2 \) production was from one or more of the five sites highlighted in orange in Fig. 1b.

We also incubated the mitochondria with 400 \( \mu \text{M} \) malate, which will be oxidized to oxaloacetate by malate dehydrogenase, or directly oxidized to the enol form of oxaloacetate by succinate dehydrogenase itself (43). Oxaloacetate is a potent inactivator of succinate dehydrogenase (17). The presence of malate eliminated much of the observed \( \text{H}_2\text{O}_2 \) production (Fig. 3a); the residual rate probably came from site I\(_F\) through oxidation of NADH generated by malate dehydrogenase.

The most telling result came from the use of the site II\(_Q\) inhibitor, atpenin A5. At 1 \( \mu \text{M} \), atpenin A5 fully inhibited uncoupled respiration with succinate as substrate and had no effect on uncoupled respiration with glycerol 3-phosphate as substrate (Fig. 3c), consistent with its known properties as a specific inhibitor of site II\(_Q\) (44). However, atpenin A5 did not fully inhibit \( \text{H}_2\text{O}_2 \) production. In the presence of 1 \( \mu \text{M} \) atpenin A5 about 40–50% of \( \text{H}_2\text{O}_2 \) production remained (Fig. 3a), showing that a substantial proportion of the observed signal was from sites upstream of the atpenin A5 inhibition site (and downstream of the malonate inhibition site), i.e. from within complex II. Fig. 3d shows a succinate titration of \( \text{H}_2\text{O}_2 \) production in the presence of 1 \( \mu \text{M} \) atpenin A5 to test whether the effect of atpenin A5 was to shift the position of the peak without inhibiting the maximum rate of \( \text{H}_2\text{O}_2 \) production. In the presence of atpenin A5 there was a slight left-shift in the peak, indicating that a lower succinate concentration was required to generate the peak rates. The rate of \( \text{H}_2\text{O}_2 \) production at the optimal succinate concentration in the presence of atpenin A5 was >50% of the rate at the peak in the absence of inhibitor, showing that >50% of the signal was from complex II. Furthermore, since atpenin A5 inhibits site II\(_Q\), this superoxide or \( \text{H}_2\text{O}_2 \) must have been produced at site II\(_F\).

Analysis of the Effect of Atpenin A5 on the Observed \( \text{H}_2\text{O}_2 \) Production in the Presence of Rotenone and Myxothiazol following Addition of Succinate—The observation that atpenin A5 did partially inhibit \( \text{H}_2\text{O}_2 \) production suggests that not all the observed \( \text{H}_2\text{O}_2 \) arose from site II\(_Q\) of complex II, but up to 50% may have been generated by site II\(_O\) or any of the other three downstream sites highlighted in orange in Fig. 1b. However, the oxaloacetate occupancy and succinate dehydrogenase activity of complex II are known to be very dependent upon the incubation conditions (18), so an alternative possibility is that binding of atpenin A5 at site II\(_Q\) decreased the enzymatic activity at site II\(_F\), indirectly decreasing \( \text{H}_2\text{O}_2 \) production from site II\(_F\).

To test this possibility, we measured the succinate-DCPIP oxidoreductase activity of site II\(_F\) of succinate dehydrogenase in the absence and presence of atpenin A5 (Fig. 4a). We took advantage of a unique property of the enzyme: at 15 °C, succi-
nate added at 10 mM in the activity assay does not compete off inhibitory oxaloacetate and change the activation state of succinate dehydrogenase set up in the original 37 °C incubation at different succinate concentrations (17) (see “Experimental Procedures”). Fig. 4 indicates that higher concentrations of succinate in the 37 °C incubation led to higher activation states in the subsequent 15 °C activity assays, reflecting lower occupancy of site IIF by oxaloacetate or a succinate-mediated release of a kinetic limitation. The behavior was the same when atpenin A5 was present in the 37 °C incubation, except that the succinate dehydrogenase activity was decreased by 25–45% at each succinate concentration. Similar decreases in succinate dehydrogenase activity have been observed in mitochondria incubated with carboxins and TTFA (46). When the rates of \( \text{H}_2\text{O}_2 \) production in Fig. 3d were recalculated by normalizing to the activation state of succinate dehydrogenase in the absence and presence of atpenin A5, the peak rates of \( \text{H}_2\text{O}_2 \) production were not different (Fig. 4b). This result indicates that essentially none of the observed \( \text{H}_2\text{O}_2 \) production came from sites downstream of the atpenin A5 inhibition site, and site IIF was the only source of \( \text{H}_2\text{O}_2 \) production during oxidation of succinate in the presence of rotenone and myxothiazol.

