Superoxide Is Produced by the Reduced Flavin in Mitochondrial Complex I

A SINGLE, UNIFIED MECHANISM THAT APPLIES DURING BOTH FORWARD AND REVERSE ELECTRON TRANSFER

Kenneth R. Pryde and Judy Hirst
From the Medical Research Council Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge, CB2 0XY, United Kingdom

NADH:ubiquinone oxidoreductase (complex I) is a major source of reactive oxygen species in mitochondria and a contributor to cellular oxidative stress. In isolated complex I the reduced flavin is known to react with molecular oxygen to form predominantly superoxide, but studies using intact mitochondria contend that superoxide may result from a semiquinone species that responds to the proton-motive force (Δp) also. Here, we use bovine heart submitochondrial particles to show that a single mechanism describes superoxide production by complex I under all conditions (during both NADH oxidation and reverse electron transfer). NADH-induced superoxide production is inhibited by complex I flavin-site inhibitors but not by inhibitors of ubiquinone reduction, and it is independent of Δp. Reverse electron transfer (RET) through complex I in submitochondrial particles, driven by succinate oxidation and the Δp created by ATP hydrolysis, reduces the flavin, leading to NAD+ and O2 reduction. RET-induced superoxide production is inhibited by both flavin-site and ubiquinone-reduction inhibitors. The potential dependence of NADH-induced superoxide production (set by the NAD+ potential) matches that of RET-induced superoxide production (set by the succinate potential and Δp), and they both match the potential dependence of the flavin. Therefore, both NADH- and RET-induced superoxide are produced by the flavin, according to the same molecular mechanism. The unified mechanism describes how reactive oxygen species production by complex I responds to changes in cellular conditions. It establishes a route to understanding causative connections between the enzyme and its pathological effects and to developing rational strategies for addressing them.

Complex I (NADH:ubiquinone oxidoreductase) is a major entry point for electrons to the respiratory chain in mammalian mitochondria (1). It oxidizes NADH to NAD+, reduces ubiquinone to ubiquinol, and uses the redox potential difference to translocate protons across the mitochondrial inner membrane. The translocated protons contribute to the proton motive force (Δp) that supports ATP synthesis and many transport processes. Complex I is a significant source of cellular reactive oxygen species (ROS) also (2). Thus, complex I is associated with many mitochondrial pathologies and neurodegenerative diseases as a result of both decreases in its catalytic capability and increases in its ROS production (3, 4). Defining and characterizing the sites and mechanisms of ROS production by complex I provide a route to understanding causative connections between the enzyme and its pathological effects and to developing rational strategies for addressing them.

In isolated complex I there is substantial evidence that ROS are produced only by the reduced flavin mononucleotide, the cofactor that oxidizes NADH (5). In the presence of NADH only, isolated complex I from Bos taurus heart mitochondria reduces O2 to predominantly superoxide; less than 10% of the O2 is reduced directly to H2O2. The superoxide that is produced dismutates rapidly to produce H2O2 (the dismutation step is not rate-limiting) so that the rate of superoxide (and H2O2) production by complex I can be quantified accurately by measuring the rate of H2O2 production (5). The rate of superoxide production by the flavin in complex I is set by a pre-equilibrium with NADH and NAD+. The NAD+/NADH ratio determines the population of complex I molecules that have a fully reduced flavin; the nucleotide concentrations determine the binding site occupancy. Only those molecules that have a reduced flavin in a nucleotide-free binding site react with O2 in a slow, rate-determining step to form superoxide (and H2O2); the reaction is unaffected by ubiquinone oxidation or the binding of Q-site inhibitors (5, 6). Thus, the flavin-site mechanism explains how the composition of the mitochondrial NAD+ pool may affect ROS production, and clear links between NAD(P)H oxidation state and ROS production have been identified in mitochondria (7, 8). Furthermore, expression of the yeast alternative NADH dehydrogenase in Drosophila confers increased lifespan; the NAD+/NADH ratio is raised, and ROS production and the aging-associated decline in respiratory capacity are mitigated (9).

In contrast, many studies on intact mitochondria have suggested that complex I produces ROS (usually considered as superoxide but detected as H2O2 diffusing out of the mitochondria) by a mechanism involving an iron-sulfur cluster that is

*This work was supported by the Medical Research Council.

†To whom correspondence should be addressed. Tel.: 44-1223-252810; E-mail: jh@mrc-mbu.cam.ac.uk.

2The abbreviations used are: ROS, reactive oxygen species; ACMA, amino-6-chloro-2-methoxyacridine; DPI, diphenyleneiodonium; RCR, respiratory control ratio; RET, reverse electron transfer; SMP, submitochondrial particle; SOD, superoxide dismutase; cyt c, cytochrome c; APAD, 3-acetylpyridine adenine dinucleotide.
adjacent to the ubiquinone binding site (Q-site) and/or ubisemiquinone intermediates (for review, see Refs. 2 and 10). Originally, the observation that complex I Q-site inhibitors such as rotenone increase \( \Delta \phi \) production from mitochondria respiring on NADH-linked substrates was used to propose the involvement of the Q-site (11). However, this observation is readily explained by the flavin-site mechanism, as inhibiting NADH oxidation causes NADH to accumulate and lowers the NAD\(^+\)/NADH ratio (lowers the NAD\(^+\) potential). Two further observations have been proposed to be inconsistent with the flavin-site mechanism and to support a semiquinone-based (or related) mechanism (11–13). First, higher rates of \( \Delta \phi \) production are observed during reverse electron transport (RET) by mitochondria than during NADH oxidation. RET refers to the reduction of NAD\(^+\) by complex I, driven by succinate oxidation to produce ubiquinol (to supply the electrons), and by a substantial \( \Delta \psi \) (to overcome the unfavorable redox potential difference) between ubiquinol and NAD\(^+\). The idea is that the flavin can be fully reduced during NADH oxidation; this sets a maximum rate of \( \Delta \phi \) production from the flavin that should not be exceeded during RET (13). Second, \( \Delta \psi \) has two components that represent the difference in charge (\( \Delta \psi \)) and pH (\( \Delta \phi \)) on each side of the membrane. In mitochondria, their relative contributions can be manipulated, and during both NADH oxidation and RET, \( \Delta \psi \) production appears to accelerate as \( \Delta \phi \) increases. The idea is that the flavin is less intimately linked to proton translocation than the Q-site, so it should not be significantly affected by \( \Delta \phi \) (12).

