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 ($\Delta p$) also. Here, we use bovine heart submitochondrial particles (SMPs) 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 $\Delta p$. Reverse electron transfer (RET) through complex I in SMPs, driven by succinate oxidation and the $\Delta p$ created by ATP hydrolysis, reduces the flavin, leading to $\text{NAD}^+$ and $\text{O}_2$ 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 $\Delta p$), 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, and establishes a route to understanding causative connections between the enzyme and its pathological effects, and to developing rational strategies for addressing them.
slow, rate-determining step to form superoxide (and \( \text{H}_2\text{O}_2 \)); 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 \( \text{H}_2\text{O}_2 \) diffusing out of the mitochondria) by a mechanism involving an iron-sulfur cluster that is adjacent to the ubiquinone binding site (Q-site), and/or ubisemiquinone intermediates (reviewed in (2,10)). Originally, the observation that complex I Q-site inhibitors such as rotenone increase \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \Delta p \) (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 \( \text{H}_2\text{O}_2 \) production from the flavin that should not be exceeded during RET (13). Second, \( \Delta p \) has two components that represent the difference in charge (\( \Delta \psi \)) and pH (\( \Delta p \)) on each side of the membrane. In mitochondria, their relative contributions can be manipulated, and, during both NADH oxidation and RET, \( \text{H}_2\text{O}_2 \) production appears to accelerate as \( \Delta p \) 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 \( \Delta p \) (12).

Here, we use submitochondrial particles (SMPs, inverted membrane vesicles) from *B. taurus* heart mitochondria to bridge the gap between studies of superoxide and \( \text{H}_2\text{O}_2 \) production on the isolated enzyme and on intact mitochondria. Like intact mitochondria, our SMPs sustain a substantial \( \Delta p \) during energy-linked forward (NADH or succinate:O\(_2\) oxidoreduction) electron transport, and they catalyze \( \Delta p \)-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 \( \text{H}_2\text{O}_2 \) detection are unaffected by the mitochondrial antioxidant defenses. Our results define an integrated and unified mechanism for superoxide and \( \text{H}_2\text{O}_2 \) 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-Aldrich, unless otherwise stated.

*The preparation of SMPs from B. taurus heart mitochondria* — Mitochondria were prepared from *B. taurus* heart tissue as described previously (14), and stored as ~5 g pellets at \(-20 \, ^\circ\text{C}\). A single pellet was thawed overnight at \(4 \, ^\circ\text{C}\), resuspended in 10 mM Tris-SO\(_4\), 250 mM sucrose at pH \( 7.0 \) (final volume 120 mL, pH corrected at \(20 \, ^\circ\text{C}\), buffer A), and refrozen. 40 mL of suspension (thawed at \(4 \, ^\circ\text{C}\) ) were centrifuged (11,300 g, 12 min., 4 °C), the dark red supernatant was discarded and the pellet resuspended in buffer A to 40 mL; this step helps remove saturated fats, as well as soluble proteins. The pH was adjusted to 9 (15) on ice by the dropwise addition of 2.5 M Tris, and the sample incubated on ice for 15 min., then recentrifuged (37,900 g, 12 min., 4 °C); this step aims to dissociate the inhibitor protein, II\(_{1}\), from ATP synthase (16). The supernatant was discarded and the pellet twice resuspended in buffer A to 40 mL and recentrifuged (11,300 g, 12 min., 4 °C).
Then, 10 mM MgSO₄ was added because it improves the respiratory control ratio (RCR) values of the preparation (17,18) and the sample sonicated on ice (Sonicator 3000, Misonix, New York, 19 mm probe, ten 15 s bursts with 1 min. intervals, 150 W). The sonicated material was centrifuged (27,100 g, 20 min., 4 °C), and the pellet discarded. Then, 1 mM NADH was added and the sample incubated for 1 hour on ice; this step aims to activate any complex I which has undergone the ‘deactive’ transition (15). The SMPs were collected by centrifugation (82,000 g, 30 min., 4 °C), then twice resuspended to 4 mL in buffer A and recentrifuged, to remove the nucleotides. Finally, the SMPs were resuspended to ~7 mg protein mL⁻¹ (determined using the Pierce bicinchoninic acid (BCA) assay) and stored as aliquots at ~−20 °C. The yield was typically ~30 mg protein of SMPs.

**Catalytic activity measurements** — All kinetic measurements were performed in 10 mM Tris-So₄ and 250 mM sucrose at pH 7.5, 32 °C, in an Ocean Optics DH-2000-BAL diode array spectrometer, a Molecular Devices spectramax plus 384 plate reader, or a Shimadzu RF-5301 PC spectrofluorometer.