**H\(_2\)O\(_2\) Production by Complex II during the Reverse Reaction in the Presence of Rotenone, with and without Myxothiazol**—Complex II is known to be fully catalytically reversible depending on the experimental conditions (47). A second test of the ability of complex II to generate superoxide or \( \text{H}_2\text{O}_2 \) is to reduce the Q pool by another pathway in the presence of rotenone and myxothiazol and measure whether \( \text{H}_2\text{O}_2 \) production during the reverse reaction is sensitive to inhibitors of complex II. Under these conditions, if the observed \( \text{H}_2\text{O}_2 \) is generated by any of the three sites other

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**FIGURE 3. Effect of inhibitors of complex II on \( \text{H}_2\text{O}_2 \) production in the presence of rotenone and myxothiazol and on respiration rate.** a, rates of \( \text{H}_2\text{O}_2 \) production caused by addition of 400 \( \mu\text{M} \) succinate in the presence of 4 \( \mu\text{M} \) rotenone and 2 \( \mu\text{M} \) myxothiazol (first bar), and in the additional presence of 500 \( \mu\text{M} \) malonate (second bar), 1 \( \mu\text{M} \) malate (third bar), or 1 \( \mu\text{M} \) atpenin A5 (fourth bar) \((n=4)\). Higher concentrations of each inhibitor did not have a greater inhibitory effect. b, effect of different concentrations of malonate on the titration shown in Fig. 2 (mean values shown, \( n = 3 \)). c, effect of atpenin A5 on uncoupled respiration on \( \alpha/\beta \) glycerol 3-phosphate (27 \( \mu\text{M} \)) and on succinate (5 \( \mu\text{M} \)). 1 \( \mu\text{M} \) FCCP and 4 \( \mu\text{M} \) rotenone were present in all experiments. d, effect of 1 \( \mu\text{M} \) atpenin A5 on the titration shown in Fig. 2 \((n = 4)\). Higher concentrations of atpenin A5 did not have a greater inhibitory effect. Data are means ± S.E., except in b where only means are shown. *, significantly different from rate without inhibitor \((p < 0.05)\). **Closed symbols:** uninhibited complex II, **open symbols:** malonate- or atpenin A5-inhibited complex II.
ROS Production by Complex II

![Graph](image)

**FIGURE 4. Correction for the effect of atpenin A5 on the activation state of complex II.** a, effect of succinate concentration and atpenin A5 on the activation state of complex II. Intact mitochondria were incubated at 37 °C for 5 min in the presence of rotenone (4 μM), myxothiazol (2 μM), and a range of succinate concentrations as indicated, in the absence (closed symbols) or presence (open symbols) of 1 μM atpenin A5. Following incubation, the mitochondria were lysed at 15 °C and the rate of PM5-linked reduction of DCPIP induced by 10 mM succinate was measured to report the activation state achieved in the prior incubation (see “Experimental Procedures”). b, data of Fig. 3d replotted after normalization to the activation state of complex II derived from a. Normalized H$_{2}$O$_{2}$ production = nmol H$_{2}$O$_{2}$·min$^{-1}$·mg mitochondrial protein$^{-1}$ in the initial incubation/nmol DCPIP reduced min$^{-1}$·mg mitochondrial protein$^{-1}$ in the subsequent activity assay. Data are means ± S.E. (n = 3); standard errors were propagated to give the normalized standard error. A Student’s t test indicated that there was no significant difference between the normalized maximum rates of H$_{2}$O$_{2}$ production.

