Research Paper

Sites of reactive oxygen species generation by mitochondria oxidizing different substrates

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Abstract

Mitochondrial radical production is important in redox signaling, aging and disease, but the relative contributions of different production sites are poorly understood. We analyzed the rates of superoxide/\(\text{H}_2\text{O}_2\) production from different defined sites in rat skeletal muscle mitochondria oxidizing a variety of conventional substrates in the absence of added inhibitors: succinate; glycerol 3-phosphate; palmitoyl-carnitine plus carnitine; or glutamate plus malate. In all cases, the sum of the estimated rates accounted fully for the measured overall rates. There were two striking results. First, the overall rates differed by an order of magnitude between substrates. Second, the relative contribution of each site was very different with different substrates. During succinate oxidation, most of the superoxide production was from the site of quinone reduction in complex I (site I2), with small contributions from the flavin site in complex I (site I1) and the quinol oxidation site in complex III (site IIIQo). However, with glutamate plus malate as substrate, site I1 made little or no contribution, and production was shared between site I2, site IIIQo, and 2-oxoglutarate dehydrogenase. With palmitoyl-carnitine as substrate, the flavin site in complex II (site IIb) was a major contributor (together with sites I1 and IIIQo), and with glyceraldehyde 3-phosphate as substrate, five different sites all contributed, including glyceraldehyde 3-phosphate dehydrogenase. Thus, the relative and absolute contributions of specific sites to the production of reactive oxygen species in isolated mitochondria depend very strongly on the substrates being oxidized, and the same is likely true in cells and in vivo.

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Introduction

An increasing number of hypotheses propose that production of mitochondrial reactive oxygen species (ROS) plays a crucial role in different areas of physiology and pathology [1–4]. Despite this, we know very little about which mitochondrial sites in the electron transport chain and associated metabolic enzymes are responsible for physiological or pathological ROS production under native conditions (i.e. in the absence of added inhibitors).

It is well established that isolated mitochondria can produce hydrogen peroxide \(\text{H}_2\text{O}_2\) in vitro [5,6]. Since the earliest observations by Chance and colleagues [7], this field has expanded considerably and many characteristics of mitochondrial superoxide/\(\text{H}_2\text{O}_2\) production have been revealed. ROS are produced by the leak of electrons from donor redox centers to molecular oxygen. The mitochondrial electron transport chain (Fig. 1) consists of several complexes containing multiple redox centers that normally facilitate transfer of electrons to their final acceptor, molecular oxygen, which is reduced by four electrons to water at complex IV. Premature single electron reduction of molecular oxygen earlier in the chain forms the superoxide radical \(\text{O}_2^–\) and divalent reduction forms \(\text{H}_2\text{O}_2\). Superoxide dismutase-2 in the matrix converts superoxide to \(\text{H}_2\text{O}_2\), which can escape and be assayed in the surrounding medium. The general term ‘ROS’ can refer to several different species, but in this context we use it to refer only to superoxide or \(\text{H}_2\text{O}_2\).

Using inhibitors to manipulate the redox states of particular sites and prevent superoxide generation from others, at least ten different sites of superoxide/\(\text{H}_2\text{O}_2\) production in the electron transport chain and associated enzymes (Krebs cycle, β-oxidation etc.) have been identified in mammalian mitochondria (Fig. 1).
Fig. 1. Isopotential groups, electron flow, and endogenous reporters of superoxide/H$_2$O$_2$ production at different sites in mitochondria. The three planes represent different isopotential groups of redox centers, each operating at about the same redox potential ($E_h$): NADH/NAD$^+$ at $E_h \sim -280$ mV, QH$_2$/Q at $E_h \sim +20$ mV, and cytochrome c at $E_h \sim +320$ mV [31]. The normal flow of electrons from substrate dehydrogenases through NADH to oxygen is indicated by the large green arrows dropping down through the isopotential planes. The enzymes that feed electrons into each isopotential group are represented as ovals, and relevant inhibitors are drawn with blunted arrows. Electrons from NAD-linked substrates enter the NADH/NAD$^+$ pool at $E_h \sim -280$ mV through appropriate NAD oxidoreductases (NOR) including 2-oxoglutarate dehydrogenase (OGDH) and malate dehydrogenase (MDH) and flow into complex I (site I$F$). They then drop down via site I$O$ to QH$_2$/Q at $E_h \sim +20$ mV in the next isopotential pool, providing the energy to pump protons and generate protonmotive force (pmf). Q oxidoreductases (QOR) including complex II and mitochondrial glycerol 3-phosphate dehydrogenase (mgPDPH) can also pass electrons into the Q pool. Electrons flow from QH$_2$ through complex III to cytochrome c at $E_h \sim +320$ mV in the next isopotential pool, again pumping protons and generating pmf. Ascorbate plus NAD/N,N,N-tetramethyl-phenylenediamine (TMPD) and cytochrome c oxidoreductases including sulfite oxidase can also pass electrons to cytochrome c. Finally, electrons flow through complex IV to oxygen at $E_h \sim +600$ mV, generating pmf. The redox state of NAD$^+$ (outlined in blue), measured as NAD$^+$P$^+$H using autofluorescence, reports the redox state of the first isopotential group. The redox state of cytochrome b$_{566}$ (outlined in blue), measured using absorbance spectroscopy, reports the redox state of the second isopotential group [21]. Red dots are sites of superoxide/H$_2$O$_2$ production. Named sites are the flavin/lipoate of 2-oxoglutarate dehydrogenase (OGDH), the complex I flavin and Q-binding sites (I$F$ and IQ, respectively), the flavin site of complex II (II$F$), the flavin/quinone site of mgPDPH (G$_{70}$, Q), and the outer quinol-binding site of complex III (IIIQ$_{o}$). Other sites, both known and unknown, are indicated by unlabeled red dots. These include less well-described sites such as pyruvate dehydrogenase, the ETF/ETF:QOR system, proline dehydrogenase, and dihydroorotate dehydrogenase.

