Complex I is the Major Site of Mitochondrial Superoxide Production by Paraquat

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Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; PQ) is widely used as a redox cycler to stimulate superoxide production in organisms, cells, and mitochondria. This superoxide production causes extensive mitochondrial oxidative damage, however, there is considerable uncertainty over the mitochondrial sites of paraquat reduction and superoxide formation. Here we show that in yeast and mammalian mitochondria, superoxide production by paraquat occurs in the mitochondrial matrix, as inferred from manganese superoxide dismutase-sensitive mitochondrial DNA damage, as well as from superoxide assays in isolated mitochondria, which were unaffected by exogenous superoxide dismutase. This paraquat-induced superoxide production in the mitochondrial matrix required a membrane potential that was essential for paraquat uptake into mitochondria. This uptake was of the paraquat dication, not the radical monocation, and was carrier-mediated. Experiments with disrupted mitochondria showed that once in the matrix paraquat was principally reduced by complex I (mammals) or by NADPH dehydrogenases (yeast) to form the paraquat radical cation that then reacted with oxygen to form superoxide. Together this membrane potential-dependent uptake across the mitochondrial inner membrane and the subsequent rapid reduction to the paraquat radical cation explain the toxicity of paraquat to mitochondria.

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; PQ) is used to increase superoxide (O2−) flux when investigating oxidative stress (reviewed in Refs. 1 and 2). The paraquat dication (PQ2+) accepts an electron from a reductant to form the paraquat monocation radical (PQ•−), which then rapidly reacts with O2 (k = 7.7 × 109 M−1 s−1) (3) to produce O2− and regenerate PQ2+ (Fig. 1A). This redox cycling is a proximal cause of PQ toxicity, as indicated by the protection against PQ by superoxide dismutase (SOD) overexpression or administration of SOD mimetics (4–8), and by the PQ hypersensitivity caused by SOD deficiency (9–11).

PQ has been used to generate O2− in systems ranging from isolated mitochondria (12–15) and cultured mammalian cells (4, 14, 16, 17), to whole organisms including Saccharomyces cerevisiae (18–21), Caenorhabditis elegans (22, 23), Drosophila melanogaster (9, 24–26), and rodents (6, 11, 27). In many of these studies, PQ increases mitochondrial oxidative damage; for example, mitochondrial expression of human peroxiredoxin 5 protects yeast more effectively against PQ toxicity than expression in the cytosol (19); flies overexpressing catalase in mitochondria are resistant to PQ, whereas enhancement of cytosolic catalase was not protective (24); RNA interference silencing of MnSOD (the isoform of superoxide dismutase located in the mitochondrial matrix) in flies causes hypersensitivity to PQ (9), mice heterozygous for MnSOD show greater sensitivity to PQ than wild-type (11), and mitochondrial swelling is one of the earliest ultrastructural changes upon PQ exposure in vivo (28, 29). Therefore the interaction of PQ with mitochondria is an important component of its toxicity, and PQ is used in experimental models of Parkinson disease to generate mitochondrial oxidative damage (30). Consequently, there is considerable interest in identifying the sites of PQ2+ reduction associated with mitochondria and in determining whether O2− production by PQ occurs within mitochondria, or if it takes place outside and then passes into the matrix. The very negative reduction potential of PQ (PQ2+/PQ, E0 = −446 mV) (1) severely restricts its pool of possible intracellular reductants. However, there are a number of proposed sites for PQ2+ reduction both inside and outside mitochondria including NAD(P)H-dependent flavoenzymes, such as microsomal NADPH-cytochrome P450 reductase (31, 32), NADH-cytochrome b5 oxidoreductase and NADH-coenzyme Q oxidoreductase of the mitochondrial outer membrane (28, 33), and complex I of the mitochondrial inner membrane (34). Here we show that PQ2+ is taken up across the mitochondrial inner membrane by a carrier-mediated and membrane potential (Δψm)-dependent process, and that once in the matrix PQ2+ is reduced to PQ•− by complex I in mammalian mitochondria and by NADPH dehydrogenases in yeast. The PQ•− then reacts with oxygen to form O2− and cause mitochondrial oxidative stress.

EXPERIMENTAL PROCEDURES

Chemicals—Coelenterazine (CLZ; 2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-8-benzyl-imidazo[1,2-a]pyrazin-3-(7H)-one) was from Calbiochem. Amplex Red was from Molecular Probes. 1-Methyl-4-phenylpyridinium iodide (MPP+) was from...
Production was inferred from the rate of aconitase in mitochondrial preparations mixed with 10 mg/ml cultures as described previously (37, 38). The protein concentration was determined by the Biuret method with BSA as a standard (41). Rat liver and heart mitochondrial incubations were performed in KCl buffer (120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.2, KOH). When required, mitochondria were disrupted with a sonicator (Misonix 3000, setting 3) for 3 × 5-s periods at 30-s intervals on ice. Bovine heart mitochondrial membranes were prepared by mechanical disruption of isolated bovine heart mitochondria (42), and were resuspended in KP buffer (50 mM KP, 1 mM EGTA, 100 μM diethylenetriaminepentaacetic acid, 100 μM neocuprine, pH 7.2, KOH).

