The 2-Oxoacid Dehydrogenase Complexes in Mitochondria Can Produce Superoxide/Hydrogen Peroxide at Much Higher Rates Than Complex I*

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**Background:** At the redox potential of NADH/NAD+, at least four mitochondrial sites produce superoxide/H_2O_2.

**Results:** We compare their capacities in situ in isolated mitochondria.

**Conclusion:** Maximum capacities of complexes were 2-oxoglutarate dehydrogenase > pyruvate dehydrogenase > branched-chain 2-oxoacid dehydrogenase > complex I.

**Significance:** H_2O_2 production from 2-oxoacid dehydrogenases can be considerable but may previously have been misattributed to complex I.

Several flavin-dependent enzymes of the mitochondrial matrix utilize NAD+ or NADH at about the same operating redox potential as the NADH/NAD+ pool and comprise the NADH/NAD+ isopotential enzyme group. Complex I (specifically the flavin, site I_F) is often regarded as the major source of matrix superoxide/H_2O_2 production at this redox potential. However, the 2-oxoglutarate dehydrogenase (OGDH), branched-chain 2-oxoacid dehydrogenase (BCKDH), and pyruvate dehydrogenase (PDH) complexes are also capable of considerable superoxide/H_2O_2 production. To differentiate the superoxide/H_2O_2-producing capacities of these different mitochondrial sites in situ, we compared the observed rates of H_2O_2 production over a range of different NAD(P)H reduction levels in isolated skeletal muscle mitochondria under conditions that favored superoxide/H_2O_2 production from complex I, the OGDH complex, the BCKDH complex, or the PDH complex.

The rates from all four complexes increased at higher NAD(P)H/NAD(P)+ ratios, although the 2-oxoacid dehydrogenase complex produced superoxide/H_2O_2 at high rates only when oxidizing their specific 2-oxoacid substrates and not in the reverse reaction from NADH. At optimal conditions for each system, superoxide/H_2O_2 was produced by the OGDH complex at about twice the rate from the PDH complex, four times the rate from the BCKDH complex, and eight times the rate from site I_F of complex I. Depending on the substrates present, the dominant sites of superoxide/H_2O_2 production at the level of NADH may be the OGDH and PDH complexes, but these activities may often be misattributed to complex I.

Mitochondria may generate superoxide anion radical ("superoxide") or H_2O_2 from at least 10 distinct sites in the electron transport chain and associated pathways (such as the Krebs cycle and β-oxidation). Respiratory complexes I and III are usually described as the principal producers (1–9), but many other mitochondrial enzymes can also reduce oxygen prematurely, most notably complex II (10).

In complex I there are two sites of superoxide production: the flavin in the NADH-oxidizing site (site I_F) and the ubiquinone-reducing site (site I_Q) (11). In complex III, superoxide arises from the quinol-oxidizing site (site III_{QO}) (12–14). In complex II, the flavin site of complex II (site II_F) generates superoxide and/or H_2O_2 (10). Other sites include mitochondrial glycerol-3-phosphate dehydrogenase (15), the electron transferring flavoprotein/ETF:ubiquinone oxidoreductase system of fatty acid β-oxidation (16), dihydroorotate dehydrogenase (15, 17), and the dihydrolipoyldehydrogenase of 2-oxoacid dehydrogenase complexes (18): 2-oxoglutamate dehydrogenase (OGDH) complex

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4 The abbreviations used are: site I_F, flavin site of NADH-oxidizing site of complex I; site I_Q, ubiquinone-reducing site of complex I; site I_QO, flavin site of NADH-oxidizing site of complex I; site III_{QO}, ubiquinone-reducing site of complex III; OGDH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; BCKDH, branched-chain 2-oxoacid (or α-ketoacid) dehydrogenase; KIV, 3-methyl-2-oxopentanoate (α-ketoisovalerate); KIC, 4-methyl-2-oxopentanoate (α-ketoisocaproate); E_2, operating redox potential; Q, ubiquinone; OH_{2}, ubiquinol: E_1, 2-oxoacid dehydrogenase; E_2, dihydrolipoyldehydrogenase; ETF, electron transferring flavoprotein.

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(19–21), branched-chain 2-oxoacid dehydrogenase (BCKDH) complex (22), and pyruvate dehydrogenase (PDH) complex (20, 23). Proline dehydrogenase has also been implicated (24). Most studies on mitochondrial production of superoxide and H$_2$O$_2$ have concentrated on the maximum capacities of one or other of these sites when electron flow is blocked by electron transport chain inhibitors, but techniques to assay the rates from several different sites during normal electron flow are now becoming available (15, 16, 25–27).