Each of these sites has been at least partially characterized. The sites that are often invoked as the most important mitochondrial superoxide producers are in respiratory complexes I and III [5,6]. In complex I there are two sites: the flavin in the NADH-oxidizing site (site I$F$) and the ubiquinone-reducing site (site I$O$) [8]. In complex III, the ubiquinone is thought to arise from the quinol oxidizing site (site IIIQ$_{o}$) [9–11]. However, other sites of superoxide/H$_2$O$_2$ production have also been defined, including 2-oxoglutarate dehydrogenase (OGDH) [12,13]; pyruvate dehydrogenase (PDH) [14]; complex II (site II$F$) [15]; and glycerol 3-phosphate dehydrogenase (mgPDPH) [16]. In addition, there are suggestions that other less well-described sites may also be involved in H$_2$O$_2$ production: the electron transferring flavoprotein/ETF-Q oxidoreductase (ETF/ETF:QOR) system of fatty acid β-oxidation [17,18]; proline dehydrogenase [19]; and dihydroorotate dehydrogenase [16,20].

Despite our understanding of the superoxide/H$_2$O$_2$-producing capacities of mitochondrial enzymes in vitro, we know very little about the native ROS-producing behavior of mitochondria in vivo or in situ. This is because the standard way to implicate a specific ROS-producing site in a particular phenotype is to inhibit or genetically modify the site, and observe the change in ROS signal or in the downstream phenotype. However, this approach is fundamentally flawed, because blocking a site of electron transport will invariably interrupt normal electron flow and alter the redox states of other sites in the electron flow pathway, which can dramatically alter their rates of ROS production, leading to unreliable or incorrect conclusions. This raises the question: how can the individual contributions from a complex suite of superoxide/H$_2$O$_2$-producing sites be assessed within intact mitochondria under native conditions? To address this question, we developed a novel method of estimating the rates of superoxide generation from two specific sites (I$F$ and IIIQ$_{o}$) by determining the dependence of superoxide production from each site (defined using inhibitors) on the redox state of its electron donor (reported by the redox states of NAD$^+$P$^+$H and cytochrome b$_{566}$, respectively), then measuring the redox state of the reporter under native conditions in the absence of added inhibitors to predict the contribution of the reported site to overall H$_2$O$_2$ production [21].

In the present study, we extend this approach of using endogenous reporters under native conditions to encompass many more superoxide/H$_2$O$_2$-producing sites and a greater variety of substrates. We determine the contributions of each site to overall H$_2$O$_2$ production by isolated skeletal muscle mitochondria oxidizing four different substrate combinations in the absence of inhibitors: (a) succinate, (b) glycerol 3-phosphate, (c) palmitoyl carnitine plus carnitine, and (d) glutamate plus malate. The results show that the absolute and relative contribution of each site differs greatly with different substrates.

Materials and methods

Animals, mitochondrial preparation, and reagents

Female Wister rats (Harlan Laboratories), age 5–8 weeks, were fed chow ad libitum and given free access to water. Mitochondria from hind limb skeletal muscle were isolated at 4 °C in Chappell–Perry buffer (CP1; 100 mM KCl, 50 mM Tris, 2 mM EGTA, pH 7.1 at 25 °C) by standard procedures [22]. The animal protocol was approved by the Buck Institute Animal Care and Use Committee, in accordance with IACUC standards. All reagents were from Sigma except Amplex UltraRed, which was from Invitrogen.

