Inhibitors of the quinone-binding site allow rapid superoxide production from
mitochondrial NADH:ubiquinone oxidoreductase (complex I)

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Running Title: Superoxide Production by Complex I
SUMMARY

Neither the route of electron transport nor the sites or mechanism of superoxide production in mitochondrial complex I has been established. We examined the rates of superoxide generation (measured as hydrogen peroxide production) by rat skeletal muscle mitochondria under a variety of conditions. The rate of superoxide production by complex I during NADH-linked forward electron transport was less than 10% of that during succinate-linked reverse electron transport even when complex I was fully reduced by pyruvate plus malate in the presence of the complex III inhibitor, stigmatellin. This asymmetry was not explained by differences in protonmotive force or its components. However, when inhibitors of the quinone-binding site of complex I were added in the presence of ATP to generate a pH gradient, there was a rapid rate of superoxide production by forward electron transport that was as great as the rate seen with reverse electron transport at the same pH gradient. These observations suggest that quinone-binding site inhibitors can make complex I adopt the highly radical-producing state that occurs during reverse electron transport. Despite complete inhibition of NADH:ubiquinone oxidoreductase activity in each case, different classes of quinone-binding site inhibitor (rotenone, piericidin and high concentrations of myxothiazol) gave different rates of superoxide production during forward electron transport (the rate with myxothiazol was twice that with rotenone) suggesting that the site of rapid superoxide generation by complex I is in the region of the ubisemiquinone binding sites and not upstream at the flavin or low potential FeS centres.
KEY WORDS: electron transport chain, mitochondria, skeletal muscle, reactive oxygen species, hydrogen peroxide

ABBREVIATIONS: ROS, reactive oxygen species; Q, ubiquinone; Δp, protonmotive force; Δψ, membrane potential; ΔpH, pH gradient; PHPA, p-hydroxyphenyl acetic acid; SOD, superoxide dismutase; TPMP, triphenylmethylphosphonium; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; SMPs, submitochondrial particles; ROS, reactive oxygen species
INTRODUCTION

It is well established that the mitochondrial electron transport chain produces superoxide ($O_2^-$), as the result of single electron leaks to oxygen during electron transport from reduced substrates to complex IV (1). Superoxide is converted into hydrogen peroxide by the action of manganese superoxide dismutase (MnSOD) in the mitochondrial matrix, or by the action of copper/zinc superoxide dismutase (Cu/ZnSOD) in the cytosol. If superoxide is not removed from the mitochondrial matrix, as in the case of MnSOD nullizygous mice, severe pathologies arise and lifespan is curtailed to about 10 days (2). If superoxide is not removed from the cytosol, as in the case of Cu/ZnSOD nullizygous mice, the effects are not lethal, although an increase in sensitivity to oxidative stress is apparent (3). Thus there is intense interest in superoxide and the other reactive oxygen species (ROS) it gives rise to (such as hydrogen peroxide and hydroxyl radical), as ROS clearly play a role in a variety of pathological disorders and perhaps ageing (4, 5).

The sites of superoxide production within the mitochondrial electron transport chain in vitro have been localized to complexes I and III (6). Complex I produces superoxide to the matrix side of the mitochondrial membrane exclusively whereas complex III appears to produce superoxide to both the matrix and intermembrane space in roughly equal amounts (7-9). The relative importance of each complex to total superoxide production is debated; during reverse electron transport from succinate to NAD, complex I produces superoxide at very high rates (10-15), but the physiological relevance of reverse electron transport in unclear. During forward electron transport, however (which is clearly physiological), both complexes produce superoxide at relatively low rates, unless inhibitors such as rotenone (for complex I) or antimycin A (for complex III) are present. Under these inhibited conditions with
forward electron transport, (which are not physiological) the superoxide production rates are relatively high. Whatever the relative importance of the complexes to total superoxide production in vivo, compared to complex III, very little is known about the mechanism of complex I superoxide production.