Here, we use submitochondrial particles (SMPs, inverted membrane vesicles) from \textit{B. taurus} heart mitochondria to bridge the gap between studies of superoxide and \( \Delta \phi \) production on the isolated enzyme and on intact mitochondria. Like intact mitochondria, our SMPs sustain a substantial \( \Delta \psi \) during energy-linked forward (NADH or succinate:O\(_2\) oxidation) electron transport, and they catalyze \( \Delta \psi \)-driven reverse electron transport. Like the isolated enzyme, our SMPs allow precise control over the availability of substrates and the conditions for the active site chemistry, and superoxide and \( \Delta \phi \) detection are unaffected by the mitochondrial antioxidant defenses. Our results define an integrated and unified mechanism for superoxide and \( \Delta \phi \) production by the reduced flavin in complex I that applies under all conditions and that is relevant to understanding how rates of ROS production by complex I in intact mitochondria are determined.

**EXPERIMENTAL PROCEDURES**

All chemicals were supplied by Sigma unless otherwise stated.

\textit{The Preparation of SMPs from B. taurus Heart Mitochondria—} Mitochondria were prepared from \textit{B. taurus} heart tissue as described previously (14) and stored as \( \sim 5 \) g pellets at \( -20^\circ \text{C} \). A single pellet was thawed overnight at \( 4^\circ \text{C} \), resuspended in 10 \text{mM} Tris-SO\(_4\), 250 \text{mM} sucrose at pH 7.0 (final volume 120 \text{ml}, pH corrected at \( 20^\circ \text{C} \), buffer A), and refrozen. 40 \text{ml} of suspension (thawed at \( 4^\circ \text{C} \)) were centrifuged (11,300 \( \times g \), 12 min, 4 \( ^\circ \text{C} \)), the dark red supernatant was discarded, and the pellet was resuspended in buffer A to 40 \text{ml}; this step helps remove saturated fats as well as soluble proteins. The pH was adjusted to 9 on ice by the dropwise addition of 2.5 \text{mTris} (15), and the sample was incubated on ice for 15 min then centrifuged (37,900 \( \times g \), 12 min, 4 \( ^\circ \text{C} \)); this step aims to dissociate the inhibitor protein, IF\(_1\), from ATP synthase (16). The supernatant was discarded, and the pellet was twice resuspended in buffer A to 40 \text{ml} and centrifuged (11,300 \( \times g \), 12 min, 4 \( ^\circ \text{C} \)). Then 10 \text{mM} MgSO\(_4\) was added because it improves the respiratory control ratio (RCR) values of the preparation (17, 18), and the sample was sonicated on ice (Sonicator 3000, Misonix, 19 \text{mm probe}, ten 15 \text{s bursts with 1 min intervals, 150 Watts}). The sonicated material was centrifuged (27,100 \( \times g \), 20 min, 4 \( ^\circ \text{C} \)), and the pellet was discarded. Then, 1 \text{mM} NADH was added, and the sample was incubated for 1 \text{h} on ice; this step aims to activate any complex I that has undergone the “deactive transition” (15). The SMPs were collected by centrifugation (82,000 \( \times g \), 30 \text{min}, 4 \( ^\circ \text{C} \)) then twice resuspended to 4 \text{ml} in buffer A and centrifuged to remove the nucleotides. Finally, the SMPs were resuspended to \( \sim 7 \) mg of protein ml\(^{-1}\) (determined using the Pierce bicinchoninic acid (BCA) assay) and stored as aliquots at \( -20^\circ \text{C} \). The yield was typically \( \sim 30 \) mg of protein per \text{SMPs}.

\textit{Catalytic Activity Measurements—} All kinetic measurements were performed in 10 \text{mM} Tris-SO\(_4\), 250 \text{mM} sucrose at pH 7.5, 32 \( ^\circ \text{C} \), in an Ocean Optics DH-2000-BAL diode array spectrophotometer, a Molecular Devices spectramax plus 384 plate reader, or a Shimadzu RF-5301 PC spectrofluorometer.

NADH:O\(_2\) oxidoreduction (100 \muM NADH) and NADH:fumarate oxidoreduction (100 \muM NADH, 40 \muM fumarate, 40 \muM KCl) were followed at 340–380 nm (\( \epsilon_{\text{NADH}} = 4.81 \text{mM}^{-1} \text{cm}^{-1} \)). Succinate:O\(_2\) oxidoreduction (20 \muM succinate) was followed using a Clark electrode. ATP hydrolysis was followed using a coupled assay to detect ADP (19); ADP drives the conversion of phosphoenolpyruvate (200 \muM) to pyruvate by pyruvate kinase (40 \muM ml\(^{-1}\)), then pyruvate reduction was coupled to NADH oxidation (200 \muM) by lactate dehydrogenase (50 \muM ml\(^{-1}\)). Rotenone was used to prevent NADH oxidation, and the reaction was initiated with 200 \muM ATP + MgSO\(_4\) and followed at 340–380 nm. ATP hydrolysis traces were biphasic due to a known lag phase from the expulsion of bound ADP (20); reported rates are for the second, linear phase. Succinate: NAD\(^+\) oxidoreduction (RET, in 10 \text{mM} succinate, 1 \text{mM} NAD\(^+\), 1 \text{mM} ATP + MgSO\(_4\), 40 \muM KCl) was followed at 340–380 nm; assay traces exhibited a short lag phase before becoming linear due either to ATP hydrolysis (see above) or to the reactivation of small quantities of deactive enzyme (15). NADH:ferricyanide, hexammineruthenium (HAR), and 3-acylpyridine adenine dinucleotide (APAD\(^+\)) oxidoreduction (100 \muM NADH, 1 \text{mM} ferricyanide, 3.5 \text{mM} HAR, 1 \text{mM} APAD\(^+\), rotenone) were followed as described previously (6). Cytochrome c:O\(_2\) oxidoreduction (100 \muM bovine heart cytochrome c, reduced using excess sodium dithionite and purified using a PD10 Sephadex G-25 column) was monitored via the oxidation of the reduced cytochrome c (cyt c) (see below). The fluorescence of 500 \text{nm} amino-6-chloro-2-methoxyacridine (ACMA) was measured using excitation and emission wavelengths of 435 and 477 \text{nm}, respectively, typically in the presence of \( \sim 100 \mu\text{g} \) of protein ml\(^{-1}\) SMPs.