than complex II indicated in orange in Fig. 1b it will be insensitive to malonate and atpenin A5; if it comes from site IIQ$_{F}$ it will be inhibited by atpenin A5 but not by malonate, and if it comes from site II$_{F}$ it will be fully inhibited by both malonate and atpenin A5. Therefore, we next explored the ability of complex II to generate H$_{2}$O$_{2}$ with electrons flowing in the reverse direction, from QH$_{2}$.

Electrons were fed into the Q pool through glycerol-3-phosphate dehydrogenase in the presence of rotenone and myxothiazol to prevent superoxide and H$_{2}$O$_{2}$ production from sites I$_{O}$, I$_{F}$, and IIIQ$_{O}$. Oxidation of glycerol-3-phosphate under these conditions is thought to generate superoxide or H$_{2}$O$_{2}$ primarily from glycerol-3-phosphate dehydrogenase (48–50). However, Fig. 5a shows that the observed rate of H$_{2}$O$_{2}$ production during oxidation of glycerol 3-phosphate was inhibited ∼70% by atpenin A5 (Fig. 5a). Since atpenin A5 does not inhibit respiration with glycerol 3-phosphate as substrate but is a specific inhibitor of complex II (Fig. 3c), this shows that complex II generated H$_{2}$O$_{2}$ at a substantial rate during the reverse reaction, when electrons entered complex II from the Q pool.

Fig. 5a shows that malonate was as effective as atpenin A5 at inhibiting H$_{2}$O$_{2}$ production under these conditions. Since site IIQ$_{F}$ was uninhibited in the presence of malonate, this result shows that ROS production by complex II during the reverse reaction was entirely from site II$_{F}$.

We have observed a similar atpenin A5 and malonate sensitivity of glycerol 3-phosphate-driven H$_{2}$O$_{2}$ production in rat brown adipose tissue mitochondria in the presence of rotenone and myxothiazol, suggesting that H$_{2}$O$_{2}$ can also be produced by complex II in mitochondria isolated from other tissues.5

The experiments above show that complex II can generate H$_{2}$O$_{2}$ at relatively high rates in the presence of multiple electron transport chain inhibitors and in the absence of proton-motive force. Importantly, it can also do so in the absence of such constraints. During normal, uninhibited glycerol 3-phosphate oxidation (with rotenone present to prevent reverse electron transport through complex I), a significant portion of the observed H$_{2}$O$_{2}$ production was sensitive to malonate and atpenin A5 (Fig. 5b). These data indicate that H$_{2}$O$_{2}$ production by complex II is not contingent upon extreme unphysiological constraints or the presence of complex III inhibitors.

Fig. 5c summarizes the results from Fig. 4 and Fig. 5a. During the forward reaction, H$_{2}$O$_{2}$ production from complex II was inhibited by malonate but not by atpenin A5. During the reverse reaction it was inhibited by both compounds. These results show that superoxide or H$_{2}$O$_{2}$ from complex II is produced entirely from site II$_{F}$ and not from site IIQ$_{F}$.

**Maximum Rates of H$_{2}$O$_{2}$ Production from Complex II**—It is of note that complex II can produce superoxide or H$_{2}$O$_{2}$ at rates similar to those that we associate with the major mitochondrial producers, complex I and complex III. Fig. 6 shows the maximum attainable rates from sites I$_{F}$, I$_{Q}$, II$_{F}$, and IIIQ$_{O}$, after correction for matrix peroxidase activity (using the CNDB correction described under “Experimental Procedures”). For this correction, we assumed that 100% of the superoxide or H$_{2}$O$_{2}$ from complex II was produced to the matrix. This assumption was based on observations that the rate sensitive to exogenous superoxide dismutase was small and that no superoxide was detected externally in the acetylated cytochrome c reduction assay (36) (data not shown). The maximum rate of superoxide or H$_{2}$O$_{2}$ production by site II$_{F}$ was ∼3 times higher than the maximum rate of superoxide production from site I$_{F}$. It was similar to the maximum rate from site I$_{Q}$ and about half of the maximum rate of superoxide production from site IIIQ$_{O}$.