In mitochondria, superoxide is produced by the single electron reduction of oxygen by an electron carrier within the electron transport chain. In complex III the electron carriers are cytochromes b₃, b₄, and c₁, the Rieske iron-sulphur centre, and the semiquinones at centres ‘i’ and ‘o’. The main reductant of oxygen to produce superoxide at complex III has been identified as the semiquinone at centre ‘o’, consistent with the Q cycle mechanism of the complex (16). In complex I, the electron carriers are the flavin, the iron-sulphur centres N₁a, N₁b, N₂, N₃, N₄, N₅ and an unknown number of semiquinones (17). The reductant of oxygen to produce superoxide in this enzyme is not known and published reports are highly conflicting. On thermodynamic grounds, centre N₁a (12) and the flavin (18) were suggested as the main superoxide producing sites. Based on inhibitor studies, the flavin (11, 19), centre N₂ (20) and the iron-sulphur proteins and semiquinones in general (21) were suggested as sites of superoxide production. It is feasible that all these sites produce superoxide, and that production rates by different sites are tissue or condition specific. We recently demonstrated that superoxide production rates by complex I during reverse electron transport are highly dependent on the pH gradient across the mitochondrial inner membrane (22). All previous studies of complex I ROS production used conditions in which the pH gradient was either zero or very low, so probably missed the site of maximal superoxide production by the complex. It has been suggested that the main site of superoxide production within complex I is a semiquinone (6). As stated above, superoxide production rates by complex I during
reverse electron transfer are much greater than during forward electron transfer. By investigating this asymmetry, we have found conditions in which it is abolished. Our results suggest strongly that the major site of superoxide production in complex I is the quinone binding site; it is most likely a semiquinone.
EXPERIMENTAL PROCEDURES

Materials - Piericidin A was a kind gift from Dr Mauro Degli Esposti (University of Manchester, UK). All other chemicals were purchased from Sigma.

Measurement of Mitochondrial Superoxide Production - Mitochondria from skeletal muscle of female Wistar rats (aged between 5 and 8 weeks) were isolated by differential centrifugation as described (23). Superoxide production rate was assessed by measurement of hydrogen peroxide generation rate, determined fluorometrically by measurement of oxidation of p-hydroxyphenylacetic acid (PHPA) coupled to the enzymatic reduction of H$_2$O$_2$ by horseradish peroxidase. Mitochondria (0.35 mg mitochondrial protein.ml$^{-1}$) were incubated at 37°C in standard buffer containing 120 mM KCl, 3 mM HEPES, 1 mM EGTA, 0.3% BSA (w/v) (pH 7.2 at 37°C). All incubations also contained 50 µg.ml$^{-1}$ PHPA, 4 U.ml$^{-1}$ horseradish peroxidase, 30 U.ml$^{-1}$ superoxide dismutase (SOD) and 1.875 µM triphenylmethylphosphonium (TPMP$^+$) bromide. The reaction was initiated by addition of respiratory substrates, after 1 min the increase in fluorescence at an excitation of 320 nm and emission of 400 nm was followed on a computer-controlled Shimadzu RF5301 spectrofluorometer for 2-3 min. Appropriate corrections for background signals were applied (7), and standard curves generated using known amounts of H$_2$O$_2$ were used to calculate the rate of H$_2$O$_2$ production in nmol.min$^{-1}$.mg mitochondrial protein$^{-1}$. Essentially all the superoxide from complex I is generated on the matrix side of the inner membrane, then converted by endogenous processes to H$_2$O$_2$ which leaks out and is measured in the assay (7). Certain compounds employed in the experiments (such as myxothiazol and ATP) caused significant quenching of the fluorescent signals, therefore careful
calibration with standard curves generated for all conditions was essential to obtain the correct rates of H$_2$O$_2$ production.

**Measurement of Mitochondrial Protonmotive Force (Δp)** - Mitochondrial membrane potential, Δψ, was determined using an electrode sensitive to TPMP$^+$ as described (24). Skeletal muscle mitochondria were incubated under the same conditions as for superoxide production at 37°C in standard buffer with PHPA, horseradish peroxidase and SOD. The electrode was calibrated by sequential 0.375 µM additions of TPMP$^+$ up to 1.875 µM. The reaction was initiated by addition of respiratory substrate and Δψ was measured upon reaching the steady state (approximately 1 min). The chemical component of protonmotive force, ΔpH, was then measured as the change in ΔΨ after ΔpH was converted to Δψ following addition of 100 nM nigericin. After each run, the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was added to 2 µM to release the TPMP$^+$ and allow correction for any small drift in the TPMP$^+$ electrode. Potentials were calculated as described (24), on the basis that Δp = Δψ + ΔpH (all in mV, giving positive signs to electrical potentials that were positive outside and pH gradients that were acid outside).