\textit{H\(_2\)O\(_2\) and Superoxide Production Measurements—} \textit{H\(_2\)O\(_2\)} production was quantified using the horseradish peroxidase.

**Mechanism of Superoxide Production by Complex I**
**Mechanism of Superoxide Production by Complex I**

<table>
<thead>
<tr>
<th>Catalytic reaction</th>
<th>Rate of reaction (μmol min⁻¹ mg protein⁻¹)</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NADH-O₂ oxidoreduction</td>
<td>Coupled (+Δp) 0.246 ± 0.011</td>
<td>Uncoupled (Δp = 0) 0.743 ± 0.018</td>
</tr>
<tr>
<td>2. Succinate-O₂ oxidoreduction</td>
<td>0.148 ± 0.015</td>
<td>0.264 ± 0.024</td>
</tr>
<tr>
<td>3. NADH:fumarate oxidoreduction</td>
<td>0.052 ± 0.001</td>
<td>0.053 ± 0.001</td>
</tr>
<tr>
<td>4. Succinate:NAD⁻ oxidoreduction (RET)</td>
<td>0.133 ± 0.002</td>
<td>0.002 ± 0.002</td>
</tr>
<tr>
<td>5. ATP hydrolysis</td>
<td>0.893 ± 0.109</td>
<td>1.353 ± 0.043</td>
</tr>
</tbody>
</table>

*Δp was collapsed using 10 μg ml⁻¹ gramicidin.
*Observed rates of NADH-O₂ and succinate:NAD⁻ oxidoreduction varied by up to 2-fold between preparations and were comparable to a range of published values recorded in varying conditions (see for example Refs. 15 and 30). Conversely, the rates of reactions that are catalyzed only by the flavin site in complex I did not vary significantly.
*When SMPs were prepared without the NADH incubation to activate them during the preparation (15) several wash steps could be omitted, and the RCR values were significantly higher (5–6 for NADH-O₂ oxidoreduction). These SMPs were not used extensively here because their rates of RET are significantly diminished unless they are pre-activated with NADH, a procedure that results in nucleotides being present in the assay; they were used only for comparison with our standard preparation, in experiments to quantify NADH-induced H₂O₂ production (see “Results”).
*Activating the complex I with NADH (15) immediately before the RET assay typically increased the rate by 20%, suggesting that ~80% of the complex I is already in the active form.

(HRP)-dependent oxidation of Amplex Red (10 μM, Invitrogen) to resorufin, monitored at 557–620 nm (ε = 51.6 ± 2.5 mM⁻¹ cm⁻¹ at pH 7.5) (5, 7). The HRP concentration was 2 units ml⁻¹ and increased to 8 units ml⁻¹ when 400 μM KCN was present; further increasing the concentration did not affect the observed rate. NADH-induced H₂O₂ production was determined in 30 μM NADH (5, 21); E_set was set by varying the NAD⁺ concentration. When necessary, the NADH was re-purified by ion exchange chromatography under anaerobic conditions (22). Re-purification was crucial for redox potential titrations and absolute rate measurements (for example, detection of NADH-induced H₂O₂ production by rotenone-inhibited complex I increased from 4.83 ± 0.04 to 5.20 ± 0.09 nmol min⁻¹ mg of protein⁻¹, but it did not affect comparative measurements or catalytic rate measurements. Unless stated otherwise, rates of NADH-induced H₂O₂ production reported here were determined using re-purified NADH. RET-induced H₂O₂ production was measured in 10 mM succinate, 1 mM ATP + MgSO₄ and 400 μM KCN; E_set was set by varying the fumarate concentration. 50 μM acetylated cyt c was used for the direct detection of superoxide production by complex I (ε = 18.0 mM⁻¹ cm⁻¹, 550–541 nm) (5, 23) in 30 μM NADH (NADH-induced) or in 10 mM succinate, 1 mM ATP + MgSO₄ and 400 μM KCN (RET-induced).

Catalase was from bovine liver (2–8 units ml⁻¹), and superoxide dismutase (SOD) was the CuZn enzyme from bovine erythrocytes (2 units ml⁻¹). SMP preparations were checked to make sure they did not have the ability to remove H₂O₂ and superoxide from solution and, thus, affect their detection; the rates of H₂O₂ and superoxide production by xanthine oxidase (Fluka, 0.94 miunits ml⁻¹ + 10 μM xanthine) (24) were measured in the presence and absence of SMPs (140 μg ml⁻¹). The SMPs made no significant difference to the detection (by the Amplex Red system) of the H₂O₂ produced (in the presence or absence of SOD), so they do not have any significant ability to remove H₂O₂. SMPs also did not affect the detection of standard concentrations of H₂O₂ added directly to assay solutions. In the presence of KCN, SMPs made no significant difference to the detection (by acetylated cyt c) of the superoxide produced, so they did not have any significant ability to remove superoxide from the solution either; they do not contain detectable SOD activity. The KCN was added to prevent the reoxidation of reduced cyt c by cytochrome c oxidase, a reaction that decreases detection of the superoxide.