**Measurement of Mitochondrial Membrane Potential**—Mitochondrial membrane potential (ΔΨm) was measured from the uptake of TPMP+ (43). Yeast mitochondria (0.1–0.4 mg of protein ml−1) were incubated for 3 min at 30 °C in 1 ml of mannitol buffer with substrate and 1 μM TPMP+ including 25 nCi ml−1 of [3H]TPMP+, and then pelleted by centrifugation (2 min at 16,000 g). Radioactivity in the pellet and supernatant was measured by liquid scintillation analysis (44), and the ΔΨm was derived from the Nernst equation (43). The mitochondrial matrix volume was taken as 1.8 μl of protein−1, and data were corrected for the 60% of TPMP+ assumed to be membrane-bound (published in Ref. 45; calculated according to the method in Ref. 43).

**Superoxide and H2O2 Assays**—Aconitase activity was measured spectrophotometrically by a coupled enzyme assay (46), and O2− production was inferred from the rate of aconitase inactivation (15). O2− was also assayed by chemiluminescence of CLZ in a luminometer (Berthold AutoLumatPlus LB 953) over 5 min with cumulative readings for 5 s every 30 s (47, 48) and was expressed as relative light units s−1. Samples were incubated in a 1-ml volume at 30 °C with 2 μM CLZ. H2O2 efflux from heart mitochondria was assayed using a fluorometer (Shimadzu RF-5301) by incubating heart mitochondria at 37 °C in a stirred 2.5-ml volume with 5 units ml−1 horseradish peroxidase and 50 μM Amplex Red (15), and was calibrated against H2O2 standards.

**Yeast Mitochondrial DNA Damage Assays**—Cytoplasmic petite mutants were identified by the colorimetric tetrazolium overlay technique (49). Yeast were cultured overnight in 5 ml of YPD medium, from A600 ~ 0.1 until A600 ~ 10. An aliquot was diluted into H2O, spread onto YPD plates, and incubated at 30 °C for 2 days until colonies formed. Plates were then overlaid with 20 ml of 1.5% (w/v) agar dissolved in 67 mM NaP, pH 7.0, and supplemented with 0.1% (w/v) 2,3,5-triphenyltetrazolium chloride, incubated for a further 1 h at 30 °C and then scored for red (respiration-competent) or white (petite) colonies. Approximately 5,000 colonies were scored for each condition.

Mitochondrial DNA (mtDNA) point mutations were measured by the ethryrocytosis resistance (EryR) assay, based on the principle that specific point mutations in the mtDNA-encoded 21S ribosomal gene can confer EryR (50, 51). Wild-type yeast (strain CEN.PK2-1C) were streaked onto YPG agar and incubated at 30 °C for 2–3 days until single colonies formed. Yeast cultures were then prepared by inoculating 5 ml of YPG with a guishable from that of freshly isolated mitochondria (data not shown). Mitochondria from rat liver and rat heart were prepared fresh by homogenization and differential centrifugation (40). The protein concentration was determined by the Biuret method with BSA as a standard (41). Rat liver and heart mitochondrial incubations were performed in KCl buffer (120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.2, KOH). When required, mitochondria were disrupted with a sonicator (Misonix 3000, setting 3) for 3 × 5-s periods at 30-s intervals on ice. Bovine heart mitochondrial membranes were prepared by mechanical disruption of isolated bovine heart mitochondria (42), and were resuspended in KP buffer (50 mM KP, 1 mM EGTA, 100 μM diethylenetriaminepentaacetic acid, 100 μM neocuprine, pH 7.2, KOH).

**Interaction of Paraquat with Mitochondria**

Paraquat (PQ2+) undergoes univalent reduction to generate the paraquat radical (PQ·), then reacts rapidly with O2 to produce superoxide (O2−). The PQ concentration gradient (0–1 mM), incubated for 1 week on each plate. Yeast were cultured in the following liquid media: YPG (1% yeast extract, 2% peptone, 2% glucose), and YPG (0.01–10 mM) and incubated at 30 °C with shaking at 250 rpm. Radioactivity in the pellet and supernatant was measured by liquid scintillation analysis (44), and the ΔΨm was derived from the Nernst equation (43). The mitochondrial matrix volume was taken as 1.8 μl of protein−1, and data were corrected for the 60% of TPMP+ assumed to be membrane-bound (published in Ref. 45; calculated according to the method in Ref. 43).