**Determination of NADH:Q$_2$ Oxidoreductase Activity** - Complex I activity was assessed by monitoring the disappearance of 25 µM NADH with 125 µM coenzyme Q$_2$ as an electron acceptor. NADH was monitored fluorometrically at excitation and emission wavelengths of 365 and 450 nm respectively under identical incubation and buffer conditions to those used for measurement of H$_2$O$_2$ and Δp. To allow NADH to react with complex I, the mitochondria were broken open by 3 freeze-thaw cycles.
prior to assay. The concentrations of rotenone, piericidin and myxothiazol required to achieve maximal inhibition of complex I were determined by titration.

Statistics - Values are given as means ± standard error with n being the number of separate mitochondrial preparations. The significance of differences between means was assessed by unpaired Student’s t test, p values < 0.05 were taken to be significant.

RESULTS

Comparison of superoxide production by complex I during forward and reverse electron transport

A series of experiments (Figure 1) was conducted to examine production of superoxide during forward and reverse electron transport by complex I. As shown in Figure 1a, with pyruvate + malate as substrates, the rate of H₂O₂ production from the entire electron transport chain was low, only 0.03 nmol.min⁻¹.mg protein⁻¹. A similar rate was found for another combination of forward electron transport linked substrates: glutamate + malate (not shown). With succinate as substrate the rate of superoxide production was some 100 fold greater (Figure 1a), and was localised almost entirely at complex I during reverse electron transport (22). The high rate of superoxide production during reverse electron transport into complex I is very sensitive to the pH gradient across the inner mitochondrial membrane (22), since conversion of ΔpH to Δψ by the addition of nigericin strongly inhibited superoxide production (Figure 1a). There was no significant difference in ΔpH between mitochondria oxidising succinate and pyruvate + malate (Table 1), so the differences in superoxide production between forward and reverse electron transport are not simply due to differences in ΔpH. Nigericin did not significantly alter superoxide
production rate with pyruvate + malate (Figure 1a), showing that with forward electron transport, ΔpH has no detectable effect on superoxide production rate under these conditions.

It might be that the complex I superoxide-producing site is more reduced during reverse electron transport, when electrons are forced into the complex using the high Δp (and perhaps the high ubiquinone reduction state) generated by succinate oxidation, than it is during forward electron transport, when Δp may be a little lower (Table 1) and when the ubiquinone pool may be more oxidized. To test this possibility, we added the complex I inhibitors rotenone, piericidin or myxothiazol, to allow the complex to become fully reduced by electrons from pyruvate + malate. Myxothiazol is a centre ‘o’ inhibitor of complex III, but at high concentrations it is also an effective inhibitor of complex I (20, 25). Addition of any one of these inhibitors did increase H₂O₂ production from pyruvate + malate, with myxothiazol having the largest effect, followed by piericidin then rotenone (Figure 1b). The differences between inhibitors were not due to different levels of inhibition of complex I: Figure 2 shows that superoxide production was near maximal for each inhibitor, and Figure 3 shows that each caused maximal inhibition of NADH:Q₂ oxidoreductase activity at the concentration used. However, even with myxothiazol, H₂O₂ production rates were still only one third of those seen with reverse electron transport, so the reduction state of superoxide-generating sites upstream of the sites where these inhibitors work in complex I may contribute to the difference in superoxide production between reverse and forward electron transport, but cannot fully explain it.