**Inhibition of the Respiratory Chain Enzymes and Dissipation of Δp—Rotenone (23 μM) and piericidin A (10 μM) were used to inhibit complex I (>99% inhibition of NADH-O₂ oxidoreduction) and NADH-OH (2.5 μM, >99%) (25) and ADP-ribose (5 mM, >80%) (26) to competitively inhibit the complex I flavin site. Diphenyleneiodonium chloride (DPI) is a noncompetitive inhibitor that reacts irreversibly with the reduced flavin (27); the DPI concentration and incubation time were adjusted for the experimental conditions (high NADH concentrations prevent DPI from accessing the reduced flavin). Malonate (a competitive inhibitor of succinate oxidation, 20 mM, >99% inhibition of succinate:O₂ oxidoreduction) (28) and carboxin (20 μM) were used to inhibit complex II. Carboxin binds to the quinone site in complex II (29); its solubility in ethanolic stock solutions limits its concentration in the assay to 20 μM (~60% inhibition). All inhibitors were checked for artifacts in the Amplex Red assay; antimycin A, a Qi site inhibitor of complex III, was used at 500 nM (~94% inhibition of NADH-O₂ oxidoreduction) because higher concentrations interfere with the Amplex Red assay; myxothiazol was not used because it also inhibits complex I (11) and because it leads to artificial H₂O₂ detection. 400 μM KCN was used to inhibit cytochrome c oxidase (~98% inhibition of NADH-O₂ oxidoreduction); the KCN concentration was minimized to minimize HRP inhibition. Gramicidin (mixture of forms A, B, C, and D, 10–40 μg ml⁻¹ depending on the SMP concentration) was used to dissipate Δp. FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) was not used because its effect is concentration dependent; as the concentration increases, the rate of NADH-O₂ oxidoreduction first increases, but before a maximum rate is attained it decreases again.
Mechanism of Superoxide Production by Complex I

By including an NADH activation step in our preparation (see "Experimental Procedures"), our SMPs were optimized for a maximum rate of RET without requiring nucleotides to be added to the assay buffer to activate the deactive form of complex I in situ (15). In addition, we avoided stimulating RET by adding poorly defined and complicating couplers or activators, such as oligomycin (15) or fatty-acid-free bovine serum albumin (which interferes with both the ACMA and Amplex Red detection systems also) (15). Finally, all the catalytic activities in Table 1 are sensitive to the canonical respiratory chain inhibitors rotenone and piericidin A (complex I), malonate and carboxin (complex II), antimycin A (complex III), and cyanide (complex IV), and RET is abolished by uncoupling agents also.

NADH-induced Superoxide and H₂O₂ Production by Complex I in SMPs—Fig. 1 shows a set of experiments that monitored both NADH-induced H₂O₂ production and NADH oxidation by complex I in SMPs. The addition of NADH initiates H₂O₂ production. If catalysis (energy-transducing NADH:O₂ oxidoreduction) is prevented by rotenone (Fig. 1A), then H₂O₂ production is constant, although NAD⁺ decreases its rate significantly, to the catalase-insensitive background level observed upon the addition of a complex I flavin-site inhibitor such as DPI (see Table 2). Alternatively, during the catalytic conversion of NADH to NAD⁺, H₂O₂ production gradually slows (Fig. 1B); the effect was observed in both the presence of Δp and when Δp = 0. Importantly, the initial rates observed during catalysis match the rate observed when catalysis was inhibited (although the curvature makes them difficult to quantify). Therefore, H₂O₂ production is not stimulated by ubiquinone reduction at the Q-site, for example by the formation of a short-lived semiquinone intermediate (even in the presence of a substantial Δp), or by rotenone binding.

Observed rates of NADH-induced H₂O₂ production were linearly dependent on SMP concentration and were ~80% sensitive to catalase (see Table 2). They were stimulated <10% by SOD (see Table 2) because (as observed previously for purified complex I (5)) even in the absence of SOD the dismutation of superoxide to H₂O₂ is not rate-limiting. The catalase-insensitive rate matches the rates observed in DPI and high concentrations of NAD⁺ (see Table 2). It is decreased (but not abolished) in the absence of SMPs and abolished by the absence of HRP or NADH. Thus, it probably represents a direct interaction between NADH and the Amplex Red detection system (21).

Table 2 presents a side-by-side comparison of NADH-induced H₂O₂ and superoxide production by complex I inhibited by rotenone. Superoxide production rates were measured using the reduction of acetylated cyt c (5); Table 2 shows that there is a significant DPI-, NAD⁺-, and SOD-insensitive component. However, subtraction of the background rates (for both H₂O₂ and superoxide production) led to a ratio of 1.71 ± 0.12 superoxides per H₂O₂. The ratio is lower than 2 (the value expected if complex I only produces superoxide directly) for two reasons. First, the reduced flavin site in purified complex I was shown previously to convert up to 10% of the O₂ consumed directly to H₂O₂ (5). Second, the apparent rate of superoxide production by SMPs is decreased slightly by reoxidation of the reduced cyt


**Mechanism of Superoxide Production by Complex I**

### TABLE 2

Side-by-side comparison of measurements of H$_2$O$_2$ and superoxide production by a typical SMP preparation

<table>
<thead>
<tr>
<th>NADH-induced reactions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obs. value$^1$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Superoxide</td>
<td>19.1 ± 0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RET-induced reactions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obs. value$^1$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>Superoxide</td>
<td>2.97 ± 0.02</td>
</tr>
</tbody>
</table>

$^1$ The observed (Obs.) values are rates of resorufin formation or rates of acetylated cytochrome c reduction and are reported in nmol min$^{-1}$ mg of protein$^{-1}$. The conditions were 30 μM NADH and 23 μM rotenone (NADH-induced) and 10 mM succinate, 2 mM ATP + MgSO$_4$, and 400 μM KCN (RET-induced).

$^2$ Values are expressed as a percentage of the observed value. NAD$^+$: 1 μM NAD$^+$ was added to set the potential to −0.29 V; at this potential superoxide/H$_2$O$_2$ formation by the reduced flavin is minimized (5) (see text). Cat, catalase; Rot, rotenone.

$^3$ The corrected (Corr.) values (nmol min$^{-1}$ mg of protein$^{-1}$) are the observed values adjusted for the background rate (the background rates used here are the DPI insensitive rates). Note that the corrected value reported for NADH-induced H$_2$O$_2$ production is slightly lower than the value reported in the text because the NADH used here was not re-purified before use (see "Experimental Procedures").