NADH:O₂ oxidoreduction (100 µM NADH) and NADH:fumarate oxidoreduction (100 µM NADH, 40 mM fumarate, 400 µM KCN) were followed at 340–380 nm (ε_{NADH} = 4.81 mM⁻¹ cm⁻¹). Succinate:O₂ oxidoreduction (20 mM 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 (PEP, 200 µM) to pyruvate by pyruvate kinase (40 µg mL⁻¹), then pyruvate reduction was coupled to NADH oxidation (200 µM) by lactate dehydrogenase (50 µg mL⁻¹). Rotenone was used to prevent NADH oxidation, and the reaction was initiated with 200 µM ATP+MgSO₄ 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, 10 mM succinate, 1 mM NAD⁺, 1 mM ATP+MgSO₄, 400 µM KCN) 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, hexaamminemethenium (HAR) and APAD⁺ oxidoreduction (100 µM NADH, 1 mM ferricyanide, 3.5 mM HAR, 1 mM APAD⁺, rotenone) were followed as described previously (6). Cytochrome c:O₂ oxidoreduction (100 µM 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 cyt c (see below). The fluorescence of 500 nM amino-6-chloro-2-methoxyacridine (ACMA) was measured, using excitation and emission wavelengths of 435 and 577 nm, respectively, typically in the presence of ~100 µg protein mL⁻¹ SMPs.

**H₂O₂ and superoxide production measurements** — H₂O₂ production was quantified using the horseradish peroxidase (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, 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 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 cytochrome c (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 U mL⁻¹), and superoxide dismutase (SOD) was the CuZn enzyme from bovine erythrocytes (2 U mL⁻¹). SMP preparations were checked to make sure they
did not have the ability to remove H$_2$O$_2$ and superoxide from solution and thus affect their detection; the rates of H$_2$O$_2$ and superoxide production by xanthine oxidase (Fluka, 0.94 nU 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 assay solutions). In the presence of KCN, SMPs contain detectable SOD activity. The KCN was superoxide from the solution either; they do not they do not have any significant ability to remove H$_2$O$_2$. SMPs also did not affect the detection of standard concentrations of H$_2$O$_2$ added directly to assay solutions. In the presence of KClN, SMPs made no significant difference to the detection (by acetylated cyt c) of the superoxide produced, so they do not have any significant ability to remove superoxide from the solution either; they do not contain detectable SOD activity. The KClN 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:O$_2$ 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 non-competitive 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:O$_2$ 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 artefacts in the Amplex Red assay: antimycin A, a Q$_1$ site inhibitor of complex III, was used at 500 nM (~94% inhibition of NADH:O$_2$ 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 artefactual H$_2$O$_2$ detection. 400 μM KCN was used to inhibit cytochrome c oxidase (~98% inhibition of NADH:O$_2$ oxidoreduction); the KCN concentration was minimized to minimize HRP inhibition. Gramicidin (mixture of forms A, B, C and D, 10 to 40 μg mL$^{-1}$ depending on the SMP concentration) was used to dissipate Δp. FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhyrazzone) was not used because its effect is concentration dependent: as the concentration increases the rate of NADH:O$_2$ oxidoreduction first increases, but before a maximum rate is attained it decreases again.

**Results**

**SMPs as a model system for studying energy transduction in the inner mitochondrial membrane** — Table 1 summarizes the kinetic properties of a typical SMP preparation. NADH:O$_2$ and succinate:O$_2$ oxidoreduction, and ATP hydrolysis, are all energy-conserving reactions (coupled to proton translocation), so their rates increase significantly when Δp is dissipated. NADH:fumarate oxidoreduction is also energy conserving, but it is probably limited kinetically by quinol:fumarate oxidoreduction. Both the reaction rates and their RCRs (the ratio of the rates in the presence and absence of Δp, that reflect the ability of the system to work against Δp, as well as how well the vesicles are sealed) are comparable to values described previously (see for example (15,30)), and consistent with a population of catalytically active, well-coupled particles.

Importantly, both ATP hydrolysis and NADH:fumarate oxidoreduction can be ‘reversed’ by using Δp to drive them away from equilibrium. Initial experiments showed that NADH oxidation can drive ATP synthesis to more than 90% completion (quantified by the luciferase assay, 200 μM ADP, 10 mM KPi, 10 mM MgSO$_4$, 70 μg mL$^{-1}$ IF$_1$ (31)), showing that NADH oxidation supports a substantial Δp. NAD$^+$ reduction driven by succinate oxidation (to reduce the ubiquinone pool) and ATP hydrolysis (to create Δp) proceeds by RET through complex I, and the fact that it achieves a significant rate (see Table 1) demonstrates that a substantial Δp is sustained (32). 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 H$_2$O$_2$ production by complex I in SMPs** — Figure 1 shows a set of experiments that monitored both NADH-induced H$_2$O$_2$ production and NADH oxidation by complex I in SMPs. The addition of NADH initiates H$_2$O$_2$ production. If catalysis (energy-transducing NADH:O$_2$ oxidoreduction) is prevented by rotenone (Figure 1A) then H$_2$O$_2$ production is constant, although NAD$^+$ decreases its rate significantly, to the catalase-insensitive background level observed upon addition of a complex I flavin-site inhibitor such as DPI (see Table 2). Alternatively, during the catalytic conversion of NADH to NAD$^+$, H$_2$O$_2$ production gradually slows (Figure 1B); the effect is observed in both the presence of $\Delta$p, and when $\Delta$p = 0. Importantly, the initial rates observed during catalysis match the rate observed when catalysis is inhibited (although the curvature makes them difficult to quantify). Therefore, H$_2$O$_2$ 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 $\Delta$p), or by rotenone binding.