It may be that the main site of superoxide production from complex I is the Q binding site itself. If so, then blocking this site with Q-type inhibitors (rotenone,
piericidin or high concentrations of myxothiazol) may inhibit superoxide production during forward electron transport, and the high rates seen with succinate cannot be achieved. To test this possibility, a condition is required in which the Q binding site within complex I is fully reduced with forward electron transport but open (i.e. uninhibited). One way to achieve this condition would be to use a low concentration of myxothiazol, so that only centre ‘o’ of complex III is inhibited. The concentration of myxothiazol required to fully inhibit complex III in our system was 0.625 µM (not shown), but this concentration also inhibited complex I activity by about 40% (Figure 3). Hence the Q binding sites were not fully open at this relatively low myxothiazol concentration under the conditions employed. Another centre ‘o’ inhibitor of complex III is stigmatellin (26), which at 80 nM did not inhibit complex I activity at all (not shown) but did inhibit complex III, as Δp was zero (Table I). We tested the effects of stigmatellin and two other different combinations of substrates and inhibitors that produce an open Q binding site but reduced complex I during forward electron transport; the results are shown in Figure 1c. The first other condition was pyruvate + malate plus potassium cyanide, which will cause maximal reduction of all complexes upstream of complex IV. The second other condition employed pyruvate + malate plus antimycin A (which gives complex I superoxide plus maximal superoxide production from complex III) minus the rate with succinate plus rotenone plus antimycin A (which gives maximal superoxide from complex III only). None of the three conditions tested caused the rate of superoxide production by complex I with forward electron transport to approach the rates seen with succinate alone. In the presence of stigmatellin, the rate of superoxide production was less than 10% of the rate during reverse electron transport with succinate seen in Figure 1a. Therefore a
fully reduced and open Q binding site with forward electron transport is not sufficient to give high rates of superoxide production by complex I.

**The effect of ΔpH on superoxide production during forward electron transport**

Under the conditions of pyruvate + malate + inhibitor shown in Figure 1, ΔpH and Δψ are zero (Table 1) because there is no proton pumping by the electron transport chain. As shown previously, H₂O₂ production by complex I is particularly sensitive to ΔpH during reverse electron transport (22), so could the remaining difference in superoxide production rate between forward and reverse electron flow be caused by the lack of ΔpH during inhibited forward electron transport? The effects of ΔpH were tested using hydrolysis of added ATP to produce ΔpH and Δψ (Table 1). As shown in Figure 4a, ATP addition did elevate rates of H₂O₂ production with all three complex I inhibitors, and these increases were abolished by addition of nigericin, which brings ΔpH to zero while raising Δψ. In the presence of pyruvate + malate plus myxothiazol and ATP, ΔpH was about 10 mV (Table 1) and the rate of H₂O₂ production was about 1.6 nmol.min⁻¹.mg protein⁻¹ (Figure 4a), which is the same as the rate achieved by reverse electron transport from succinate at the same ΔpH (22). The increases in H₂O₂ production seen upon addition of ATP were prevented when oligomycin was present in the medium (not shown), indicating that the effects of ATP were mediated by the ATPase. In support of these conclusions, we found that ATP hydrolysis could be replaced by succinate oxidation: the rate of H₂O₂ production was high when succinate was added in the presence of pyruvate + malate and either rotenone or piericidin (not shown).

Experiments with pyruvate + malate plus either stigmatellin, KCN or antimycin A with ATP are shown in Figure 4b. It can be seen that imposition of a pH gradient did not permit generation of superoxide at high rates in the absence of Q site
inhibitors. Therefore, a reduced, open Q binding site in the presence of ΔpH during forward electron transport is not sufficient to generate the large amounts of superoxide seen during reverse electron transport. However, complex I with its Q binding site inhibited by myxothiazol (or, to a lesser extent, by piericidin or rotenone) in the presence of ΔpH during forward electron transport is able to generate superoxide at the same rate as it does during reverse electron transport from succinate in the absence of any Q site inhibitor.

The effect of matrix pH on superoxide production during forward electron transport

Using the protocol previously described (22), we checked that the strong inhibitory effect of nigericin on H$_2$O$_2$ production in the presence of pyruvate + malate, myxothiazol and ATP (Figure 4a) was not simply due to changes in internal pH (Figure 5). At any internal pH, the rate of H$_2$O$_2$ production was greater in the absence of nigericin than in its presence. Thus, like superoxide generation during reverse electron transport, superoxide generation during forward electron transport under these conditions is dependent on ΔpH.

DISCUSSION

In agreement with previous studies (10-15), we show that superoxide production by complex I during reverse electron transport is huge compared to forward electron transport under similar conditions. This asymmetry of superoxide production by complex I has not previously been investigated in detail, and no mechanistic explanation of it has been offered in the literature. We characterized superoxide production by complex I and addressed two questions: under what conditions can complex I produce superoxide during forward electron transport at the high rates seen during reverse electron transport in intact mitochondria (in other
Superoxide Production by Complex I

words, can the asymmetry be explained) and which site in complex I generates the majority of the superoxide?