Complex I is often credited as the primary site of matrix superoxide production when electron flow from Krebs cycle intermediates into the rest of the respiratory chain is inhibited by the addition of rotenone to block site I$_O$ (1–9). However, other sites within the matrix can also be important producers of superoxide/H$_2$O$_2$ under these conditions (19, 20, 23). Together with site I$_P$, these matrix sites (including the OGDH, BCKDH, and PDH complexes) comprise the NADH/NAD$^+$ isopotential group, i.e., they operate at the relatively low redox potential of the NADH/NAD$^+$ pool. The matrix sites are flavoenzymes. Reduced flavins are good electron donors to oxygen (28), as shown for several isolated flavoenzymes (19, 20, 29, 30). The relative contribution of each site in the NADH/NAD$^+$ isopotential group to superoxide/H$_2$O$_2$ production by isolated mitochondria has not been measured. This may be because they probably all respond to the reduction state of the NADH pool, making it hard to assign individual activities.

The objective of the present study was to measure the relative maximum capacities for superoxide/H$_2$O$_2$ production of the matrix NADH/NAD$^+$-linked enzyme complexes OGDH, BCKDH, PDH, and complex I under optimal conditions in situ in isolated muscle mitochondria. We show that complex I is not the highest capacity matrix superoxide/H$_2$O$_2$-producing enzyme in the presence of rotenone. Instead, the OGDH complex has the greatest capacity followed by the PDH complex and the BCKDH complex. Site I$_P$ of complex I has the lowest capacity in this isopotential group.

**EXPERIMENTAL PROCEDURES**

**Animals, Mitochondria, and Reagents**—Female Wistar rats (Harlan Laboratories), age 5–8 weeks, were fed chow ad libitum and given free access to water. Mitochondria were isolated from hind limb skeletal muscle at 4 °C in Chappell-Perry buffer (100 mM KCl, 50 mM Tris, 2 mM EGTA (pH 7.4 at 4 °C)) by standard procedures (31). The animal protocol was approved by the Buck Institute Animal Care and Use Committee (IACUC) in accordance with IACUC standards. All reagents were from Sigma except Amplex UltraRed (Invitrogen) and aperin A5 (Santa Cruz).

Complex I-deficient mutant (Ndufa1$^{SSSA/Y}$) and wild-type control (Ndufa1$^{+/+}$) male mice were used at 24 weeks of age. Mutant mice were developed by Ndufa1$^{SSSA}$ allele knock-in at the native locus by homologous recombination on the X chromosome on the 129/Sv genetic background. Animals were fed ad libitum and given free access to water. Mitochondria were isolated from hind limb skeletal muscle as above (31), except tissue was disrupted with no more than five strokes in a glass-Teflon homogenizer. Mitochondria were resuspended in CP1, and protein was measured by the biuret method.

**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 horseradish peroxidase and Amplex UltraRed (9). Mitochondria (0.3 mg of protein·mL$^{-1}$) were suspended at 37 °C in non-phosphorylating medium containing 120 mM KCl, 5 mM Hepes, 5 mM KH$_2$PO$_4$, 2.5 mM MgCl$_2$, 1 mM EGTA, 0.3% (w/v) bovine serum albumin (pH 7.0 at 37 °C), 5 units·mL$^{-1}$ horseradish peroxidase, 25 units·mL$^{-1}$ superoxide dismutase, 50 μM Amplex UltraRed, and 1 μM oligomycin. For measurement of H$_2$O$_2$ production from the PDH complex, the medium also contained 1 mM dichloroacetic acid and 450 mM free Ca$^{2+}$ achieved by addition of 575 μM total Ca$^{2+}$, calculated using the program MaxChelator). Reactions were monitored fluorometrically in a Varian Cary Eclipse spectrophotometer ($\lambda_{\text{excitation}}$ = 560 nm, $\lambda_{\text{emission}}$ = 590 nm) with constant stirring and calibrated with known amounts of H$_2$O$_2$ in the presence of all relevant substrates, as some substrates quenched the fluorescence (9).

H$_2$O$_2$ production rates in Fig. 11 were corrected for losses of H$_2$O$_2$ caused by peroxidase activity in the matrix to give a better estimate of actual superoxide/H$_2$O$_2$ production rates. Rates were mathematically corrected to those that would have been observed in these mitochondria after pretreatment with 1-chloro-2,4-dinitrobenzene to deplete glutathione and decrease glutathione peroxidase and peroxiredoxin activity as described (27, 32) using an empirical equation,

$$v_{\text{CONH}} = v_{\text{control}} + \left(1.43v_{\text{control}}(0.55 + v_{\text{control}})\right)$$

where rates are in nmol H$_2$O$_2$·min$^{-1}$·mg of protein$^{-1}$.