5 A. L. Orr, C. L. Quinlan, I. V. Perevoshchikova, and M. D. Brand, submitted manuscript.
DISCUSSION

The results presented above show that complex II in isolated mitochondria can generate superoxide or H$_2$O$_2$ at high rates when ubiquinone reoxidation through complex I and complex III is prevented and succinate concentration is low (Fig. 2). These rates approach or exceed the maximum rates achieved by complex I or complex III (Fig. 6). Complex II generates superoxide or H$_2$O$_2$ from site IIF but not site IIQ. It can do so in both the forward reaction from succinate and the reverse reaction from QH$_2$ (Fig. 5c), even in the absence of complex III inhibitors (Fig. 5b).

Figs. 2 and 3 show that the observed rates of H$_2$O$_2$ production from complex II first rise to a maximum and then fall as succinate concentration is increased. The rise in H$_2$O$_2$ production is readily explained as the result of increasing reduction of the flavin species that reacts with oxygen to form superoxide or H$_2$O$_2$. There are at least three possible explanations of the fall in observed H$_2$O$_2$ production as succinate concentration is increased further: (a) the species that reacts with oxygen is the flavin semiquinone, which decreases in concentration as succinate concentration exceeds a threshold and over-reduces it to FADH$_2$; (b) at higher succinate concentrations binding of succinate to the active site alters the midpoint potential of the species that reacts with oxygen, making it a poorer reductant; and (c) at higher succinate concentrations succinate occupancy of the active site hinders access of oxygen. Explanation (a) has not been tested, but is not considered likely. Explanation (b) is supported by evidence that the midpoint potential of the FAD in succinate dehydrogenase is negative (~ −120 to −40 mV) at

1 μM atpenin A5 were present where indicated. The final three bars are the normalized rates of H$_2$O$_2$ production in the reverse reaction. Data from a at 27 mM glycerol 3-phosphate (G3P) were normalized to the activity of the enzyme incubated under identical conditions (as described for Fig. 4). The rate in the presence of 500 μM malonate was made zero by definition. Data are means ± S.E. (n = 3). *, significantly different from rate without malonate or atpenin A5 (p < 0.02).
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When substrate or substrate analogues are in the substrate binding site, the non-ROS-producing species "FAD-suc", "FADH-succinate" (top) or exit from the Q pool and reduce the flavin (bottom). ROS production is only possible when the site is reduced but not occupied with substrate or substrate analog (i.e. succinate (succ), fumarate (fum), oxaloacetate (oxa), malonate (mal)). When substrate or substrate analogues are in the substrate binding site, the non-ROS-producing species "FAD-suc", "FADH-succinate", or generically "FAD-X" or "FADH-X" are created. Presumably, the semireduced species (FADH) can also bind substrate or substrate analogues, but that is not represented graphically.

In summary, when complex III is inhibited and rapid reoxidation of the Q pool is prevented, mitochondrial complex II generates superoxide (or H$_2$O$_2$) at site II$_f$ in both the forward and reverse reactions. It is capable of high rates of production comparable to other mitochondrial sites. We also observe significant rates of H$_2$O$_2$ production in the reverse reaction in the absence of complex III inhibitors. This observation suggests that complex II may be a relevant producer in vivo when multiple substrates feed electrons into the Q pool, but there is limited energetic demand (e.g. resting muscle). We also hypothesize that low substrate occupancy and relatively high reduction state may occur under other in vivo conditions such as hypoxia, and that complex II may emerge as a physiologically relevant mitochondrial ROS producer.

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