$^4$ The complex I rate (min$^{-1})$ is the rate of superoxide or H$_2$O$_2$ production by complex I (the corrected value has been adjusted for the complex I concentration, vesicle orientation, and for RET, for the presence of uncoupled particles, see "Results").

$^5$ The ratios between the rates of superoxide and H$_2$O$_2$ production are 1.71 (NADH-induced) and 1.62 ± 0.46 (RET). Note that the addition of KCN to the NADH-induced superoxide measurements causes the ratio to increase slightly to ~1.9, because the (slow) reoxidation of reduced cytochrome c by cytochrome c oxidase is prevented (see "Results").

$^6$ The complex I rate (min$^{-1}$) is the rate of superoxide or H$_2$O$_2$ production by complex I (the corrected value has been adjusted for the complex I concentration, vesicle orientation, and for RET, for the presence of uncoupled particles, see "Results").

### FIGURE 2.

NADH-induced H$_2$O$_2$ production in the presence of various respiratory chain inhibitors, in the presence and absence of Δp and ΔpH. H$_2$O$_2$ production was induced by 30 μM NADH and measured using the HRP-Amplex Red system; Δp was imposed by ATP hydrolysis. ΔpH was increased using 20 mM KC1 or decreased using 20 mM KC1 and 2 mM nigericin (see "Results"). See "Experimental Procedures" for conditions.

---

c by cytochrome c oxidase. The addition of KCN prevents the reoxidation reaction, and the measured superoxide:H$_2$O$_2$ ratio increases to ~1.9 (note that this value also includes correction for a slow, superoxide-independent change to the cyt c spectrum caused by KCN). The data in Table 2 show that measuring H$_2$O$_2$ production by complex I is a reliable method of quantitating the total rate of superoxide and H$_2$O$_2$ production together and that the H$_2$O$_2$ results predominantly from superoxide dismutation. Most of the data presented here are measurements of rates of H$_2$O$_2$ production; thus, they are termed “H$_2$O$_2$ production” but include both the H$_2$O$_2$ produced by superoxide dismutation and the relatively small amount of H$_2$O$_2$ produced directly.

Fig. 2 (Δp = 0) shows that complex I produces H$_2$O$_2$ at the same rate in the presence of rotenone or piericidin A, inhibitors of ubiquinone reduction by complex I, but in the presence of DPI (27), ADP-ribose (6, 26) and NADH-OH (25), inhibitors of NADH oxidation, the rate is much lower. Antimycin A leads to a higher rate due to a known contribution from complex III (33), as does KCN (the KCN effect was abolished by carboxin so the extra H$_2$O$_2$ originates from complex II); both effects were abolished by rotenone.

Fig. 3 compares H$_2$O$_2$ production by isolated complex I in the absence of ubiquinone (5) with that by rotenone-inhibited SMPs (the most analogous condition), inhibitors of ubiquinone reduction by complex I, but in the presence of DPI (27), ADP-ribose (6, 26) and NADH-OH (25), inhibitors of NADH oxidation, the rate is much lower. Antimycin A leads to a higher rate due to a known contribution from complex III (33), as does KCN (the KCN effect was abolished by carboxin so the extra H$_2$O$_2$ originates from complex II); both effects were abolished by rotenone.

The rate of H$_2$O$_2$ production by rotenone-inhibited complex I in SMPs (5.20 ± 0.09 nmol of min$^{-1}$ mg of protein$^{-1}$) after correction for the NADH-OH insensitive rate; see Fig. 2) was adjusted for the orientation of the vesicles and the complex I content. First, the NADH/APAD$^+$ and cytochrome c:O$_2$ oxidoreductase activities were determined before and after solubilization of the SMPs with 0.5% dodecylmaltoside. In a typical experiment the NADH/APAD$^+$ oxidoreductase activity (a reaction catalyzed by the complex I flavin (35)) increased from 1.10 ± 0.01 to 1.30 ± 0.02 μmol of min$^{-1}$ mg of protein$^{-1}$, and the cytochrome c:O$_2$ oxidoreductase activity increased from 1.83 ± 0.01 to 8.37 ± 0.21 μmol min$^{-1}$ mg of protein$^{-1}$. There-
The potential of zero net rate, at which $E$ was varied (by varying the four substrate concentrations according to the Nernst equation), and the potential of zero net rate, at which $-2\Delta E = 4\Delta$ (each two-electron NADH oxidation translocates four protons) was identified. Fig. 4A presents a typical titration that gave $\Delta \varphi = 0.157 V$; $\Delta \varphi$ varied by less than 10 mV between preparations. Our value is similar to the $\Delta \varphi$ produced by ATP hydrolysis in mitochondria, which was reported to increase NADH-induced $H_2O_2$ production by rotenone- and piericidin A-inhibited complex I ($-0.15 V$) (11); clearly, our value is high enough that any $\Delta \varphi$-dependent effects on complex I observed in mitochondria should be observed here also. Fig. 2 ($\Delta \varphi = 0.16 V$) shows how $\Delta \varphi$ affects NADH-induced $H_2O_2$ production by SMPs in the presence of various inhibitors. With rotenone or piericidin A (to prevent quinone reduction by complex I directly) or KCN and carbinoxin (to prevent complex I turnover indirectly), $H_2O_2$ production is unaffected by $\Delta \varphi$. As expected, ADP-ribose and NADH-OH essentially abolish $H_2O_2$ production. In addition, NADH-induced $H_2O_2$ production from SMPs that had higher RCR values is independent of $\Delta \varphi$. Thus, these results provide no evidence to