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Interaction of Paraquat with Mitochondria

**FIGURE 2. Effect of PQ on yeast cultures and induction of mtDNA damage.** A, wild-type (CEN.PK2-1C) yeast were cultured in either YPD (glucose) or YPG (glycerol) medium, with a range of PQ concentrations (0.01–100 μM). The initial cell density was adjusted to A595 = 0.1, and growth was measured spectrophotometrically after 48 h. Data are expressed as a percentage of the appropriate controls without PQ and are the mean ± S.D. of three independent experiments. Statistical significance was calculated with a Student’s two-tailed t test. N/S, not significant; p > 0.05; *, p < 0.05; **, p < 0.01.

The PQ-sensitive strain was detected by following an increase in A440 over time with a spectrophotometer (DW-2000 SLM-Aminco), equipped with stirring and thermostatted at 30 °C. The suspension was rendered anaerobic by purging with argon and sealing the 3-ml quartz cuvette. Following a 5–10 min incubation, samples were subjected to absorbance readings (500–700 nm), first while still under anaerobic conditions and then following exposure to air. Difference spectra ± O2 were then determined to diagnose PQ radical formation.

**Mitochondrial Uptake of PQ**—Ion-selective electrodes were constructed and used as described (53, 54), except that the plasticizer dioctyl phthalate was replaced by 2-fluoro-2-nitrodiphenyl ether (55), which greatly improved membrane selectivity for PQ. Electrodes were characterized for either PQ or MPP+ by filling and soaking overnight in a 10 μM aqueous solution. Incubations were performed in a stirred 3-ml chamber, thermostatted at 30 °C. The PQ-electrode response was linear with the log10[PQ2+], with a slope of 29.4 ± 1.0 mV per decade (mean ± S.D.; n = 5, with at least three different PQ-electrodes), which is consistent with the 30.02 mV predicted by the Nernst equation for a dication species at 30 °C. Alternatively, uptake of PQ by mitochondria was measured by centrifuging the incubations (2 min at 16,000 × g) and quantifying the amount of PQ in the mitochondrial pellet, either by EPR (under anaerobic conditions following conversion to PQ+) with excess sodium dithionite), or by using radiolabeled [14C]PQ with liquid scintillation analysis. In some cases, dual isotope counting ([1H]TPMP and [14C]PQ) was performed to control for any effect of PQ uptake on Δψm.

**RESULTS AND DISCUSSION**

PQ Increases Mitochondrial Matrix O2 within Intact Yeast—Growth of wild-type yeast on the non-fermentable substrate glycerol was more PQ-sensitive than on the fermentable substrate glucose (Fig. 2A), indicating that PQ disrupted mitochondrial function. This disruption was due to O2− within mitochondria, as growth of yeast lacking mitochondrial SOD (Δsod2) on the non-fermentable substrate lactate was far more PQ-sensitive than that of the wild-type strain (Fig. 2B) (56). To see if PQ caused damage in the mitochondrial matrix, we stud-
increased the formation of petite colonies in the ∆sod2 yeast strain and not the wild type (Fig. 2D). Together these data confirm that PQ causes mitochondrial oxidative damage by increasing matrix O$_2^\ast$.

Matrix O$_2^\ast$ Production by PQ in Isolated Yeast Mitochondria Requires a Membrane Potential and a Respiratory Substrate—We next tested the effect of PQ on O$_2^\ast$ production in isolated yeast mitochondria as measured by the O$_2^\ast$-specific chemiluminescence of the membrane-permeant probe CLZ. In the presence of the respiratory substrate ethanol, PQ caused a dramatic dose-dependent increase in O$_2^\ast$ production by ∆sod2 mitochondria, but not wild-type mitochondria (Fig. 3A). Surprisingly, this O$_2^\ast$ production was completely blocked by abolishing the mitochondrial membrane potential (∆ψm) with the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Fig. 3A). Addition of exogenous SOD did not affect PQ-induced O$_2^\ast$ production (Fig. 3B), consistent with PQ generating O$_2^\ast$ inside the mitochondrial matrix.

The CLZ assay did not detect matrix O$_2^\ast$ production in wild-type yeast mitochondria (Fig. 3A), because CLZ cannot compete for O$_2^\ast$ against MnSOD ($k \sim 10^7$ and $10^9$ m$^{-1}$ s$^{-1}$, respectively) (58, 59). Therefore to assess O$_2^\ast$ formation in wild-type yeast mitochondria, we measured the inactivation of the matrix enzyme aconitase, which is significantly more reactive with O$_2^\ast$ ($k \sim 10^9$ m$^{-1}$ s$^{-1}$) (60) than CLZ and will not react with O$_2^\ast$ outside the mitochondrial matrix. Aconitase inactivation also showed a dose-dependent increase in O$_2^\ast$ production with PQ within isolated yeast mitochondria that required a respiratory substrate, was blocked by FCCP (Fig. 3C), and was unaffected by exogenous SOD (Fig. 3D).