Observed rates of NADH-induced H$_2$O$_2$ production were linearly dependent on SMP concentration and were $\sim$80% sensitive to catalase (see Table 2). They were stimulated <10% by SOD (see Table 2), because (as observed previously for purified complex I (5)) even in the absence of SOD, the dismutation of superoxide to H$_2$O$_2$ 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 H$_2$O$_2$ and superoxide production by complex I inhibited by rotenone. Superoxide production rates were measured using the reduction of acetylated cytochrome c (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 H$_2$O$_2$ and superoxide production), leads to a ratio of 1.71 $\pm$ 0.12 superoxides per H$_2$O$_2$. 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 O$_2$ consumed directly to H$_2$O$_2$ (5). Second, the apparent rate of superoxide production by SMPs is decreased slightly by reoxidation of the reduced cyt c by cytochrome c oxidase. The addition of KCN prevents the reoxidation reaction, and the measured superoxide:H$_2$O$_2$ ratio increases to $\sim$1.9 (note that this value also includes correction for a slow, superoxide-independent change to the cyt c spectrum caused by KCN). The data in Table 2 show that measuring H$_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.

Figure 2 ($\Delta$p = 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.

Figure 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), as a function of the NAD$^+$ potential. Because the flavin reacts much more rapidly with NADH and NAD$^+$ than with O$_2$, the curves in Figure 3 are essentially redox titrations
of the flavin cofactor (5). The data from the two systems match closely, and the values determined for the flavin’s reduction potential (−0.353 V (isolated complex I) and −0.363 V (SMPs, Δp = 0)) are close to the independent value from EPR (−0.365 V) (34). These data confirm that the flavin-site mechanism described previously for NADH-induced H$_2$O$_2$ production by isolated complex I (5,6) applies equally to complex I in SMPs.

The rate of H$_2$O$_2$ production by rotenone-inhibited complex I in SMPs (5.20 ± 0.09 nmol min$^{-1}$ mg protein$^{-1}$, following correction for the NADH-OH insensitive rate, see Figure 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 min$^{-1}$ mg protein$^{-1}$, and the cytochrome c:O$_2$ oxidoreductase activity from 1.83 ± 0.01 to 8.37 ± 0.21 µmol min$^{-1}$ mg protein$^{-1}$. Therefore, the SMP preparations used are 81 ± 5% in the N-side out orientation (reversed with respect to mitochondria). Second, two methods were used to show that complex I comprises approximately 10% of the total protein in SMPs. First, the intensities of the bands observed in Western blots of the 24 kDa and 51 kDa complex I subunits, from a range of SMP and complex I concentrations, were compared, indicating that complex I comprises 9.1 ± 2.6% of the total protein. The proteins were separated by SDS-PAGE, then transferred to nitrocellulose membranes, labeled with polyclonal antibodies raised against the overexpressed B. taurus subunits, and detected by enhanced chemiluminescence. Second, comparison of the intensities of the g$_z$ components of the N2 EPR signal (36) exhibited by samples reduced by 10 mM NADH and inhibited by 10 µM piericidin A, from intact complex I and SMPs, indicated that 10.1 ± 0.5% of the protein present in SMPs is complex I. Correcting for the orientation and amount of complex I present (~9.6% of the total protein) gives a turnover rate for NADH-induced H$_2$O$_2$ production (per complex I) of ~64 min$^{-1}$. The value (per complex I) is approximately twice as high as the corresponding value from isolated complex I (5,6), but the rates of three other flavin-catalyzed reactions (6), the NADH:ferricyanide, NADH:APAD$^+$ and NADH:hexaammineruthenium oxidoreductase reactions, are all approximately twice as high in SMPs, compared to isolated complex I, also. The reason for the difference is not known, but it is likely that SMPs are the most physiologically relevant, because isolating the complex I from SMPs decreased its activity to that of the directly purified enzyme.