Superoxide/H$_2$O$_2$ production

Rates of superoxide/H$_2$O$_2$ production were measured collectively as rates of H$_2$O$_2$ production, as two superoxide molecules are dismutated by endogenous or exogenous superoxide dismutase to yield one H$_2$O$_2$. H$_2$O$_2$ was detected using the horseradish peroxidase
and Amplex UltraRed detection system [22]. Mitochondria (0.3 mg protein ml$^{-1}$) were suspended under non-phosphorylating conditions in medium at 37 °C containing 120 mM KCl, 5 mM Hepes, 5 mM K$_2$HPO$_4$, 2.5 mM MgCl$_2$, 1 mM EGTA, and 0.3% (w/v) bovine serum albumin (pH 7.0 at 37 °C), together with 5 U ml$^{-1}$ horseradish peroxidase, 25 U ml$^{-1}$ superoxide dismutase, 50 μM Amplex UltraRed and, except for experiments with palmitoylcarnitine, 1 μg ml$^{-1}$ oligomycin. Reactions were monitored fluorometrically in a Shimadzu RF5301-PC or Varian Cary Eclipse spectrophotometer ($\lambda_{excitation}=560$ nm, $\lambda_{emission}=590$ nm) with constant stirring, and calibrated with known amounts of H$_2$O$_2$ [22].

To correct for losses of H$_2$O$_2$ caused by peroxidase activity in the matrix and give a better estimate of superoxide/H$_2$O$_2$ production rates, all H$_2$O$_2$ production rates were mathematically corrected to the rates that would have been observed in these mitochondria after pre-treatment with 1-chloro-2,4-dinitrobenzene (CDNB) to deplete glutathione and decrease glutathione peroxidase and peroxiredoxin activity, as described in Refs. [21,23], using an empirical equation

$$V_{CDNB} = V_{control} × (100 × V_{control}) / (72.6 + V_{control})$$

(1)

(rates in pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$).

**NAD(P)H redox state**

Experiments were performed using 0.3 mg mitochondrial protein ml$^{-1}$ at 37 °C in parallel with measurements of H$_2$O$_2$ production and cytochrome b$_{566}$ redox state in the same medium with the same additions. The reduction state of endogenous NAD(P)H was determined by autoluminescence (most of the autoluminescence signal is from NADH bound in the matrix, and NADPH hardly changes in our experiments, but for full disclosure we use “NAD(P)H”) [21,23] using a Shimadzu RF5301-PC or Varian Cary Eclipse spectrofluorometer at $\lambda_{excitation}=365$ nm, $\lambda_{emission}=450$ nm. NAD(P)H was assumed to be 0% reduced after 5 min without added substrate and 100% reduced with 5 mM malate plus 5 mM glutamate and 4 μM rotenone. Intermediate values were determined as % reduced NAD(P)H relative to the 0% and 100% values.

**Cytochrome b$_{566}$ redox state**

Experiments were performed at 1.5 mg mitochondrial protein ml$^{-1}$ in parallel with measurements of H$_2$O$_2$ production and NAD(P)H redox state in the same medium. The reduction state of endogenous cytochrome b$_{566}$ was measured with constant stirring at 37 °C in an Olis DW-2 dual wavelength spectrophotometer as the absorbance difference at 566–575 nm [11,21]. Cytochrome b$_{566}$ was assumed to be 0% reduced after 5 min without added substrate and 100% reduced with saturating substrates plus antimycin A. Intermediate values were determined as % reduced b$_{566}$ relative to the 0% and 100% values.

**Definition of sites and calibration of endogenous reporters**

Site I$_F$ was defined as the site producing superoxide (measured as H$_2$O$_2$) in the presence of malate to reduce NAD to NADH, and rotenone to inhibit reoxidation of complex I by the Q-pool. Any H$_2$O$_2$ arising from reverse flow from the NADH pool into NAD oxidoreductases such as OGDH and PDH will appear in the analysis as a component of site I$_F$ (Fig. 2a). To decrease the contributions of forward electron flow at OGDH and PDH to H$_2$O$_2$ production...
assigned to site I, we added 1.5 mM aspartate to remove endogenous 2-oxoglutarate by transamination and 2.5 mM ATP to decrease carbon flows at various points in the Krebs cycle, particularly succinate thiokinase. This change to our previous protocol [18,21] strongly decreased our earlier estimates of the rates from site I. The rate of superoxide/H$_2$O$_2$ production from site I defined by inhibitors in this way was measured as a function of the redox state of NAD(P)H at different malate concentrations between 0.02 mM and 5 mM (Fig. 2b). The data were arbitrarily fitted by non-linear regression to a single exponential, to give the parameter values in the following equation:

$$v_{H_2O_2} \triangleq NAD(P)H = 0.88 \times e^{\frac{0.04}{c(NAD(P)H)}} + 26.5$$

(2)

where $v_{H_2O_2}$ is the rate of H$_2$O$_2$ production. This equation was used to predict the rate of superoxide/H$_2$O$_2$ production from site I at any observed NAD(P)H redox state in the absence of inhibitors in subsequent experiments.