**Mechanism of Superoxide Production by Complex I**

- **A.** The rate of NADH:fumarate oxidoreduction, recorded during ATP hydrolysis ($1M$ ATP + MgSO$_4$) to generate $\Delta \varphi$ and with 400 $\mu$M KCN to inhibit respiration depends on the difference between the NAD$^+$ and fumarate potentials, $\Delta E = -0.335 V + 0.020 - RT/2F\ln([NADH]/[fumarate])/([NAD$^+$]/[fumarate])$, set using [NADH] = 0.1 mM, [NAD$^+$] = 1 mM, [succinate] = 0.5 mM, and [fumarate] = 0.025–40 mM. The rate is 0 when $-2\Delta E = 4\Delta$. When $-2\Delta E$ is close to $4\Delta$, the data vary linearly with potential, but at high and low potential the rates are determined kinetically; the points with arrows are in principal at infinite potential ([NADH] = [fumarate] = 0 shown at $0.18 V$). Similar titrations varying the succinate, NAD$^+$, and NADH concentrations gave, overall, $\Delta \varphi = 0.16 \pm 0.005 V$. B, the fluorescence of ACMA (500 nm) is quenched upon the addition of $100 \mu$M ATP + MgSO$_4$ to SMPs (112 mg ml$^{-1}$) in varying KCN concentrations. 2 nm gramicidin (a K$^+$/H$^+$ exchanger) or $20 \mu$g ml$^{-1}$ gramicidin (a cation transport-per) abolish the quenching effect. Note that the ACMA fluorescence is affected by ATP (the final value varies). See “Experimental Procedures” for conditions.

**FIGURE 3. NADH-induced $H_2O_2$ production by complex I in SMPs with and without $\Delta \varphi$ compared with that by isolated complex I.** The three curves have been normalized independently. The data are plotted using $E_{\text{cat}} = -0.335 V - RT/2F\ln([NADH]/[NAD^+])$ (using $30 \mu$M NADH and variable NAD$^+$) and fit by the Nernst equation for a 2-electron cofactor with 2 distinct potentials described by their average value, $E_{av}$, and their separation, $\Delta E$. Blue, isolated complex I ($E_{av} = -0.353, \Delta E = 0.079 V$) (5). Green, complex I in SMPs inhibited by rotenone ($\Delta \varphi = 0 V$) ($E_{av} = -0.366, \Delta E = 0.079 V$). Black, complex I in SMPs inhibited by rotenone with $\Delta \varphi = 0.16 V$ (generated by ATP hydrolysis) ($E_{av} = -0.366, \Delta E = 0.060 V$). The NADH-OH independent rates, measured in NADH only (see Fig. 2), have been subtracted from the SMP data sets. See “Experimental Procedures” for conditions.

**FIGURE 4. Measurement of $\Delta \varphi$ in SMPs hydrolyzing ATP and evaluation of $\Delta \varphi$.** A, the rate of NADH:fumarate oxidoreduction, recorded during ATP hydrolysis ($1M$ ATP + MgSO$_4$) to generate $\Delta \varphi$ and with 400 $\mu$M KCN to inhibit respiration depends on the difference between the NAD$^+$ and fumarate potentials, $\Delta E = -0.335 V + 0.020 - RT/2F\ln([NADH]/[fumarate])/([NAD^+]/[fumarate])$, set using [NADH] = 0.1 mM, [NAD$^+$] = 1 mM, [succinate] = 0.5 mM, and [fumarate] = 0.025–40 mM. The rate is 0 when $-2\Delta E = 4\Delta$. When $-2\Delta E$ is close to $4\Delta$, the data vary linearly with potential, but at high and low potential the rates are determined kinetically; the points with arrows are in principal at infinite potential ([NADH] = [fumarate] = 0 shown at $0.18 V$). Similar titrations varying the succinate, NAD$^+$, and NADH concentrations gave, overall, $\Delta \varphi = 0.16 \pm 0.005 V$. B, the fluorescence of ACMA (500 nm) is quenched upon the addition of $100 \mu$M ATP + MgSO$_4$ to SMPs (112 mg ml$^{-1}$) in varying KCN concentrations. 2 nm gramicidin (a K$^+$/H$^+$ exchanger) or $20 \mu$g ml$^{-1}$ gramicidin (a cation transport-per) abolish the quenching effect. Note that the ACMA fluorescence is affected by ATP (the final value varies). See “Experimental Procedures” for conditions.
support the participation of a second (non-flavin) site of H$_2$O$_2$ production.

To investigate the effects on H$_2$O$_2$ production of modulating the ΔpH component of Δp, a fluorescent aminoacridine dye (ACMA) was used to evaluate ΔpH. The fluorescence of ACMA is quenched when it partitions into the SMP lumen in response to ΔpH (37, 38). Fig. 4B shows how the ACMA fluorescence is quenched by SMPs catalyzing ATP hydrolysis (NADH and succinate oxidation provide similar traces). The ATP concentration was kept to a minimum (100 μM ATP + MgSO$_4$) because ATP itself quenches the fluorescence. Under our standard conditions (0 mM KCl) the fluorescence is quenched by only ~20%, and ΔpH is relatively low. For perfectly sealed particles, transferring just a few protons into the lumen is sufficient to produce a large Δψ without affecting the internal pH, so Δp = Δψ and ΔpH = 0. However, when other ions cross the membrane they collapse Δψ (the impediment to proton uptake) so protons accumulate inside; Δψ is converted to ΔpH. Thus, permeant anions were added to the external solution to induce conversion of Δψ to ΔpH. Thiocyanate, an intrinsically membrane permeable anion, could not be used because it dramatically quenches the ACMA fluorescence directly. However, both chloride and nitrate gave similar results (37, 38); it is possible that they are conducted across the membrane by an anion transporter (39). Fig. 4B shows that chloride induces the formation of ΔpH with a rate and magnitude dependent on its concentration (37). Conversely, in the presence of K$^+$, ΔpH can be significantly decreased (perhaps abolished) by nigericin, a K$^+$/H$^+$ exchanger. 20 mM KCl (Δψ to ΔpH) and 20 mM KCl + 2 mM nigericin (ΔpH to Δψ) were chosen as standard conditions here because they do not affect Δp significantly (determined as in Fig. 4A), or decrease the rate of RET by more than 10%.