**Conditions of high rates of superoxide production by complex I**

In terms of conditions, firstly, we demonstrate that a pH gradient across the mitochondrial inner membrane during forward (Figures 4a and 5) or reverse (Figure 1a and 22) electron transfer is required for high rates of superoxide production. The pH gradient can be generated by proton pumping by substrate oxidation (in the case of succinate) or by ATP hydrolysis via the ATPase (when the electron transport chain is inhibited).

Secondly, we show that a relatively reduced complex I (as a whole) may be required but is not sufficient for high superoxide generation rates. During reverse electron transport an overall high reduction state is achieved by the high Δp forcing electrons from succinate into complex I. However, with forward electron transport, if a reduced complex I was sufficient for high superoxide production, then large rates would have been observed with pyruvate + malate plus stigmatellin, KCN or [(antimycin A) minus (succinate + rotenone + antimycin A)]. This was not the case: even when ATP was added to generate ΔpH, the superoxide production rates remained low under these conditions compared to the rates seen with succinate.

Thirdly, we have found that direct Q site inhibition is required for high rates of superoxide production by complex I during forward electron transport. Only in the presence of either rotenone, piericidin or myxothiazol (and ΔpH) was the superoxide production rate high with forward electron transport (Figure 4a). All three Q site inhibitors fully inhibited NADH:Q oxidoreductase activity, but myxothiazol was more effective at inducing superoxide production than piericidin, which in turn was more effective than rotenone. This situation appears analogous to the case with
complex III. In its native (uninhibited) state, complex III produces superoxide at low rates, but in the presence of antimycin A the rate increases dramatically (7, 11, 13, 15). During forward electron transport, complex I produces superoxide at very low rates in its native state, but the rate can increase 10-30 fold in the presence of Q site inhibitors.

Our findings are in good agreement with a previous report on superoxide production in heart submitochondrial particles (SMPs) (20). Using NADH to generate forward electron transport, a very low superoxide production rate was observed. This rate increased by about 3-fold when mucidin was used to block centre ‘o’ of complex III. However 10-fold increases in rate were seen in the presence of various complex I Q site inhibitors, and from our results it is likely these rates would have been even higher in the presence of ΔpH.

Therefore, the conditions that explain the asymmetry of superoxide production between forward and reverse electron transport are very specific. The asymmetry is not simply due to differences in the overall redox state of complex I or differences in protonmotive force or its components. High rates of superoxide production, in the presence of ΔpH, were only achieved with the native enzyme during reverse electron transport (22), or in the presence of Q site inhibitors during forward electron transport. The simplest explanation of these observations is that there is a particular state of complex I that leads to high superoxide production, and this state can be accessed either during reverse electron transport, or during forward electron transport when an inhibitor occupies the Q binding site. The fact that different rates of superoxide production were obtained with the three different Q site inhibitors is consistent with the concept of two or three classes of complex I inhibitor with different degrees of overlap in a large Q binding pocket (25, 27). Occupation of one
of these binding sites by an inhibitor would trigger superoxide production by promoting the reaction with oxygen of some reductant either within the Q site (such as a semiquinone), or elsewhere within complex I.

*Sites of superoxide production by complex I*

Which sites in complex I generate superoxide at high rates? If the main or only site of the high superoxide production we observe in the presence of ATP is upstream of the Q binding site (i.e. from iron sulphur centres N1a, N1b, N3, N4, N5 or flavin), then, for a simple linear chain of electron carriers, addition of any Q site inhibitor should result in the same rate of superoxide production. This was not the case; we observed different increases (myxothiazol > piericidin > rotenone) in the rate of superoxide production from pyruvate + malate in both the absence (Figure 1) and presence (Figure 4a) of ATP. In addition, if sites upstream of Q generate superoxide, then the production rates from complex I should be similar with the Q site inhibitors and with stigmatellin, KCN or antimycin A. This was clearly not the case (Figure 4b). We conclude, therefore, that the sites upstream of Q must produce superoxide at low rates compared to the Q site itself, as illustrated in Figure 6.