Superoxide production by site IIIQo was measured as H$_2$O$_2$ production in the presence of succinate to reduce the Q pool, malonate to keep total succinate plus malonate at 5 mM and inhibit superoxide/H$_2$O$_2$ formation from site IIQ without fully inhibiting succinate oxidation [15], and rotenone to inhibit superoxide formation from site I (Fig. 2c). Site IIIQo was defined as the component of the observed H$_2$O$_2$ production under these conditions that was sensitive to myxothiazol (a specific inhibitor of site IIIQo) after correction for the small difference in rates from site I before and after myxothiazol addition (calculated from parallel measurements of NAD(P)H and application of the calibration curve in Fig. 2b). The rate of superoxide/H$_2$O$_2$ production from site IIIQo defined by inhibitors in this way was measured as a function of the redox state of cytochrome $b_{566}$ at different succinate: malonate ratios ranging from 75% to 100% succinate at 5% increments (total dicarboxylate concentration 5 mM) (Fig. 2d). The data were arbitrarily fitted in the same way as for site I to give the parameter values using the following equation:

$$v_{H_2O_2} \triangleq b_{566} = 5.22 \times e^{\frac{0.064}{c(b_{566})}} - 5.22$$

(3)

This equation was used to predict the rate of superoxide/H$_2$O$_2$ production from site IIIQo at any observed cytochrome $b_{566}$ redox state in the absence of inhibitors in subsequent experiments. See Ref. [21] for more extensive discussion and descriptions.

Statistics

When using the calibration curves in Fig. 2 to calculate rates of H$_2$O$_2$ production at a given reduction state of the reporter, the error in the measurements during calibration was taken into account. This error was calculated by standard methods of error propagation through all the steps, as detailed in [21].

The significance of differences between reported and experimentally measured rates of H$_2$O$_2$ production in each experimental condition was tested using Welch's t-test. Because error propagation was used to capture uncertainty in the calibration curves, individual data-points could not be used for statistical analysis. Instead we used the traits describing the population of data (mean, SEM based on error propagation and number of observations) to calculate if differences were significant ($p < 0.05$).

Results and discussion

The aim of the present study was to determine the contributions of different sites of superoxide/H$_2$O$_2$ production to the total observed H$_2$O$_2$ generation by mitochondria during oxidation of different substrates in the absence of inhibitors. Electrons leak from the respiratory chain to generate superoxide or H$_2$O$_2$ at two different redox potentials ($E_b$): at the isopotential group of redox carriers around the NADH/NAD$^+$ pool at $E_b$~−280 mV and at the isopotential group of redox carriers around the QH$_2$/Q pool at $E_b$~+20 mV (Fig. 1). At each isopotential group, an important determinant of the rate of superoxide or H$_2$O$_2$ production is the redox state: a more reduced carrier generally leaks electrons to oxygen at a faster rate. We exploited this relationship to create assays of the rate of superoxide/H$_2$O$_2$ production from different sites, either by defining the site precisely with inhibitors and determining its dependence on the redox state of the appropriate pool as described in Materials and methods (Fig. 2), or by inhibiting the site and determining the change in superoxide/H$_2$O$_2$ production after correction for secondary changes in the redox states of the two pools. Specifically, to predict rates from each site with different respiratory substrates in the absence of inhibitors, we measured the redox states of the endogenous reporters NAD(P)H and cytochrome $b_{566}$ as proxies of the redox states of the two isopotential groups, and determined the rates of superoxide/H$_2$O$_2$ production from sites I and IIIQo, respectively, using the calibration curves in Fig. 2. Assessing the contribution of other sites was a little more complicated, so we first provide an example of how the endogenous reporter calibration curves and the careful use of inhibitors can be used to quantify the sites of H$_2$O$_2$ production during oxidation of the substrate succinate.