A similar pattern of O$_2^\ast$ production was obtained by both the CLZ
Interaction of Paraquat with Mitochondria

![Graphs showing the effect of paraquat (PQ) on mitochondrial 
H₂O₂ production](image)

**FIGURE 4.** H₂O₂ production by PQ in mammalian mitochondria: effect of respiratory substrate, uncoupler, and respiratory inhibitors. A–D, H₂O₂ production from rat heart mitochondria was determined fluorometrically by the Amplex Red assay. Heart mitochondria (0.2 mg of protein ml⁻¹) were incubated at 37 °C in KCl buffer supplemented with 0.01% (w/v) BSA. Substrate (5 mM succinate or 5 mM glutamate/malate) and PQ (0.1 or 1 mM) were added as indicated. Data are representative of typical traces, repeated at least twice. A and B, effect of the uncoupler FCCP (1 μM). C and D, effect of the complex I inhibitor rotenone (4 μg ml⁻¹). E, rates of H₂O₂ efflux from rat heart mitochondria, determined from the above traces. Data are the mean ± S.D. of three to four determinations.

The interaction of PQ with mammalian and yeast mitochondria was similar to that of yeast mitochondria. Interest-ingly, the rate of PQ-induced aconitase inactivation was greater for heart mitochondria respiring on succinate than on the complex I-linked substrates glutamate/malate, and the complex I inhibitor rotenone prevented O₂⁻ formation by PQ in the presence of succinate (Fig. 3E).

To assess the intriguing substrate and ∆ψₘ, dependence of PQ-induced O₂⁻ production in real time, we measured H₂O₂ efflux, derived from matrix O₂⁻, in heart mitochondria (61). In the absence of PQ, energizing heart mitochondria with glutamate/malate resulted in negligible H₂O₂ efflux (Fig. 4A) that increased substantially when succinate was the substrate (Fig. 4B). This increase with succinate was due to the high ∆ψₘ and fully reduced coenzyme Q pool driving reverse electron transport through complex I that led to O₂⁻ production (61). O₂⁻ production from succinate was blocked by abolishing the ∆ψₘ with FCCP (Fig. 4B), with the complex III inhibitor stigmatellin (data not shown), or by inhibiting electron entry from the coenzyme Q pool into complex I with rotenone (Fig. 4D).

Addition of PQ to mitochondria respiring on glutamate/malate increased H₂O₂ efflux (Fig. 4C) and this was decreased by abolishing the ∆ψₘ with rotenone (Fig. 4C) or stigmatellin (data not shown), however, these inhibitors were only effective when present from the beginning of the incubation; when they were added after H₂O₂ production was well established they increased H₂O₂ efflux (Fig. 4C and data not shown). In contrast, FCCP blocked the PQ-induced H₂O₂ efflux when added early or late in the incubation (Fig. 4A). This difference may occur because both rotenone and stigmatellin are respiratory inhibitors that cause a build-up of electrons that can be used to drive H₂O₂ efflux.

**PQ Increases O₂⁻ Production by Mammalian Mitochondria**—To see if the interaction of PQ with mammalian and yeast mitochondria was similar, we next investigated its effects on O₂⁻ production within isolated rat heart mitochondria. As with yeast mitochondria, there was a dose-dependent inactivation of aconitase by PQ that required a respiratory substrate, was blocked by FCCP (Fig. 3E), and was unaffected by exogenous SOD (Fig. 3F). Thus PQ-induced O₂⁻ production by mammalian mitochondria is similar to that of yeast mitochondria. Interestingly, the rate of PQ-induced aconitase inactivation was greater for heart mitochondria respiring on succinate than on the complex I-linked substrates glutamate/malate, and the complex I inhibitor rotenone prevented O₂⁻ formation by PQ in the presence of succinate (Fig. 3E).

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Interaction of Paraquat with Mitochondria

The movement of electrons into complex I driven by reverse electron transport. This shows that complexes II and III are not involved in the production of O$_2^\cdot$ by PQ. These findings indicate that in mammalian mitochondria respiring on succinate, O$_2^\cdot$ production by PQ occurs at complex I and requires a $\Delta\psi_{mt}$ both to cause the accumulation of PQ into mitochondria and to drive electrons from the coenzyme Q pool into complex I by reverse electron transport. Thus the main site of PQ reduction in mammalian mitochondria is likely to be on complex I.

Sites of O$_2^\cdot$ Production by PQ within Mitochondria—To further investigate the sites of PQ$^\cdot$ generation in mammalian mitochondria and to determine whether a $\Delta\psi_{mt}$ was essential for this, we measured O$_2^\cdot$ production by bovine heart mitochondrial membranes using the CLZ chemiluminescence assay. In this system, the respiratory complexes are exposed to PQ without requiring uptake into the mitochondrial matrix and reverse electron transport into complex I is not possible.