Importantly, ACMA provides only a qualitative measure of ΔpH; the extent of quenching is strongly dependent on the concentrations of both ACMA and SMPs, and our attempts to calibrate the response by soaking the SMPs in low pH buffers to create an artificial ΔpH were not successful. Furthermore, the simple volume/volume partition equation that has been applied previously (37) leads to a calculated ΔpH of 4.3 pH units in 120 mM KCl, a value that far exceeds Δp. Thus, we adopted a simple and pragmatic approach to estimate ΔpH; we assumed that the maximum quench, in 120 mM KCl, represents the limiting condition ΔpH = Δp = 0.157 V (2.6 pH units) and used a linear function (set to ΔpH = 0 in KCl and nigericin) to estimate ΔpH. Our standard conditions are, thus, ΔpH = 0.08 V and Δψ ~ 0.08 V (20 mM KCl) and ΔpH ~ 0 V and Δψ ~ 0.16 V (20 mM KCl + 2 mM nigericin). These ΔpH values are maximum estimates because RET cannot be observed in 120 mM KCl, so Δp is probably decreased, and Δψ may not be completely converted to ΔpH (40). Fig. 2 shows that altering the balance between ΔpH and Δψ while keeping Δp and the external pH constant does not affect NADH-induced H$_2$O$_2$ production by complex I.

H$_2$O$_2$ Production during RET—RET-induced H$_2$O$_2$ production was quantified in the absence of NAD$^+$ using nucleotide-free SMP preparations because bound nucleotides prevent O$_2$ access, NAD$^+$ reacts with the reduced flavin, and NADH formation leads to NADH-induced H$_2$O$_2$ production by poorly coupled particles. The observed rate of RET-induced H$_2$O$_2$ production (with Δp = 0.16 V, sufficient for substantial NAD$^+$ reduction; see Table 1) was low in comparison to the NADH-induced rate, only 0.35 ± 0.11 μmol min$^{-1}$ mg of protein$^{-1}$. Table 2 shows that the H$_2$O$_2$ detected was not affected significantly by SOD (superoxide dismutation is not rate-limiting) and that it was ~80% sensitive to catalase. However, only around half of the observed rate is DPI- and rotenone-sensitive (see Table 2 and Fig. 5), indicating that the actual rate of RET-induced H$_2$O$_2$ production by complex I is only 0.18 ± 0.06 μmol min$^{-1}$ mg of protein$^{-1}$. The rotenone-insensitive contribution does not depend on Δp, but it is sensitive to malonate (therefore, it originates at complex II and/or complex III). Importantly, the rotenone-sensitive contribution is essentially abolished by the flavin site inhibitors NADH-OH, ADP-ribose, and DPI, observations that are consistent only with RET-induced H$_2$O$_2$ production being from the flavin site in complex I. Table 2 shows also that RET-induced superoxide production corresponds to H$_2$O$_2$ production (within the error of the measurements and taking into account the high background rates of cytochrome c reduction). Therefore, during RET, complex I produces predominantly superoxide, and (as for NADH-induced H$_2$O$_2$ production) the rates of H$_2$O$_2$ detection described include both the H$_2$O$_2$ formed by superoxide dismutation and the H$_2$O$_2$ produced directly.

Two known effects decrease the rate of RET-induced H$_2$O$_2$ production in our experiments. First, because we exclude nucleotides from our experiments, not all of the complex I is in the active form. Second, due to variations in their size, integrity, and enzyme distribution, individual SMPs within a population sustain different Δp values; Δp determined as in Fig. 4A is a weighted average. Only some SMPs sustain a high enough Δp to reduce the flavin in complex I, and only those SMPs are able to reduce NAD$^+$ or O$_2$. Therefore, RET was performed in the absence of NAD$^+$ and in the presence of DPI (typically ~14 μg ml$^{-1}$ SMPs and 4 μM DPI, 32°C, 2 min) to specifically modify any reduced flavin formed (27) and inactivate only those complexes I that are RET-competent; no NAD$^+$ reduction could be observed after DPI treatment, and increasing the DPI concentration or incubation time did not affect the results. Then, rotenone was added,
and the rate of NADH:APAD\(^+\) oxidoreduction was compared with that from a control sample. In a typical preparation the rate decreased from 1.103 ± 0.007 to 0.975 ± 0.024 μmol min\(^{-1}\) mg of protein\(^{-1}\), a decrease of 11.6%; only ~10% of the complex I present is RET-competent. Correcting the rate of RET-induced \(\text{H}_2\text{O}_2\) production by complex I accordingly gives 1.51 ± 0.47 nmol min\(^{-1}\) mg of protein\(^{-1}\), or ~20 min\(^{-1}\). Thus, RET-induced \(\text{H}_2\text{O}_2\) production is approximately three times slower than NADH-induced \(\text{H}_2\text{O}_2\) production. Similarly, the corrected rate of RET-induced APAD\(^+\) reduction is approximately three times slower than the rate of NADH-induced APAD\(^+\) reduction. It is unlikely that the difference arises from kinetic limitations (APAD\(^+\) reduction is significantly faster than \(\text{O}_2\) reduction) but possible that the different mechanisms of flavin reduction (in forward and reverse) produce slightly different reduced states or that the positively charged DPI enhances the likelihood of flavin reduction. Changing the balance between \(\Delta p\) and \(\Delta p\) (using 20 mM KCl with or without nigericin, see above) while maintaining the same \(\Delta p\) and external pH, does not affect RET-induced \(\text{H}_2\text{O}_2\) production by complex I (see Fig. 5).