**Sites of superoxide/H$_2$O$_2$ production during oxidation of succinate: a worked example**

When succinate is oxidized in the absence of rotenone and other electron transport chain inhibitors, electrons flow through complex II into the Q pool. Next, there are two options (Fig. 3a). The electrons can flow forward to complex III and thermodynamically downhill to more oxidized isopotential groups at cytochrome c and H$_2$O$_2$, pumping protons at complexes III and IV and generating protonotive force. Alternatively, they can flow in reverse to complex I, driven thermodynamically uphill to the more negative isopotential group by reversal of the proton pumps in complex I driven by the protonotive force generated by proton pumping at complexes III and IV. In the process, a number of possible sites of superoxide/H$_2$O$_2$ production may be engaged, particularly sites I, IIIQo, I$_Q$ and I (Fig. 3a). However, it is generally agreed that the primary mechanism of H$_2$O$_2$ production during succinate oxidation is reverse electron transport into complex I, because this H$_2$O$_2$ production is very sensitive to rotenone, the classic Q-site inhibitor of complex I [24,25]. What is not usually discussed is that when an inhibitor such as rotenone is added there are subsequent shifts in electron distribution that invariably change the rates of superoxide/H$_2$O$_2$ production by sites both upstream and downstream of the inhibition site [21]. Therefore, the decrease in rate observed after the addition of an inhibitor is not an accurate indication of the rate from its target site before inhibition. The endogenous reporter method described here circumvents this problem: by monitoring the changes in the NADH isopotential group (through NAD(P)H) and the Q isopotential group (through cytochrome $b_{566}$), the changes in electron distribution after inhibitors are added can be quantified and corrected for.

In this worked example, we quantified the sites of H$_2$O$_2$ production during succinate oxidation by measuring the redox states of the endogenous reporters, and the changes in these redox states after addition of rotenone. From this information, we could predict the contribution of each site to the total H$_2$O$_2$ production observed during succinate oxidation. To begin, we measured the rate of H$_2$O$_2$ production during succinate oxidation in the absence of rotenone (891 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$) (Fig. 4a). In parallel, in different cuvettes, we measured the reduction states of the reporters. We observed that NAD(P)H was 89% reduced and cytochrome $b_{566}$ was 46% reduced (Fig. 4b, Table 1). From
the information, we could predict using the equations describing the calibration curves in Fig. 2 that site I$_p$ was producing 59 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ (7% of the total), and site IIIQ$_o$ was producing 91 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ (10%) (Fig. 4c, Table 1), with the remaining 741 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ coming from other sites.

To identify and quantify these other sites, we added rotenone to inhibit site I$_p$, and measured the changes in H$_2$O$_2$ production and the changes in the reduction state of the reporters (Figs. 4a and b, Table 1). After rotenone addition, the overall rate of H$_2$O$_2$ production dropped to 131 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$, and cytochrome b$_{566}$ appeared to become slightly more reduced (Fig. 4a and b, Table 1). Again, using the calibration curves in Fig. 2 we determined that site I$_p$ now appeared to produce slightly less superoxide/H$_2$O$_2$ (49 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$) and site IIIQ$_o$ appeared to produce more (124 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$) than before the addition of rotenone (Fig. 4c, Table 1). When rotenone was added, not only was reverse electron flow abolished, leading to partial oxidation of NAD(P)H as the electron supply from succinate through complex I failed, but there appeared to be a small increase in the reduction level of cytochrome b$_{566}$ as all flow was diverted through complex III. The total change in superoxide/H$_2$O$_2$ production at site I$_p$ was calculated by subtracting the rate before rotenone addition (59 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$) from the rate after rotenone addition (49 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$) to give a final change in rate from site I$_p$ of -10 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$. The equivalent calculation performed for site IIIQ$_o$ gave a total change of +33 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$. The rotenone-sensitive rate (759 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$) was then corrected for the changes in superoxide/H$_2$O$_2$ production from sites I$_p$ and IIIQ$_o$ to give a final adjusted rate of 736 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$, all assigned to site I$_q$ (it was not from site I$_p$, as has been suggested [26], because site I$_p$ superoxide production was fully corrected for using NAD(P)H as the endogenous reporter). This painted a final picture (Fig. 4d) showing that during succinate oxidation, site I$_p$ contributed 83% of the observed rate of H$_2$O$_2$ production while site I$_q$ accounted for 7% and site IIIQ$_o$ accounted for 10%. Since there was no unaccounted-for rate of H$_2$O$_2$ production, either before (881 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ observed versus 886 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ assigned, N.S.) or after addition of rotenone (131 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ observed versus 171 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$ assigned, N.S.), no other site made a substantial contribution to the total observed rate. The reason site I$_p$ did not contribute is that the conventional succinate concentration used (5 mM) was sufficiently high to effectively abolish production of superoxide/H$_2$O$_2$ by site I$_p$ [15].

There are no surprises in these conclusions (although they are more accurate than simply assigning the rotenone-sensitive signal to complex I): site I$_q$ is the dominant superoxide/H$_2$O$_2$ producer when succinate is oxidized in the absence of rotenone. However, this example illustrates clearly that during oxidation of a single substrate, superoxide/H$_2$O$_2$ are produced from multiple sites (Fig. 4d). It also clearly illustrates that site-specific inhibitors not only affect superoxide/H$_2$O$_2$ production at the site that they inhibit, but also (through changes in electron distribution) change superoxide/H$_2$O$_2$ production at other sites.