PQ did not enhance O$_2^\cdot$ production by bovine heart mitochondrial membranes respiring on succinate (Fig. 5A), although it was possible to stimulate O$_2^\cdot$ production by complex III with the inhibitor antimycin A. This confirms that complex III does not interact with PQ$^{2+}$ to produce O$_2^\cdot$. Similar results were obtained when measuring H$_2$O$_2$ efflux from freeze-thawed or sonicated rat heart mitochondria oxidizing succinate (data not shown). These findings and the FCCP- and rotenone-sensitive O$_2^\cdot$ production in intact mitochondria respiring on succinate in the presence of PQ (Fig. 4, B and D) indicate that succinate induces O$_2^\cdot$ production from PQ via complex I through $\Delta\psi_{mt}$-dependent reverse electron transport (61).

Incubation of bovine heart mitochondrial membranes with NADH led to a dramatic PQ dose-dependent stimulation of O$_2^\cdot$ production (Fig. 5B). In this situation rotenone stimulated O$_2^\cdot$ production by increasing the reduction state of complex I, consistent with the H$_2$O$_2$ assay results obtained for intact heart mitochondria (Fig. 4C). The flavoprotein inhibitor diphenyleneiodonium (DPI) prevented the PQ-induced O$_2^\cdot$ production (Fig. 5B). Furthermore, NADPH resulted in negligible O$_2^\cdot$ production compared with that by NADH (Fig. 5B). These data indicate that complex I can reduce PQ$^{2+}$ to PQ$^\cdot$ thereby producing O$_2^\cdot$, and are consistent with an earlier report with isolated complex I (34).

We cannot entirely eliminate the possibility that there are other sites of PQ$^{2+}$ reduction in mammalian mitochondria. However, the data in
Interaction of Paraquat with Mitochondria

Mitochondrial NADPH dehydrogenases in yeast mitochondria. There is no requirement for a Δψm, to reduce PQ2+ to PQ+ once the PQ is within mitochondria and when NADH is used as the substrate; however, for mammalian mitochondria respiring on succinate, PQ reduction requires a Δψm, to drive electrons from the coenzyme Q pool into complex I.

Investigating the Δψm Dependence of PQ2+ Uptake by Mitochondria—The above findings indicate that PQ2+ in the mitochondrial matrix can increase matrix O2− production in the absence of a Δψm. This suggests that the Δψm dependence of PQ-induced O2− production occurs because the Δψm, drives PQ uptake into the matrix. Many lipophilic cations and dications are taken up electrophoretically into mitochondria by direct passage through the phospholipid bilayer driven by the Δψm, in response to the Nernst equation (44, 62). Significantly, the structurally similar compound MPP+ (Fig. 1B) is accumulated by mitochondria in a Δψm-dependent manner by direct passage through the inner membrane (54). We confirmed the Δψm-dependent uptake of MPP+ into energized yeast mitochondria using a MPP+-selective electrode and showed that this uptake could be further stimulated by the lipophilic anion tetrathylborate (TPB−; Fig. 6A), which facilitates the direct passage of lipophilic cations across the membrane (63). However, similar experiments using a PQ-selective electrode showed no measurable PQ uptake into energized yeast mitochondria (Fig. 6B) or mammalian mitochondria (data not shown). Furthermore, substoichiometric amounts of TPB− did not stimulate accumulation into mitochondria (data not shown). Therefore there is no bulk uptake of PQ2+ into mitochondria, unlike the structurally related compound MPP+. The reason for this marked difference is presumably the double charge on PQ2+ relative to MPP+, which will cause a 4-fold increase in the Born energy for its movement across the membrane and thereby result in a far larger activation energy required for the movement of PQ2+ through biological membranes relative to MPP+ (64).

However, whereas the PQ-selective electrode eliminates the possibility of bulk movement of PQ2+ across the membrane, it will only detect large scale redistributions. To see if there was a low capacity uptake system that led to Δψm-dependent uptake of PQ2+ into mitochondria, we utilized more sensitive detection methods based on either EPR (Fig. 7, A and B) or radiolabeled PQ and were able to show time-dependent uptake of PQ into yeast mitochondria (Fig. 7C). This uptake was Δψm-sensitive as the presence of the uncoupler FCCP or the complex III inhibitor myxothiazol at the start of the incubation completely prevented PQ uptake (Fig. 7D). Furthermore, the K+/H+ antiporter nigericin (1 µM), which increases Δψm, by converting the mitochondrial pH gradient into a Δψm, stimulated PQ uptake (data not shown). Addition of FCCP at the end of the incubation did not lead to immediate reversal of the accumulation (Fig. 7D), unlike the situation with MPP+ (Fig. 6A). PQ uptake into yeast mitochondria was also driven by the respiratory substrates succinate and glycerol 3-phosphate (data not shown). Qualitatively similar data were obtained for mammalian mitochondria, which also showed Δψm-dependent uptake of PQ that was prevented, but not reversed by uncoupling (Fig. 7E).