The effects of Δp and ΔpH on NADH-induced H$_2$O$_2$ production — Figure 1B indicated that Δp does not affect the rate of NADH-induced H$_2$O$_2$ production when it is generated by catalytic NADH oxidation. Alternatively, the Δp generated by ATP hydrolysis is independent of the respiratory chain. To quantify the Δp from ATP hydrolysis, the potential for NADH:fumarate oxidoreduction (ΔE) was varied (by varying the four substrate concentrations, according to the Nernst equation) and the potential of zero net rate, at which −2ΔE = 4Δp (each two-electron NADH oxidation translocates four protons) was identified. Figure 4A presents a typical titration that gave Δp = 0.157 V; Δp varied by less than 10 mV between preparations. Our value is similar to the Δp produced by ATP hydrolysis in mitochondria, that was reported to increase NADH-induced H$_2$O$_2$ production by rotenone- and piericidin A-inhibited complex I (~0.15 V) (11); clearly, our value is high enough that any Δp-dependent effects on complex I observed in mitochondria should be observed here also. Figure 2 (Δp = 0.16 V) shows how Δp affects NADH-induced H$_2$O$_2$ 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 carboxin (to prevent complex I turnover indirectly), H$_2$O$_2$ production is unaffected by Δp. As expected, ADP-ribose and NADH-OH essentially abolish H$_2$O$_2$ production. In addition, NADH-induced H$_2$O$_2$ production from SMPs that had higher RCR values because they were not activated by NADH during the preparation (see Table 1) was quantified, both in the presence and absence of complex I Q-site inhibitors; the results were fully consistent with the results from our standard preparations. Finally, Figure 3 shows that the potential dependence of NADH-induced H$_2$O$_2$ production from rotenone-inhibited SMPs is
independent of Δp. Thus, these results provide no evidence to support the participation of a second (non-flavin) site of \( \text{H}_2\text{O}_2 \) production.

To investigate the effects on \( \text{H}_2\text{O}_2 \) 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). Figure 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) and 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). Figure 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 nM nigericin (ΔpH to Δψ) were chosen as standard conditions here, because they do not affect Δp significantly (determined as in Figure 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 nM 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). Figure 2 shows that altering the balance between ΔpH and Δψ, while keeping Δp and the external pH constant, does not affect NADH-induced \( \text{H}_2\text{O}_2 \) production by complex I.

\( \text{H}_2\text{O}_2 \) production during RET — RET-induced \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) production by poorly coupled particles. The observed rate of RET-induced \( \text{H}_2\text{O}_2 \) 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 protein\(^{-1}\). Table 2 shows that the \( \text{H}_2\text{O}_2 \) 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 Figure 5), indicating that the actual rate of RET-induced \( \text{H}_2\text{O}_2 \) production by complex I is only 0.18 ± 0.06 μmol min\(^{-1}\) mg 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 \( \text{H}_2\text{O}_2 \) production being from the flavin site in complex I. Table 2 shows also that RET-induced superoxide production corresponds to \( \text{H}_2\text{O}_2 \) production (within the error of the measurements, and taking into account the high background rates of cyt c
reduction). Therefore, during RET complex I produces predominantly superoxide — and (as for NADH-induced H_2O_2 production) the rates of H_2O_2 detection described include both the H_2O_2 formed by superoxide dismutation, and the H_2O_2 produced directly.

Two known effects decrease the rate of RET-induced H_2O_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 Figure 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 O₂. Therefore, RET was performed in the absence of NAD⁺, 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 which are RET competent; no NAD⁺ reduction could be observed following 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 to that from a control sample. In a typical preparation the rate decreased from 1.103 ± 0.007 to 0.975 ± 0.024 µmoles min⁻¹ mg protein⁻¹, a decrease of 11.6%; only ~10% of the complex I present is RET-competent. Correcting the rate of RET-induced H_2O_2 production by complex I accordingly gives 1.51 ± 0.47 nmol min⁻¹ mg protein⁻¹, or ~20 min⁻¹. Thus, RET-induced H_2O_2 production is approximately three times slower than NADH-induced H_2O_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 O₂ 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 Δψ and ΔpH (using 20 mM KCl, with or without nigericin, see above), while maintaining the same Δp and external pH, does not affect RET-induced H_2O_2 production by complex I (see Figure 5).

Finally, Figure 6 compares the potential dependence of RET-induced H_2O_2 production by complex I in SMPs, with that from NADH-induced H_2O_2 production in the presence of rotenone. The combination of Δp 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 Δp used to calculate the set potentials during RET is an average over the whole population of SMPs, not just over those (with the highest Δp values) that are active in the RET assays. The two datasets can be overlaid by increasing the Δp value used to calculate the set potentials during RET by 0.048/2 V, indicating that the subpopulation of RET-competent SMPs exhibit an average Δp of 0.182 V. The broadening may reflect the absence of nucleotides in the RET experiment; as discussed previously (5) nucleotide binding affects the apparent titration curve of the flavin. Importantly, the RET-induced data vary over a region of potential that is consistent with the potential of the flavin in complex I (not with the potentials of any of the other cofactors (1), or with the potential dependence of a semiquinone). All these results are fully consistent with RET-induced H_2O_2 production being only from the reduced flavin in complex I.