Some reports (11, 12, 18-20) suggest that these upstream sites are mainly responsible for complex I superoxide production. It is difficult to compare the results from different laboratories, as the tissues used, methods of mitochondrial and SMP isolation, ROS detection systems, buffer components and correction for background signals all vary. However, one consistent factor appears to be that all of these previous studies used conditions in which ∆pH was either zero (the mitochondria or SMPs were de-energized) or low (e.g. due to the presence of phosphate in the medium (22)). Therefore, superoxide production was probably not maximal in these studies, and they missed the site of high superoxide production discussed here. Their conclusions may
be valid for the minor sites of superoxide production, but would not be relevant to the major site of complex I superoxide production analysed here.

As discussed above, complex III only generates superoxide at high rates when antimycin A is present. This behaviour is consistent with the Q cycle and is explained by the formation of a semiquinone at centre ‘o’ of the complex that reduces oxygen to superoxide (28). By analogy, perhaps the requirement for Q site inhibitors for maximum superoxide production by complex I reflects Q cycle-type behaviour in this complex too and suggests that the reductant of oxygen to produce superoxide is a semiquinone. The coupling mechanism of complex I remains unknown, but analogies to the Q-cycle in complex III have been proposed (29). Other models have been suggested that are consistent with Q cycle–type behaviour in the enzyme (30).

Where within the Q site could superoxide be produced? EPR studies have detected at least three ubisemiquinone species within complex I, termed SQ\textsubscript{Ni}, SQ\textsubscript{Ns} and SQ\textsubscript{Nx} for fast relaxing, slow relaxing and very slow relaxing semiquinone (31-33). If there is a Q cycle mechanism in complex I, then as well as the canonical quinone reducing site there must be at least one additional quinone reduction and one quinol oxidation site. Recent models of complex I propose three different semiquinone sites; two quinone reduction sites and one quinol oxidation site (6, 30). Three classes of inhibitor have been described, possibly corresponding to three semiquinone species in a large Q binding pocket (25, 27). Of these species, only SQ\textsubscript{Ni} exhibits sensitivity to Δp. Since superoxide production is very sensitive to Δp, then SQ\textsubscript{Ni} might be the reductant of oxygen in complex I. If so, SQ\textsubscript{Ni} should exhibit high sensitivity to ΔpH. In addition, both rotenone and piericidin quench the SQ\textsubscript{Ni} radical, which would explain why they quench superoxide production from succinate. Figure 7 displays a tentative model of superoxide production at the semiquinone in one of the Q reducing
Superoxide production by complex I that is consistent with the observations presented here and previously (22).

*Superoxide production as a tool to investigate the coupling mechanism of complex I*

As discussed previously (22), one of the reasons why the coupling mechanism of complex I has remained elusive is the lack of easily studied intermediates. Superoxide production by complex I is an indirect measure of the redox status of a free radical intermediate in complex I, and is fairly straightforward to measure. It is likely that this intermediate is a semiquinone and closely involved in the coupling reaction, as it is ΔpH sensitive. We suggest, therefore, that superoxide production by complex I may be a useful tool to gain mechanistic insights into complex I.

**FOOTNOTES**

*Acknowledgements* This work was supported by the Medical Research Council and the Wellcome Trust. We thank Steven Roebuck, Helen Boysen and Julie Buckingham for excellent technical assistance.
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FIGURE LEGENDS

Figure 1. Rates of H$_2$O$_2$ production by rat skeletal muscle mitochondria. The medium contained 120 mM KCl, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA (pH 7.2 and 37°C) with 50 µg PHPA.ml$^{-1}$, 4 U horseradish peroxidase.ml$^{-1}$, 30 U SOD.ml$^{-1}$ and 1.875 µM TPMP$^+$. Where indicated, 2.5 mM pyruvate and 2.5 mM malate, 4 mM succinate, 100 nM nigericin, 4 µM rotenone, 2 µM piericidin, 40 µM myxothiazol, 80 nM stigmatellin, 1 mM KCN and 2.5 µM antimycin A (AA) were added. In (c), the rate with succinate + rotenone + antimycin A was measured in a separate run, and subtracted from the rate obtained with pyruvate + malate + antimycin A. Bars show means ± S.E.M. of measurements on 3 to 10 separate mitochondrial preparations. *Significant difference ($p < 0.0001$) vs. succinate, **significant difference ($p < 0.01$) vs. succinate + nigericin. †Significant difference ($p < 0.05$) vs. pyruvate + malate, ††significant difference ($p < 0.025$) vs. pyruvate + malate + rotenone.