When the initial rate of uptake of PQ2+ into yeast mitochondria was plotted against the initial [PQ2+] (1.25–20 mM) an apparent saturation was found with half-maximal uptake at about 3.5 mM (Fig. 7F). Whereas these data are consistent with a mediated uptake system that is saturable and of low affinity and capacity, a definitive interpretation of these kinetic data is not possible. This is because incubating mitochondria with increasing PQ2+ concentrations above ~1 mM for yeast mitochondria gradually lowers the Δψm (data not shown). Consequently the decrease in uptake with increasing [PQ2+] may be due to a combination of saturation and the progressive decrease in Δψm as the PQ2+ concentration increases. This interpretation is supported by the lack of fit of these data to any of the standard transformations of Michaelis-Menten kinetics.

**FIGURE 6. Investigating mitochondrial uptake of PQ with an ion-selective electrode.** A, Δψm-dependent uptake of the structurally similar MPP+ ion (Fig. 1B) by yeast mitochondria is illustrated as a positive control. The MPP+-selective electrode was calibrated with three successive additions of 10 µM MPP+ (arrowheads). Isolated wild-type yeast mitochondria (Mitos) were incubated at 0.4 mg of protein ml−1 in mannitol buffer, energized with substrate (5 mM ethanol), and then uncoupled with FCCP (1 µM). The presence of TPB− (5 µm; dashed line) both accelerates and increases the extent of MPP+ uptake. Data are representative of typical traces. B, as for A, except that a PQ-selective electrode was calibrated with three successive additions of 10 µM PQ2+ (arrowheads). The presence of TPB− had no effect (data not shown).
Interaction of Paraquat with Mitochondria

Nature of the Mitochondrial PQ Uptake System.—The data in Fig. 7 are consistent with, but not proof of, carrier-mediated uptake of PQ into mitochondria. Carrier-mediated PQ trans-

port has been reported in lung alveolar epithelial cells by the polyamine transport system, which is inhibited by putrescine (65), and across the blood-brain barrier via the neutral amino acid transporter, which is inhibited by l-valine (66). To explore whether PQ uptake into mitochondria occurs through a car-

rier in the mitochondrial inner membrane, we focused on yeast as the activity was greater, deletion libraries were available, and many mitochondrial carriers are similar in yeast and mammals.

If uptake is carrier-mediated then structurally similar compounds such as MPP⁺, diquat, and putres-

cine (Fig. 1B) might inhibit PQ uptake. To ensure that any effects of these compounds on PQ uptake were not due to effects on Δψₘ, we measured the uptake of [¹⁴C]PQ and [³H]TPMP⁺ simultaneously by dual label scintillation counting. MPP⁺, diquat, and putrescine all decreased PQ uptake in a concentration-dependent manner without severely affecting the Δψₘ (Fig. 8A).

Neither vanadate (an inhibitor of ABC transporters) nor oligomycin (an inhibitor of ATP synthase) affected PQ uptake into mitochondria (Fig. 8B), suggesting that uptake does not depend on intrami-

tochondrial ATP. The thiol alkylating agent N-ethylmaleimide inhibits many mitochondrial transporters (e.g. Ref. 67) and it had a considerably greater effect on PQ uptake than it did on Δψₘ (Fig. 8C).

These data are consistent with a mitochondrial transporter mediating Δψₘ-dependent, but ATP-inde-

pendent, uptake of PQ into mitochondria. Possible candidates are the mitochondrial carrier family that has 35 members in yeast mitochondria (68, 69). We hypothesized that yeast lacking the putative mito-

chondial PQ carrier would show resistance to PQ on non-fermenta-

ble medium. We screened the

strains in which the genes for proven or putative mitochondrial carriers had been deleted. The 0–1 mM PQ gradient in the

plates showed the PQ sensitivity of the wild-type strain and
detected a number of strains such as Δctp1 that were clearly resistant to PQ (Fig. 8D). Of the 29 deletion strains tested, 8 were resistant to PQ; however, PQ uptake by mitochondrial isolated from these strains was indistinguishable from wild-type (Table 1). Thus deletion of these carriers protects against mitochondrial damage by PQ through other mechanisms. For example, deletion of the citrate transporter Ctp1p may protect NADH, but not by succinate or NADPH (Fig. 9C). Therefore under conditions where there was extensive O$_2^-$ production within mitochondria (Figs. 3 and 4), there was negligible reduction of PQ$_{2+}$ to PQ$^-$ and consequent O$_2^-$ production outside mitochondria. Exogenous NADH and NADPH cannot cross the intact mitochondrial inner membrane and may reduce PQ$_{2+}$ via enzymes such as NADH-cytochrome $b_5$ oxidoreduc-
tase and NADH-coenzyme Q oxidoreductase of the mitochondrial outer membrane (28, 33). However, in our intact heart mitochondrial preparations, ~90% of NADH consumption was inhibitable by rotenone (data not shown), indicating that most of the observed extramitochondrial PQ reduction by NADH was due to a small proportion of damaged, NADH-permeable mitochondria. This further limits the contribution of PQ reduction and O₂⁻ formation outside mammalian mitochondria. Thus, whereas there may be some NADH/NADPH-dependent PQ²⁺ formation within cells contributing to O₂⁻ formation, this seems likely to be far less significant than O₂⁻ formation within mitochondria.