Finally, Fig. 6 compares the potential dependence of RET-induced \(\text{H}_2\text{O}_2\) production by complex I in SMPs with that from NADH-induced \(\text{H}_2\text{O}_2\) production in the presence of rotenone. The combination of \(\Delta p\) and the ubiquinone pool potential, imposed by succinate oxidation, provides a highly reducing potential, below ~0.4 V. The RET- and NADH-induced data match well, except that the RET-induced dataset is shifted by 0.048 V relative to the NADH-induced dataset and broadened slightly. The data are shifted because the value of \(\Delta p\) used to calculate the set potentials during RET is an average over the whole population of SMPs, not just over those (with the highest \(\Delta p\) values) that are active in the RET assays. The two datasets can be overlaid by increasing the \(\Delta p\) value used to calculate the set potentials during RET by 0.048/2 V, indicat
Mechanism of Superoxide Production by Complex I

countering dependence from isolated complex I (6) with an estimated 1 mM NADH when the NAD$^+$ pool is reduced, gives \( \sim 11 \text{ min}^{-1} \) for the SMPs. Given the estimations and approximations inherent to the comparison, the two values (SMPs 11 min$^{-1}$, 32 °C and mitochondria 17 min$^{-1}$, 37 °C) are fully consistent (although NADH-dependent contributions from matrix-localized dehydrogenases cannot be excluded (44, 45)). Conversely, during active RET (while NAD$^+$ is being reduced), the level to which the flavin is reduced is influenced by \( \Delta p \) and the ubiquinone potential as well as by the NAD$^+$ potential; the level of flavin reduction is not determined by the NAD$^+$ potential alone, so the NAD$^+$/NADH ratio and superoxide production need not correlate. Fluorescence measurements performed on mitochondria during RET have suggested that superoxide production is low as NAD(P)H accumulates, only becoming high once a constant level is established (13, 46). This observation is difficult to reconcile with superoxide production by a catalytic intermediate but consistent with RET-induced superoxide production being determined by the NAD$^+$ pool composition.

Brand and Lambert (10–12) proposed, from studies on intact mitochondria, that appropriate Q-site inhibitors “turn on” NADH-induced superoxide production by a semiquinone intermediate in complex I. Here, the effects of rotenone, piericidin A, and cyanide are all the same; they all simply inhibit NADH oxidation. In mitochondria, complex I Q-site inhibitors increase NADH-induced superoxide production (by preventing NADH oxidation and lowering the NAD$^+$ potential) but abolish RET-induced superoxide production (11, 12), consistent with superoxide production on the “NADH side” of the inhibitor binding site but not with an inhibitor-sensitive semiquinone. Furthermore, DPI, a characterized flavin site inhibitor (27), blocks RET-driven superoxide production in mitochondria (47, 48) as it does in SMPs.

It has been reported that \( \Delta p \) stimulates superoxide production by complex I in mitochondria, particularly during RET (10–12, 49). Notably, Lambert and Brand (11, 12) reported that \( \Delta p \) increases the apparent rate of RET-induced superoxide production (measured as H$_2$O$_2$) from \( \sim 0.4 \) nmol of H$_2$O$_2$ min$^{-1}$ mg of protein$^{-1}$ (equal to the NADH-induced rate) to \( \sim 2.5 \) nmol of H$_2$O$_2$ min$^{-1}$ mg of protein$^{-1}$, although the effect applies equally to NAD$^+$ reduction (at the flavin). Conversely, in SMPs, both NADH- and RET-induced superoxide production are independent of \( \Delta p \), ruling out a direct effect of \( \Delta p \) on complex I itself. Importantly, SMPs allow the pH at the flavin site to be controlled precisely, independently of \( \Delta p \), but in mitochondria the matrix pH can only be inferred from \( \Delta p \), and abolishing \( \Delta p \) must decrease the matrix pH. Thus, we propose that the \( \Delta p \) effect is mediated by the matrix pH; it is known that NADH-induced superoxide production by isolated complex I increases significantly as the pH increases (5). pH-dependent kinetic and thermodynamic effects (particularly substrate and enzyme potentials) and other matrix dehydrogenases (44, 45) probably contribute also. Selivanov et al. (50) have suggested also that the apparent effects of \( \Delta p \) originate from increases in mitochondrial superoxide production as the matrix pH increases. Finally, reported rates of RET-induced superoxide production in mitochondria vary widely (summarized in Ref. 41), but they are generally considered to exceed rates of rotenone-inhibited NADH-induced superoxide production. The NAD$^+$ pool is highly reduced in both cases, but variations in the NAD$^+$ and NADH concentrations (7, 8) and especially differences in the redox status of the glutathione pool and the antioxidant capacity (42, 46) are further variables that determine the observed rates of superoxide production by both complex I and other enzymes (44, 45).

In summary, the flavin-site mechanism is sufficient to explain all extant data from studies of NADH- and RET-induced superoxide production by isolated complex I and complex I in SMPs, and these studies provide no support for any additional site. Interpreting the results of studies on isolated mitochondria is more challenging. First, although our mechanism should apply under all conditions, its direct application requires a more precise knowledge of the conditions (pH, nucleotide concentrations) that are relevant to each mitochondrial metabolic state than is currently available. Second, H$_2$O$_2$ detection in the mitochondrial systems depends on the balance between the intrinsic rate of superoxide production and the rates of detoxification (46). Finally, other matrix dehydrogenase enzymes may contribute to superoxide and H$_2$O$_2$ production by mitochondria, and the activity of complex I may be regulated or modified by additional effects such as protein modifications. Thus, the complex I flavin-site mechanism explains many of the characteristics of superoxide and H$_2$O$_2$ production by isolated mitochondria, and a more complete description will require a better understanding of both the conditions in the mitochondria, and identification and characterization of additional effects and contributions that are present in mitochondria but absent from the experimental system used here.

Acknowledgments—We thank Alexander B. Kotlyar and Gary Cecchini for NADH-OH, Martin S. King for isolating complex I, Michael P. Murphy for helpful discussions, and Aleksandra Włodek for EPR spectroscopy.

REFERENCES

Superoxide Is Produced by the Reduced Flavin in Mitochondrial Complex I: A SINGLE, UNIFIED MECHANISM THAT APPLIES DURING BOTH FORWARD AND REVERSE ELECTRON TRANSFER
Kenneth R. Pryde and Judy Hirst

J. Biol. Chem. 2011, 286:18056-18065. doi: 10.1074/jbc.M110.186841 originally published online March 10, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.186841

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 15 of which can be accessed free at http://www.jbc.org/content/286/20/18056.full.html#ref-list-1