The contributions of specific sites to superoxide/H$_2$O$_2$ production during oxidation of different substrates

The approach described above as a worked example using succinate as substrate was used to define the contributions of specific sites to superoxide/H$_2$O$_2$ production during oxidation of three additional important conventional substrates: glycerol 3-phosphate, palmitoylcarnitine plus carnitine, and glutamate plus malate. In each condition, the reduction state of the reporters was measured in parallel with H$_2$O$_2$ production. The changes in reduction state of the reporters after addition of inhibitors of complex I or complex II were measured and used to correct for consequent changes in electron distribution and superoxide/H$_2$O$_2$ production at other sites. Table 1 details the observed redox states
of the reporters with each substrate before and after addition of the inhibitors rotenone or malonate.

Fig. 5 shows the central result of the present paper: the contributions of each of the individual sites of superoxide/H$_2$O$_2$ production to overall H$_2$O$_2$ generation by isolated skeletal muscle mitochondria with each of the four substrate combinations under native conditions in the absence of added inhibitors. The first striking result is that the overall rates of H$_2$O$_2$ generation were very different with different substrates, as previously observed by [27] and others[28–30]. The second, crucial, observation is that the relative contribution of each site was very different with different substrates. Thus, the relative and absolute contributions of specific sites to the production of reactive oxygen species in isolated mitochondria depend very strongly on the substrates being oxidized.

To determine the contributions of individual sites to overall H$_2$O$_2$ generation with each substrate, the rates of H$_2$O$_2$ production at site IF and IIIQo were first estimated from the redox states of NAD(P)H and cytochrome b$_{566}$ using the calibration curves in Fig. 2. With succinate and glycerol 3-phosphate, the contribution of site IQ was then estimated from the decrease in the rate of H$_2$O$_2$ production after addition of rotenone followed by correction for changes in the rate from site IF (calculated from the redox state of NAD(P)H before and after rotenone addition) and from site IIIQo (calculated from the redox state of cytochrome b$_{566}$ before and after rotenone addition). The contributions of mGPDH (with glycerol 3-phosphate as substrate) and OGDH (with glutamate plus malate as substrate) were estimated as the difference between the sum of the rates estimated for the other sites and the observed total rate.

Sites of superoxide/H$_2$O$_2$ production during oxidation of glycerol 3-phosphate

Glycerol 3-phosphate oxidation by mGPDH results directly in Q pool reduction (Fig. 3b). From the Q pool, electrons may flow both forward (to complex III and beyond, generating protonmotive force) and in reverse (to complex I and complex II). In the process, a number of possible sites of superoxide/H$_2$O$_2$ production may be engaged, particularly mGPDH and sites II$_Q$, IIIQo, IQ and IF (Fig. 3b). Protonmotive force is required for reverse electron flow to complex I [25], but not for reversal into complex II [16]. Complex II has
been shown to generate superoxide in both the forward and reverse reactions [15].

With glycerol 3-phosphate as substrate, the total observed rate of H₂O₂ production was 622 pmol H₂O₂ min⁻¹ mg protein⁻¹. Sites Iₚ (6%) and IIIQ₉ (4%) made only modest contributions to this total rate (Fig. 5). The contributions of sites Iₚ (33%) and Iₚ (26%) (after correction for changes in sites Iₚ and IIIQ₉ following the addition of inhibitors) were more substantial. The absolute rates from sites Iₚ, IIIQ₉ and Iₚ were lower with glycerol 3-phosphate than with succinate, because the two isopotential pools were less reduced (Table 1), presumably because succinate was a better substrate than glycerol 3-phosphate in this experiment, which had sub-optimal glycerol 3-phosphate and calcium concentrations. After these assignments, 192 pmol H₂O₂ min⁻¹ mg protein⁻¹ (31% of the total) remained unaccounted for. mGPDH itself is known to generate ROS (predominantly superoxide), which are released to both the matrix and the intermembrane space [16]. In this case, we assume that the remaining observed rates of H₂O₂ production must have arisen from mGPDH, since all other known sites were already accounted for, and we assign this site by difference (31%). It is unlikely that any of the unassigned H₂O₂ generation in this condition was from uncharacterized sites, since such sites would have to be engaged during oxidation of glycerol 3-phosphate but not during oxidation of succinate (where there was no unassigned H₂O₂ production, Fig. 5), despite the less extensive reduction of the two isopotential groups with glycerol 3-phosphate as substrate (Table 1).