Extramitochondrial reduction of PQ²⁺ to PQ⁻ could contribute to O₂⁻ production within mitochondria by enhancing the net uptake of PQ into mitochondria. The PQ²⁺ dication is not taken up into energized mitochondria directly through the mitochondrial inner membrane because its double charge greatly increases its activation energy for movement through the hydrophobic core of the membrane (44, 64). However, the Born energy component of this activation energy is proportional to the square of its charge and for the PQ²⁺ radical cation will be 4-fold lower than the PQ²⁺ dication. The structure and charge of the PQ⁻ monocation is very similar to the MPP⁻ monocation (Fig. 1B), which is taken up directly through the membrane (see Ref. 54 and Fig. 6A). Thus the PQ⁻ cation could probably accumulate into mitochondria by direct movement through the membrane driven by the Δψₘ. However, its competing rapid reaction with O₂ (7.7 × 10⁸ M⁻¹ s⁻¹) even at 30 μM O₂, a plausible oxygen concentration in cells (e.g. Ref. 71), means that its half-life is only ~30 μs, potentially making this pathway negligible. To see if PQ⁻ uptake by energized mitochondria could contribute to net PQ uptake, we incubated energized yeast mitochondria + NADPH, to enhance PQ⁻ formation outside mitochondria (Fig. 9B). Under these conditions, no increase in net PQ uptake was observed (data not shown), even when PQ⁻ was generated in the vicinity of mitochondria. To summarize, the increase in mitochondrial O₂⁻ caused by PQ is predominantly due to O₂⁻ generated within mitochondria.

**CONCLUSIONS**

Here we have demonstrated how PQ can act to increase intramitochondrial O₂⁻ production (Fig. 10). The major mode of O₂⁻ production requires the Δψₘ-dependent uptake of the PQ²⁺ dication by energized mitochondria. This uptake does not seem to involve movement of the dication through the phospholipid bilayer, but instead is carrier-mediated by a putative PQ carrier protein or proteins, which require a Δψₘ to drive PQ accumulation into the mitochondria. Once within mitochondria, the PQ is largely retained, even after the Δψₘ has been abolished. This Δψₘ dependence of PQ uptake into mito-

### TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>ORF</th>
<th>Description</th>
<th>PQ resistance against wild-type</th>
<th>PQ uptake by isolated mitochondria</th>
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<td>AAC1</td>
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<td>YMR070W</td>
<td>YMR070W</td>
<td>Putative ABC transporter</td>
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<td>ND</td>
</tr>
</tbody>
</table>

*a* Deleted gene.

*b* Corresponding open reading frame.

*c* Carrier/transporter function/substrate.

*d* Yes, same as wild type; ++/+++/+++/, more resistant to PQ than wild-type (low/medium/high).

*e* ND, not determined.

**Interaction of Paraquat with Mitochondria**

Experiments were performed as described under “Experimental Procedures.” A typical plate is shown in Fig. 8D. The information for Footnotes a–c was compiled from the Saccharomyces Genome Database (yeastgenome.org) (68). Candidates have been grouped as belonging to the mitochondrial carrier family and other transporters (mainly ABC transporters). To assess PQ resistance growth of mutant strains was compared against the isogenic wild-type control on each plate. Note that it was not possible to screen all mitochondrial carriers and transporters, because some deletion strains are lethal and others are non-viable on non-fermentable media. Mitochondria were isolated from selected strains, and PQ uptake was FCCP was tested by the EPR technique. In all cases, mitochondria isolated from carrier mutants showed Δψₘ-dependent uptake of PQ that was indistinguishable from that of wild-type mitochondria.
Interaction of Paraquat with Mitochondria

The paraquat dication PQ$_2^+$ enters mitochondria via a putative carrier-mediated pathway driven by the mitochondrial membrane potential ($\Delta\psi_m$). Once inside the matrix, PQ$_2^+$ is reduced to the monocation radical PQ$^+$ at complex I in the respiratory chain by electrons donated from NADH. Alternatively, in the presence of a $\Delta\psi_m$, PQ$_2^+$ can be generated at complex I by reverse electron transport whereby electrons from the CoQ pool (e.g., from the substrate succinate) are driven into complex I by the $\Delta\psi_m$. By analogy with the structurally similar MPP$^+$, PQ$_2^+$ generated outside mitochondria may be taken up into the matrix by direct passage across the membrane. However, the short lifetime of PQ$^+$ at physiological $[O_2]$ probably limits the contribution of this process to PQ uptake into mitochondria.