Discussion

A unified mechanism for superoxide production by complex I under all conditions, during both forward and reverse electron transfer — When ubiquinone reduction is prevented, and Δp = 0, complex I in SMPs behaves like isolated complex I (5): superoxide production is from the reduced flavin and determined by the NAD⁺ and NADH concentrations. [Note that, for simplicity, we refer to superoxide production in our discussion, as it is the major species produced directly by complex I]. When ubiquinone reduction is allowed, superoxide production slows in response to NADH oxidation; Q-site inhibitors do not stimulate it, but flavin-site inhibitors prevent it. No change in the behavior is
observed when $\Delta p \sim 0.16$ V, or when $\Delta p H$ is manipulated (at constant $\Delta p$ and external $pH$). Therefore, there is no evidence for NADH-induced superoxide production from any site other than the reduced flavin. RET-induced superoxide production by complex I in SMPs is slower than NADH-induced superoxide production, though much of the difference can be attributed to heterogeneity in the SMPs, particularly to variation in $\Delta p$. The heterogeneity is a disadvantage of the SMP system: it can be described and taken into account, but not fully characterized, and it offsets both the kinetics and thermodynamics of RET experiments. RET-induced superoxide production has a potential dependence consistent with the reduced flavin (not with a semiquinone), it is abolished by both Q-site and flavin-site inhibitors, and it is unaffected by manipulating $\Delta p H$ (at constant $\Delta p$ and external $pH$). All the results are fully consistent with RET-induced superoxide production from the reduced flavin in complex I and they provide no support for any additional site.

**Comparison of data from SMPs and intact mitochondria** — The response of NADH-induced superoxide production to the NAD$^+$ potential in the matrix has been observed in intact mitochondria, either by poising the NAD$^+$ potential (7) or by correlating the NAD(P)H fluorescence to superoxide production under a range of conditions (8,13) (though fluorescence measurements evaluate the relative (NADH+NADPH) concentration, not the NAD$^+$ potential or absolute NADH concentration). The maximal rate of NADH-induced superoxide production by mitochondria (when complex I is inhibited and the NAD$^+$ pool is reduced) is typically equivalent to 0.4 nmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ (summarized in (41)). Correcting for the mitochondrial antioxidant defenses (42) raises the value to $\sim$1 nmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$, and the fact that $\sim$6% of the protein in *B. taurus* heart mitochondria is complex I (determed by comparison of the N2 EPR signal intensities in mitochondria (disrupted by either freezing or sonication and reduced by NADH) and an isolated complex I standard) gives a turnover number of $\sim$17 min$^{-1}$. Finally, O$_2$ reduction by the reduced flavin is blocked by nucleotide binding, most strongly by NADH (6,43), and using the NADH-concentration dependence from isolated complex I (6), with an estimated 1 mM NADH when the NAD$^+$ pool is reduced, gives $\sim$11 min$^{-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 (though 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 coworkers proposed, from studies on intact mitochondria, that appropriate Q-site inhibitors ‘turn on’ NADH-induced superoxide production by a semiquinone intermediate in complex I (10-12). 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 $\Delta p H$ stimulates superoxide production by complex I in mitochondria, particularly during RET (10-12,49). Notably, Lambert and Brand reported that $\Delta p H$ increases the apparent rate of RET-induced superoxide production (measured as H$_2$O$_2$) from $\sim$0.4 nmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ (equal to the NADH-induced rate) to $\sim$2.5 nmol H$_2$O$_2$ min$^{-1}$ mg
protein$^1$ — though the effect applies equally to NAD$^+$ reduction (at the flavin) (11,12). Conversely, in SMPs, both NADH- and RET-induced superoxide production are independent of $\Delta$pH, ruling out a direct effect of $\Delta$pH on complex I itself. Importantly, SMPs allow the pH at the flavin site to be controlled precisely, independently of $\Delta$pH, but in mitochondria the matrix pH can only be inferred from $\Delta$pH, and abolishing $\Delta$pH must decrease the matrix pH. Thus, we propose that the $\Delta$pH 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 and coworkers have suggested also that the apparent effects of $\Delta$pH originate from increases in mitochondrial superoxide production as the matrix pH increases (50). Finally, reported rates of RET-induced superoxide production in mitochondria vary widely (summarized in (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) which 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 better understanding of both the conditions in the mitochondrion, and identification and characterisation of additional effects and contributions that are present in mitochondria, but absent from the experimental system used here.