Figure 2. Inhibitor titrations of superoxide production by complex I with pyruvate + malate as substrates. Incubation conditions as Figure 1. Points represent means ± S.E.M. of measurements on three separate mitochondrial preparations. Top panel: rotenone (triangles) and piericidin (diamonds), bottom panel: myxothiazol.

Figure 3. Inhibition of complex I NADH:Q$_2$ oxidoreductase activity by rotenone, piericidin and myxothiazol. Incubation conditions as Figure 1. Points represent means ± S.E.M. of measurements on three separate mitochondrial preparations. Top panel: rotenone, middle panel: piericidin, bottom panel: myxothiazol. Complex I activity was defined as 100% at zero inhibitor concentration.
Figure 4. Effects of ATP and nigericin on rates of \( \text{H}_2\text{O}_2 \) production in mitochondria respiring on pyruvate and malate with different inhibitors. Incubation conditions as Figure 1. ATP was added at 2 mM and nigericin at 100 nM. Bars show means ± S.E.M. of measurements on 3 to 10 separate mitochondrial preparations. *Significant \((p < 0.05)\) effect of ATP. **Significant \((p < 0.05)\) effect of nigericin. ***Significant difference \((p < 0.05)\) vs. rotenone + ATP. AA: antimycin A, S: succinate, R: rotenone, nig: nigericin.

Figure 5. Effect of internal (matrix) pH \((\text{pH}_{in})\) on \( \text{H}_2\text{O}_2 \) production rate in the presence of pyruvate + malate, myxothiazol and ATP. Incubation conditions as Figure 3, but buffer pH was adjusted from 6.8 – 7.6 in steps of 0.4 pH units. \( \text{pH}_{in} \) in the absence of nigericin was determined in parallel incubations using the TPMP’ electrode, as described in experimental procedures. In the presence of nigericin \( \text{pH}_{in} = \text{pH}_{out} \). Points represent means ± S.E.M. of measurements on three separate mitochondrial preparations. Open symbols, nigericin absent; closed symbols, plus 100 nM nigericin.

Figure 6. Sites of high rates of superoxide production in complex I during forward electron transport. Data from Figure 4, with ATP present. Total superoxide production from reduced but uninhibited complex I (forward electron transport with stigmatellin) is small (all sites within the box). Only in the presence of Q site inhibitors does the rate of superoxide production reach that during reverse electron transport at similar \( \Delta \text{pH} \) values. If sites upstream of Q produced superoxide at significant rates then the rate with stigmatellin (or KCN or antimycin) would be similar to those seen with the Q site inhibitors rotenone, piericidin or myxothiazol, so superoxide production in the presence of Q site inhibitors is shown coming from the Q binding site. *Significant \((p \)
< 0.005) difference vs. stigmatellin. **Significant difference (p < 0.05) vs. rotenone.

rot: rotenone; pier: piericidin; myx: myxothiazol.