Mitochondria may help explain findings in the literature that flies with targeted expression of human uncoupling protein 2 in the mitochondria of neurons show increased resistance to PQ (25) and that treatment of yeast cultures with the complex III inhibitor antimycin A causes PQ resistance (72), presumably because the inhibition of $\Delta\psi_m$ prevents mitochondrial uptake.

The PQ within mitochondria then goes on to produce O$_2^-$ through its reduction by intramitochondrial NAD(P)H dehydrogenases to the PQ$^+$ radical cation that reacts rapidly with O$_2$ to form O$_2$•- and regenerates the PQ$_2^+$ dication. In yeast this reduction is NADPH-dependent and involves intramitochondrial NADPH dehydrogenases. In mammalian mitochondria the only major source of reduction of PQ$_2^+$ to PQ$^+$ is complex I. Once PQ$_2^+$ is accumulated into mitochondria driven by the $\Delta\psi_m$, it can interact directly with complex I respiring on succinate to form O$_2$•- and PQ$^+$. In contrast, PQ$^+$ reduction and consequent O$_2$•- formation is enhanced by rotenone, presumably due to greater reduction of the electron donating sites upstream of the rotenone inhibition site. In contrast, mitochondria also produce extensive amounts of O$_2^-$ when respiring on succinate. However, this O$_2^-$ production does not occur in mitochondrial membranes respiring on succinate, requires a $\Delta\psi_m$, and even after significant amounts of PQ have been accumulated by mitochondria, and is inhibited by rotenone. Therefore this PQ$_2^+$ reduction is due to the action of complex I.

The requirement for a $\Delta\psi_m$ and blocking by rotenone is similar to the production of O$_2^-$ from complex I by reverse electron transport and suggests that under conditions of a reduced coenzyme Q pool and a high $\Delta\psi_m$, electrons are forced into complex I where reduction of PQ$_2^+$ takes place. Whether this is the same
site as that of $O_2^\bullet -$ production by complex I during reverse electron transport or if this site is the same for $PQ^{2+}$ reduction during NADH oxidation is currently unclear, but investigation of this point may help shed light on the mechanisms of $O_2^\bullet -$ production by complex I. That complex I is the major site of $O_2^\bullet -$ production within mitochondria exposed to PQ, and that complex I damage is a major factor in idiopathic Parkinson disease, strongly supports PQ toxicity as a useful model for Parkinson disease (73).

While this manuscript was in preparation another investigation of the interaction of PQ with mammalian mitochondria was published by Castello et al. (74). That article also reported that mitochondria are a major site of $\Delta\psi_{m}$-dependent reactive oxygen species formation by PQ. However, their suggestion that complex III in the respiratory chain is the major site of PQ reduction contrasts with our findings that complex I is the only significant site of PQ reduction within mammalian mitochondria. Furthermore, as the reduction potential for $PQ^{2+}/PQ^+$ ($-446$ mV) is far lower than the potentials spanned by complex III ($-250$ mV) and the lowest $Em_{Q_{m}}$ for a Q cycle intermediate (the ubiquinolate radical anion/ubiquinone couple) is only $-160$ mV (75), complex III is unlikely to reduce PQ. Another difference between Castello et al. (74) and our findings is their report that succinate and PQ addition to isolated mitochondria leads to $H_2O_2$ production, which is only partly inhibited by rotenone, whereas we find complete inhibition. It may be that brain mitochondrial preparations contain endogenous, NADH-linked substrates that increase rotenone-sensitive $PQ$ reduction. The other difference is that we show uptake of PQ into mitochondria, which is $\Delta\psi_{m}$-dependent in both yeast and mammalian mitochondria, whereas Castello et al. (74) report that PQ uptake by mitochondria is not dependent on $\Delta\psi_{m}$. These differences may arise from the greater sensitivity of the two methods we employed, namely the uptake of radiolabeled compound and analysis of uptake by EPR, compared with the high pressure liquid chromatography determination used by Castello et al. (74).

In conclusion, we have shown that PQ causes mitochondrial oxidative damage in mammalian systems following its $\Delta\psi_{m}$-dependent accumulation into the mitochondrial matrix. Within the matrix, PQ$^{2+}$ is reduced by complex I either via electrons from NADH-linked substrates or from the coenzyme Q pool driven into complex I by reverse electron transport. The PQ$^+$ radical thus formed rapidly reacts with $O_2$ to give $O_2^\bullet -$ and due to its short half-life, PQ$^+$ will preferentially generate $O_2^\bullet -$ close to complex I. Thus within mammalian systems mitochondria are a major site of $O_2^\bullet -$ formation and complex I is likely to be both a site and target for damage of this $O_2^\bullet -$ production.

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Helena M. Cochemé and Michael P. Murphy

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