References

1. Hirst, J. (2010) Biochem. J. 425, 327-339
2. Murphy, M. P. (2009) Biochem. J. 417, 1-13
3. Raha, S., and Robinson, B. H. (2000) Trends Biochem. Sci. 25, 502-508
4. Lin, M. T., and Beal, M. F. (2006) Nature 443, 787-795
5. Kussmaul, L., and Hirst, J. (2006) Proc. Natl. Acad. Sci. USA 103, 7607-7612
6. Birrell, J. A., Yakovlev, G., and Hirst, J. (2009) Biochemistry 48, 12005-12013
7. Kushnareva, Y., Murphy, A. N., and Andreyev, A. (2002) Biochem. J. 368, 545-553
8. Starkov, A. A., and Fiskum, G. (2003) J. Neurochem. 86, 1101-1107
9. Sanz, A. et al. (2010) Proc. Natl. Acad. Sci. USA 107, 9105-9110
10. Brand, M. D. (2010) Exp. Gerontol. 45, 466-472
11. Lambert, A. J., and Brand, M. D. (2004) J. Biol. Chem. 279, 39414-39420
12. Lambert, A. J., and Brand, M. D. (2004) Biochem. J. 382, 511-517
13. Lambert, A. J., Buckingham, J. A., and Brand, M. D. (2008) FEBS Lett. 582, 1711-1714
14. Smith, A. L. (1967) Methods Enzymol. 10, 8-86
15. Kotlyar, A. B., and Vinogradov, A. D. (1990) Biochim. Biophys. Acta 1019, 151-158
16. Beltrán, C., de Gómez-Puyou, M. T., Gómez-Puyou, A., and Darszon, A. (1984) Eur. J. Biochem. 144, 151-157

10
17. Hansen, M., and Smith, A. L. (1964) Biochim. Biophys. Acta 81, 214-222
18. Linnane, A. W., and Ziegler, D. M. (1958) Biochim. Biophys. Acta 29, 630-638
19. Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960) J. Biol. Chem. 235, 3322-3329
20. Jault, J. M., and Allison, W. S. (1993) J. Biol. Chem. 268, 1558-1566
21. Votyakova, T. V., and Reynolds, I. J. (2004) Arch. Biochem. Biophys. 431, 138-144
22. Orr, G. A., and Blanchard, J. S. (1984) Anal. Biochem. 142, 232-234
23. Azzi, A., Montecucco, C., and Richter, C. (1975) Biochem. Biophys. Res. Commun. 65, 597-603
24. Hille, R., and Massey, V. (1981) J. Biol. Chem. 256, 9090-9095
25. Kotlyar, A. B., Karliner, J. S., and Cecchini, G. (2005) FEBS Lett. 579, 4861-4866
26. Zharova, T. V., and Vinogradov, A. D. (1997) Biochim. Biophys. Acta 1320, 256-264
27. Majander, A., Finel, M., and Wikström, M. (1994) J. Biol. Chem. 269, 21037-21042
28. Kotlyar, A. B., and Vinogradov, A. D. (1984) Biochim. Biophys. Acta 784, 24-34
29. Huang, L. et al. (2006) J. Biol. Chem. 281, 5965-5972
30. Vinogradov, A. D., and Grivennikova, V. G. (2005) Biochemistry (Moscow) 70, 120-127
31. Bason, J. V., Runswick, M. J., Fearnley, I. M., and Walker, J. E. (2011) J. Mol. Biol. 406, 443-453
32. Chance, B., and Hollunger, G. (1960) Nature 185, 666-672
33. Ksenzenko, M., Konstantinov, A. A., Khomutov, G. B., Tikhonov, A. N. and Ruuge, E. K. (1983) FEBS Lett. 155, 19-24
34. Sled, V. D., Rudnitzky, N. I., Hatefi, Y., and Ohnishi, T. (1994) Biochemistry 33, 10069-10075
35. Reda, T., Barker, C. D., and Hirst, J. (2007) Biochemistry 46, 14250-14258
36. Reda, T., Barker, C. D., and Hirst, J. (2008) Biochemistry 47, 8885-8893
37. Rotenberg, H., and Lee, C.-P. (1975) Biochemistry 14, 2675-2680
38. Bashford, C. L., and Thayer, W. S. (1977) J. Biol. Chem. 252, 8459-8463
39. O'Rourke, B. (2007) Annu. Rev. Physiol. 69, 19-49
40. Sorgato, M. C., Ferguson, S. J., Kell, D. B., and John, P. (1978) Biochem. J. 174, 237-256
41. Hirst, J., King, M. S., and Pryde, K. R. (2008) Biochem. Soc. Trans. 36, 976-980
42. Treberg, J. R., Quinlan, C. L., and Brand, M. D. (2010) FEBS J. 277, 2766-2778
43. Grivennikova, V. G., and Vinogradov, A. D. (2006) Biochim. Biophys. Acta 1757, 553-561
44. Starkov, A. A. et al. (2004) J. Neurosci. 24, 7779-7788
45. Grivennikova, V. G., Kareyeva, A. V., and Vinogradov, A. D. (2010) Biochim. Biophys. Acta 1797, 939-944
46. Aon, M. A., Cortassa, S., and O'Rourke, B. (2010) Biochim. Biophys. Acta 1797, 865-877
47. Liu, Y., Fiskum, G., and Schubert, D. (2002) J. Neurochem. 80, 780-787
48. Lamert, A. J., Buckingham, J. A., Boysen, H. M., and Brand, M. D. (2008) Biochim. Biophys. Acta 1777, 397-403
49. Zoccarato, F., Cavallini, L., Bortolami, S., and Alexandre, A. (2007) Biochem. J. 406, 125-129
50. Selivanov, V. A. et al. (2008) J. Biol. Chem. 283, 29292-29300