Sites of superoxide/H₂O₂ production during oxidation of palmitoylcarnitine plus carnitine

Carnitine enhances oxidation of palmitoylcarnitine by removing inhibitory acetyl-CoA in the form of acetylcarnitine and by promoting entry of palmitoylcarnitine into the mitochondrial matrix [18]. When palmitoylcarnitine is metabolized by the β-oxidation pathway, electrons enter the respiratory chain at two sites: from acyl-CoA dehydrogenase through the electron transferring flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QOR) to the Q-pool, and from 3-hydroxyacyl-CoA dehydrogenase to NADH (Fig. 3c). Oxidation of the end product, acetyl-CoA, in the citric acid cycle leads to further electron input through the NAD-linked dehydrogenases and through complex II during oxidation of succinate. Electron entry by more than one route suggests that

Table 1
Reduction levels of reporters and corresponding calculated rates of H₂O₂ production from site Iₚ and site IIIQ₉ in the presence of different substrates and inhibitors. With each substrate, the redox states of NAD(P)H and cytochrome b₉₉₉ were measured in parallel with the measurements of overall rates of H₂O₂ production reported in Fig. 5. Eqs. (2) and (3) describing the calibration curves in Fig. 2 were used to calculate the rates of superoxide/H₂O₂ production from site Iₚ and site IIIQ₉ from these redox measurements. Concentrations of substrates and inhibitors are given in Fig. 5. All rates are in pmol H₂O₂ min⁻¹ mg protein⁻¹. Data are means ± SEM (n=4). Error values on the calculated rates of H₂O₂ production represent the propagated error as described in Materials and methods and Ref. [21].

| Substrates and inhibitors | Reduced NAD(P)H (%) | Reported rate of H₂O₂ production | Reduced cytochrome b₉₉₉ (%) | Reported rate of H₂O₂ production |
|---------------------------|---------------------|----------------------------------|--------------------------|----------------------------------|
| Succinate                 | 89 ± 1              | 59 ± 4                           | 46 ± 2                   | 91 ± 18                          |
| Succinate+rotenone        | 80 ± 2              | 49 ± 4                           | 50 ± 1                   | 124 ± 15                         |
| Glycerol 3-phosphate      | 59 ± 3              | 34 ± 6                           | 29 ± 2                   | 27 ± 7                           |
| Glycerol 3-phosphate+malonate | 59 ± 3          | 34 ± 6                           | 29 ± 2                   | 27 ± 7                           |
| Glycerol 3-phosphate+malonate+rotenone | 39 ± 2 | 31 ± 5                           | 31 ± 3                   | 33 ± 9                           |
| Palmitoylcarnitine+carnitine | 80 ± 1            | 49 ± 4                           | 42 ± 3                   | 72 ± 18                          |
| Palmitoylcarnitine+carnitine+malonate | 79 ± 1          | 48 ± 4                           | 44 ± 2                   | 80 ± 17                          |
| Glutamate+malate          | 85 ± 7              | 54 ± 9                           | 38 ± 5                   | 54 ± 18                          |

Fig. 5. Native rates of superoxide/H₂O₂ production by mitochondria oxidizing different substrates. Observed total rate of H₂O₂ production (gray bars) and sum of assigned rates of superoxide/H₂O₂ production from different sites (colored stacked bars) in the presence of different substrates as indicated: 5 mM succinate; 27 mM disodium rac-α β-glycerol phosphate (25% active optical isomer sn-glycerol 3-phosphate); 15 µM palmitoyl-L-carnitine plus 2 mM L-carnitine; and 5 mM L-glutamate plus 5 mM L-malate. With each substrate, the reduction states of NAD(P)H and cytochrome b₉₉₉ were measured in parallel with H₂O₂ production and the calibration curves in Fig. 2 were used to predict the rates of production from sites Iₚ and IIIQ₉. With succinate, 4 µM rotenone was subsequently added to allow calculation of the rate from site Iₚ as described in the text (data from Fig. 4d). With glycerol 3-phosphate, 1 mM malonate and 4 µM rotenone were subsequently added to allow calculation of the rates from sites IIIQ₉ and Iₚ respectively. The rate assigned to mGPDH was calculated by difference. With palmitoylcarnitine plus carnitine, 1 mM malonate was subsequently added to allow calculation of the rate from site Iₚ (data recalculated from [18]). With glutamate plus malate, the rate assigned to OGDH was calculated by difference (data recalculated from [21]). Results are means ± SEM (n=4–6); the error bars on the sum columns show the combined propagated errors in the total sum value. There was no significant difference between observed and assigned rates with succinate or with palmitoylcarnitine plus carnitine (Welch’s t-test; p < 0.05).
fatty acid oxidation may generate superoxide/H$_2$O$_2$ from several different sites, particularly ETF/ETF:QOR and sites IIIQo, IQ and IF (Fig. 3c). Site II$_I$ may also contribute by reverse flow from the Q pool, or by forward flow from succinate. Importantly, under this condition the acetyl-CoA generated by β-oxidation will tend to deplete inhibitory oxaloacetate through the action of citrate synthase, making site II$_I$ more prone to generate superoxide/ H$_2$O$_2$ [18].