Figure 7. Model of superoxide production during forward (top) and reverse (bottom) electron transport. The degree of overlap between putative inhibition sites (or modes of inhibition), displayed as A B and C (25, 27) is not well understood. The coupling mechanism of complex I is not known, and we do not attempt to include proton translocation steps in our model, however, it does bear resemblance to aspects of other proposed models (29, 30). During forward electron transport, electrons from NADH are passed to Q in a quinone-reducing site via the flavin (FMN) and iron sulphur centres. The resulting semiquinone (white text in black box) is then reduced by an electron in a ΔpH-dependent generating step to form QH₂. If a Q cycle mechanism operates in complex I, this electron may come from QH₂ or semiquinone at the quinol oxidation site as shown, directly or via an iron sulphur centre. In the presence of substrate and ΔpH, this reduction step occurs freely, the semiquinone has a short lifetime, and superoxide production is low. In the presence of Q site inhibitors, however, this reduction step is blocked (myxothiazol) or strongly slowed (piericidin, rotenone), so the semiquinone accumulates and superoxide production increases. This situation is analogous to antimycin A inhibition of complex III and semiquinone accumulation at centre ‘o’. During reverse electron transport electrons are passed from QH₂ to NAD until the pool is fully reduced to NADH. In the absence of inhibitors, ΔpH drives the formation of semiquinone, which can only lose its unpaired electron to oxygen because all redox centres upstream of Q are already fully reduced. In the presence of Q site inhibitors, the QH₂ to semiquinone step is blocked by the lack of an electron acceptor, and superoxide production is low.
|                        | \( \Delta \psi \) | \( \Delta \rho \text{H} \) | \( \Delta \rho \) |
|------------------------|------------------|-----------------|----------------|
|                        | (mV)             | (mV)            | (mV)          |
| succinate              | 141 ± 4          | 40 ± 5          | 181 ± 5       |
| pyruvate + malate      | 143 ± 5          | 34 ± 4          | 177 ± 5       |
| pyruvate + malate + nigericin | 177 ± 5*        | 0               | 177 ± 5       |
| pyruvate + malate + rotenone | 0 ± 0*          | 0 ± 0*          | 0 ± 0*        |
| pyruvate + malate + rotenone + ATP | 127 ± 6        | 22 ± 5          | 149 ± 2       |
| pyruvate + malate + piericidin | 0 ± 0*          | 0 ± 0*          | 0 ± 0*        |
| pyruvate + malate + piericidin + ATP | 131 ± 4        | 13 ± 4*         | 144 ± 2*      |
| pyruvate + malate + myxothiazol | 0 ± 0*          | 0 ± 0*          | 0 ± 0*        |
| pyruvate + malate + myxothiazol + ATP | 130 ± 1*       | 11 ± 1*         | 141 ± 5*      |
| pyruvate + malate + stigmatellin | 0 ± 0*          | 0 ± 0*          | 0 ± 0*        |
| pyruvate + malate + stigmatellin + ATP | 126 ± 5*       | 25 ± 8          | 151 ± 4*      |
| pyruvate + malate + KCN | 0 ± 0*          | 0 ± 0*          | 0 ± 0*        |
| pyruvate + malate + KCN + ATP | 137 ± 4        | 29 ± 1          | 166 ± 3       |
| pyruvate + malate + antimycin A | 0 ± 0*          | 0 ± 0*          | 0 ± 0*        |
| pyruvate + malate + antimycin A + ATP | 118 ± 5*       | 25 ± 1          | 144 ± 5*      |

Table 1. Values of \( \Delta \rho \text{H}, \Delta \psi \) and \( \Delta \rho \) under various conditions. Potentials were determined using a TPMP\(^+\) electrode as described in experimental procedures. Standard incubation conditions were 120 mM KCl, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA (pH 7.2 and 37°C), 50 µg PHPA.ml\(^{-1}\), 4 U horseradish peroxidase.ml\(^{-1}\), 30 U SOD.ml\(^{-1}\) and 1.875 µM TPMP\(^+\). 4mM succinate, 2.5 mM pyruvate, 2.5 mM malate, 4 µM rotenone, 2 µM piericidin, 40 µM myxothiazol, 80 nM stigmatellin, 1 µM KCN, 2.5 µM antimycin A, 2 mM ATP and 100 nM nigericin were added where
shown. Values are means ± S.E.M. of measurements on 3 to 10 separate mitochondrial preparations. We define ΔpH as zero in the presence of nigericin. Positive-outside membrane potentials and acid-outside pH gradients are given a positive sign. *Significant (p < 0.05) difference from pyruvate + malate.
Figure 1

Rate of H$_2$O$_2$ production (nmol H$_2$O$_2$.min$^{-1}$.mg protein$^{-1}$)
Figure 2

Rate of H$_2$O$_2$ production (nmol H$_2$O$_2$·min$^{-1}$·mg protein$^{-1}$)
Figure 3
Figure 4

Rate of H$_2$O$_2$ production (nmol H$_2$O$_2$.min$^{-1}$.mg protein$^{-1}$)
Figure 5
Figure 6

NADH → FMN → N1a N1b → N2 → Q QH• → Q QH2

NAD+ → N3 N4 N5

Rate of H2O2 production (nmol H2O2 min⁻¹ mg protein⁻¹)

stigmatellin  rot pier myx

* * **
Figure 7
Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I)

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J. Biol. Chem. published online July 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406576200

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