Footnotes

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The abbreviations used are: ACMA, amino-6-chloro-2-methoxyacridine; DPI, diphenyleneiodonium; RCR, respiratory control ratio; RET, reverse electron transfer; ROS, reactive oxygen species; SMP, submitochondrial particle; SOD, superoxide dismutase.
Figure Legends

1. Measurements of H$_2$O$_2$ production and NADH oxidation by complex I in SMPs. H$_2$O$_2$ production (measured by the accumulation of resorufin, y-axis scale) by SMPs (35 µg protein mL$^{-1}$) was initiated by 30 µM NADH, and NADH oxidation measured alongside (the intensity of the NADH traces has been multiplied by 0.2). A). In the presence of rotenone, H$_2$O$_2$ production and NADH oxidation were linear (black traces, 1). 1 mM NAD$^+$ (red traces, 2) decreased both rates to the background level recorded in the presence of DPI (blue traces, 3). B). The black trace (1) is reproduced from panel A. The blue trace (2) was recorded during catalytic NADH oxidation (no inhibitor) with Δp = 0 (in the presence of gramicidin). The red trace (3) was recorded during NADH oxidation in the presence of Δp. The H$_2$O$_2$ production traces (2B and 3B) curve in response to NADH oxidation. See Experimental Procedures for conditions.

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 KCl, or decreased using 20 mM KCl and 2 nM nigericin (see text). See Experimental Procedures for conditions.

3. NADH-induced H$_2$O$_2$ production by complex I in SMPs with and without Δp, compared to that by isolated complex I. The three curves have been normalized independently. The data are plotted using $E_{av} = -0.335$ V $- RT/2F \ln{\left[ \frac{[NADH]}{[NAD^+]^2} \right]}$ (using 30 µM NADH and variable NAD$^+$), and fit by the Nernst equation for a two-electron cofactor with two 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 (Δp = 0 V) ($E_{av} = -0.363$, $\Delta E = 0.079$ V). Black: complex I in SMPs inhibited by rotenone with Δp $\sim 0.16$ V (generated by ATP hydrolysis) ($E_{av} = -0.366$, $\Delta E = 0.060$ V). The NADH-OH independent rates, measured in NADH only (see Figure 2), have been subtracted from the SMP data sets. See Experimental Procedures for conditions.

4. Measurement of Δp in SMPs hydrolyzing ATP, and evaluation of ΔpH. A). The rate of NADH:fumarate oxidoreduction, recorded during ATP hydrolysis (1 mM ATP+MgSO$_4$) to generate Δp, and with 400 µM KCN to inhibit respiration, depends on the difference between the NAD$^+$ and fumarate potentials, $\Delta E = -0.335$ V + 0.020 $- RT/2F \ln{\left[ \frac{[NADH] \cdot [fumarate]}{[NAD^+]^2 \cdot [succinate]} \right]}$, using [NADH] $= 0.1$ mM, [NAD$^+$] $= 1$ mM, [succinate] $= 0.5$ mM, [fumarate] $= 0.025$ to 40 mM. The rate is zero when $-2\Delta E = 4\Delta p$. When $-2\Delta E$ is close to 4Δp 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 0.11 V, [NAD$^+$] $= [succinate] = 0$ shown at 0.18 V). Similar titrations varying the succinate, NAD$^+$ and NADH concentrations gave, overall, Δp $= 0.162 \pm 0.005$ V. B). The fluorescence of ACMA (500 nM) is quenched upon the addition of 100 µM ATP+MgSO$_4$ to SMPs (112 µg mL$^{-1}$) in varying KCl concentrations. 2 nM nigericin (a K$^+$/H$^+$ exchanger) or 20 µg mL$^{-1}$ gramicidin (a cation transporter) abolish the quenching effect. Note that the ACMA fluorescence is affected by ATP (the final value varies). See Experimental Procedures for conditions.

5. H$_2$O$_2$ production during RET, in the presence of various respiratory chain inhibitors, and in the presence and absence of Δp and ΔpH. H$_2$O$_2$ production rates were measured using the HRP-Amplex Red detection system, during the linear phases of the reactions, and normalized to the value from the standard RET condition (20 mM succinate, 1 mM ATP+MgSO$_4$, KCN). Δp was collapsed with gramicidin, and ΔpH increased using 20 mM KCl or decreased using 20 mM KCl and 2 nM nigericin (see text). See Experimental Procedures for conditions.
6. Comparison of NADH- and RET-induced H$_2$O$_2$ production by complex I in SMPs. The two curves have been normalized independently. Black: NADH-induced H$_2$O$_2$ production by complex I in SMPs inhibited by rotenone with $\Delta p \sim 0.16$ V (from Figure 3). Red: RET-induced H$_2$O$_2$ production by complex I in SMPs inhibited by KCN. The RET data are plotted using $E_{\text{Set}} = -0.020$ V $-RT/2F \ln \{[\text{succinate}]/[\text{fumarate}]\} + 2 \Delta p$ (10 mM succinate, variable fumarate concentrations) and fit by the Nernst equation for a two-electron cofactor with two distinct potentials, described by their average value, $E_{\text{av}} = -0.305$ V, and their separation, $\Delta E = -0.020$ V. The rotenone independent rates (see Figure 5) have been subtracted. The asterisk marks the 0.024 V increase in $\Delta p$ (measured at $E_{1/2}$) that is required to overlay the RET-induced data on the NADH-induced data. See Experimental Procedures for conditions.
### Table 1. Catalytic properties of a typical SMP preparation.