With palmitoylcarnitine plus carnitine as substrate, the total observed rate of H$_2$O$_2$ production was 199 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$. From the redox states of the reporters and the corrected effects of malonate on H$_2$O$_2$ production, we were able to account for the entirety of this observed H$_2$O$_2$ production. There were approximately equal contributions from sites II$_I$ (38%) and IIIQ$_o$ (36%), with site I$_F$ producing slightly less (25%) (Fig. 5). The absence of unaccounted-for H$_2$O$_2$ production indicated that neither site IQ nor the ETF/ETF:QOR system generated substantial superoxide/H$_2$O$_2$. Indeed, the ETF/ETF:QOR system does not generate measurable superoxide/H$_2$O$_2$ except under very specific conditions and in the presence of respiratory chain inhibitors [18].

**Sites of superoxide/H$_2$O$_2$ production during oxidation of glutamate plus malate**

In the final condition, the substrate pair glutamate plus malate was used to generate NADH (Fig. 3d). During oxidation of this substrate combination, malate is oxidized to oxaloacetate by malate dehydrogenase and NADH is generated. Glutamate is used to transaminase the oxaloacetate to form aspartate and 2-oxoglutarate (and some glutamate may be oxidized to 2-oxoglutarate dehydrogenase). The aspartate is exchanged for glutamate on the aspartate–aspartate antiporter, and much of the 2-oxoglutarate is exchanged for malate on the oxoglutarate–malate antiporter (although some may be oxidized by 2-oxoglutarate dehydrogenase). In this way, removal of oxaloacetate maximizes NADH generation by malate dehydrogenase [31].

With glutamate plus malate as substrate, the total observed rate of H$_2$O$_2$ production was 182 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$. Under this condition, sites I$_F$ and IIIQ$_o$ contributed equally (30% each) [21] (Fig. 5). Malonate did not significantly inhibit the rate (3% inhibition ± 5%), so site I$_F$ was not a significant contributor, presumably because it was inhibited by oxaloacetate or malate under these conditions [15]. A significant proportion (41%) of the total H$_2$O$_2$ production was unassigned, indicating that another site also contributed under this condition. We assume this was from forward flow through OGDH (Fig. 5) because some 2-oxoglutarate was likely formed during transamination of glutamate, but we cannot exclude a contribution from site IQ or uncharacterized other sites in this complex metabolic condition. In principle the contribution of site IQ could be assessed by correction for secondary changes in other defined sites, but definitive conclusions could not be drawn because of the relatively small rates of unassigned H$_2$O$_2$ production, large changes in redox states of the reporters following addition of rotenone, relatively large errors involved, and possible changes in other uncharacterized sites following addition of rotenone.

**Advantages and limitations of the approach used**

The great strength of the approach we have used here to measure the contributions of different sites to overall mitochondrial H$_2$O$_2$ production [21] is that, unlike all previous approaches, it reports rates under native conditions in the absence of added inhibitors. This gives it great potential for future studies with physiological substrate mixes in mitochondria and in intact cells and organisms. Its main limitation is the assumption that the calibration curves measured in the presence of inhibitors and particular substrates apply under native conditions using other substrates. The good agreement between calculated and measured total rates (Fig. 5) supports the validity of this assumption.

**Potential pathological and physiological implications**

The results obtained using the four different substrates described in Fig. 5 provide a very clear illustration of the remarkably different H$_2$O$_2$–producing profiles that can be attained by isolated muscle mitochondria oxidizing conventional substrates. When taken as a whole, two results are striking. (i) The native rates differed greatly between substrates. This implies that mitochondrial superoxide/H$_2$O$_2$ production rates in vivo likely depend critically on the substrate being oxidized, so physiological or pathological changes in substrate may be very important determinants of rates of radical production, even at the same overall rate of oxygen consumption. (ii) The contribution of each site differed markedly between substrates. With succinate, site IQ dominated, with relatively small contributions from sites I$_F$ and IIIQ$_o$. However, with palmitoylcarnitine plus carnitine, site II$_I$ was an important contributor, and with glycerol 3-phosphate, five sites contributed, including site II$_I$ and mGPDH. Thus, which sites contribute to superoxide and H$_2$O$_2$ production in mitochondria, in cells, and in vivo under both physiological and pathological conditions likely depends critically on the substrates being utilized.

Notably, the sites are known to differ markedly in the topology of superoxide production [6,16,27,32,33]. Essentially all superoxide/H$_2$O$_2$ from sites I$_F$, I$_Q$ and II$_I$ is directed to the matrix, but about half the superoxide from site IIIQ$_o$ and mGPDH appears in the intermembrane space. Thus, the strength of mitochondrial superoxide signaling in the cytosol (and also the amount of oxidative damage caused by superoxide and H$_2$O$_2$ in the matrix) will differ substantially between substrates, even at identical total rates of mitochondrial superoxide/H$_2$O$_2$ production.

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