| Catalytic reaction         | Rate of reaction (µmoles min⁻¹ mg protein⁻¹) | RCR |
|---------------------------|---------------------------------------------|-----|
|                           | Coupled (+Δp) | Uncoupled (Δp=0)¹                           |     |
| 1. NADH:O₂ oxidoreduction² | 0.246 ± 0.011 | 0.743 ± 0.018 | 3.02 ± 0.15³ |
| 2. succinate:O₂ oxidoreduction | 0.148 ± 0.015 | 0.264 ± 0.024 | 1.78 ± 0.24 |
| 3. NADH:fumarate oxidoreduction | 0.052 ± 0.001 | 0.053 ± 0.001 | 1.02 ± 0.03 |
| 4. succinate:NAD⁺ oxidoreduction (RET)²,⁴ | 0.133 ± 0.002 | 0.002 ± 0.002 | - |
| 5. ATP hydrolysis          | 0.893 ± 0.109 | 1.353 ± 0.043 | 1.52 ± 0.19 |

Reaction 1: 100 µM NADH; reaction 2: 20 mM succinate; reaction 3: 100 µM NADH, 40 mM fumarate, KCN; reaction 4: 1 mM NAD⁺, 10 mM succinate, 2 mM ATP+MgSO₄, KCN; reaction 5: 0.2 mM ATP+MgSO₄, rotenone.

Notes:
1. Δp was collapsed using 10 µg ml⁻¹ gramicidin.
2. Observed rates of NADH:O₂ and succinate:NAD⁺ oxidoreduction varied by up to two-fold between preparations, and were comparable to a range of published values recorded in varying conditions (see for example (15,30)). Conversely, the rates of reactions that are catalyzed only by the flavin site in complex I did not vary significantly.
3. 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 to 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 which 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 text).
4. 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.
Table 2. Side-by-side comparison of measurements of H$_2$O$_2$ and superoxide production by a typical SMP preparation.

|                      | NADH-induced reactions | RET-induced reactions |
|----------------------|------------------------|-----------------------|
|                      | Obs. value$^1$ | + DPI (%)$^2$ | + NAD$^+$ (%)$^2$ | + Cat (%)$^3$ | + SOD (%)$^3$ | Corr. value$^3$ | Complex I$^4$ |
| H$_2$O$_2$           | 5.91 ± 0.2 | 16.5 ± 1.0 | 11.5 ± 1.2 | 18.1 ± 1.7 | 108.7 ± 2.7 | 4.93 ± 0.2 | 59.2 ± 2.7 |
| Superoxide           | 19.1 ± 0.4 | 55.7 ± 0.1 | 58.5 ± 0.7 | - | 54.2 ± 1.8 | 8.5 ± 0.2 | 101.5 ± 2.4$^5$ |
|                      | Obs. value$^1$ | + DPI (%)$^2$ | + Rot (%)$^2$ | + Cat (%)$^3$ | + SOD (%)$^3$ | Corr. value$^3$ | Complex I$^4$ |
| H$_2$O$_2$           | 0.39 ± 0.01 | 49.1 ± 4.7 | 48.8 ± 2.4 | 20.0 ± 3.4 | 102.8 ± 3.5 | 0.20 ± 0.02 | 23.8 ± 2.9 |
| Superoxide           | 2.97 ± 0.02 | 89.2 ± 1.3 | 89.5 ± 1.3 | - | 88.0 ± 1.6 | 0.32 ± 0.04 | 38.5 ± 4.9$^5$ |

1. The observed (obs.) values are rates of resorufin formation or rates of acetylated cytochrome c reduction and are reported in nmoles min$^{-1}$ mg 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 mM 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 (nmoles min$^{-1}$ mg 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 Methods).
4. The complex I rate (min$^{-1}$) is the rate of superoxide or H$_2$O$_2$ reaction 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 text).
5. The ratios between the rates of superoxide and H$_2$O$_2$ production are 1.71 ± 0.12 (NADH) 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 text).
**Figure 5**

![Normalized rate of H$_2$O$_2$ production chart]

**Figure 6**

![Normalized rate of H$_2$O$_2$ production chart vs. $E_{Set}$ (V)]
Superoxide is produced by the reduced flavin in mitochondrial complex I: a single, unified mechanism that applies during both forward and reverse electron transfer

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