**NAD(P)H Redox State**—Experiments used 0.3 mg of mitochondrial protein·mL$^{-1}$ at 37 °C in parallel with measurements of H$_2$O$_2$ production in the same non-phosphorylating medium with the same additions. The reduction state of endogenous NAD(P)H was determined by autofluorescence (most of the signal is from NADH bound in the matrix, and NADPH hardly contributes in situ) in isolated muscle mitochondria. We show that complex I is not the highest capacity matrix superoxide/H$_2$O$_2$-producing enzyme in the presence of rotenone. Instead, the OGDH complex has the greatest capacity followed by the PDH complex and the BCKDH complex. Site I$_P$ of complex I has the lowest capacity in this isopotential group.

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5 C. Kim and N. Yadava, unpublished data.
**FIGURE 1. The NADH/NAD\(^{+}\) isopotential group and its component superoxide/H\(_2\)O\(_2\)-producing enzymes.** The three planes represent different isopotential groups of redox centers in mitochondrial. Each group contains multiple redox centers operating at about the same redox potential (E\(_h\)); centers around NADH/NAD\(^{+}\) at E\(_h\) \(\sim -280 \text{ mV}\), around QH\(_2\)/Q at E\(_h\) \(\sim +20 \text{ mV}\), and around cytochrome c at E\(_h\) \(\sim +320 \text{ mV}\) (34). The normal flow of electrons from substrate dehydrogenases through NADH and the respiratory complexes of the electron transport chain to oxygen are indicated by the large green arrows dropping down through the isopotential planes. Electrons from NAD-linked substrates enter the NADH/NAD\(^{+}\) pool at E\(_h\) \(\sim -280 \text{ mV}\) through NAD-linked dehydrogenases (DH) including the OGDH, BCKDH, and PDH complexes and flow into complex I (site I\(_p\)). In the absence of rotenone they then drop down via site I\(_p\) to QH\(_2\)/Q in the next isopotential pool through complex III to cytochrome c and ultimately to their final acceptor, oxygen. In the presence of rotenone (red blunted arrow) other sites of superoxide and H\(_2\)O\(_2\) production are fully oxidized and, therefore, do not leak electrons to O\(_2\), and only the sites in the NADH/NAD\(^{+}\) isopotential group are active. These sites (red dots) are site I\(_p\), the flavin of the OGDH complex (O\(_F\)), the flavin of the BCKDH complex (B\(_F\)), and the flavin of the PDH complex (P\(_F\)) (see Ref. 26).

**RESULTS AND DISCUSSION**

The aim of this study was to determine the relative maximum capacities of different matrix sites of superoxide/H\(_2\)O\(_2\) production in the NADH/NAD\(^{+}\) isopotential group in intact skeletal muscle mitochondria. Electrons leak from the respiratory chain to generate superoxide or H\(_2\)O\(_2\) at two different redox potentials (E\(_n\)): at the isopotential group of redox carriers around the NADH/NAD\(^{+}\) pool at E\(_n\) \(\sim -280 \text{ mV}\) and at the isopotential group of redox carriers around the QH\(_2\)/Q pool at E\(_n\) \(\sim +20 \text{ mV}\) (26, 27, 34) (Fig. 1). At each isopotential group, an important determinant of the rate of superoxide or H\(_2\)O\(_2\) production is the redox state; the more reduced species will generally leak electrons to oxygen at a faster rate.

The NADH/NAD\(^{+}\) isopotential group contains several enzymes, but of interest here are the superoxide/H\(_2\)O\(_2\) producing enzymes, which are typically flavoenzymes. Reduced flavins and flavoproteins generate radicals (19, 20, 28). In particular, the flavin sites of complex I (site I\(_p\)) and the OGDH complex may be important sources of matrix superoxide/H\(_2\)O\(_2\) (19, 20, 30). There is no evidence that the BCKDH complex produces superoxide or H\(_2\)O\(_2\), but it shares with the other 2-oxoacid dehydrogenase complexes a dihydrolipoamide dehydrogenase subunit that is likely a source of superoxide/H\(_2\)O\(_2\) production. Isolated PDH complex generates superoxide/H\(_2\)O\(_2\) (20, 23), but the physiological relevance is uncertain. Other members of the NADH/NAD\(^{+}\) isopotential group, such as malate dehydrogenase and isocitrate dehydrogenase, have not been shown to produce superoxide or H\(_2\)O\(_2\) (35). Here we describe conditions under which complex I and the OGDH, BCKDH, and PDH complexes are largely distinct from each other, analyze the conditions required for maximum rates in skeletal muscle mitochondria, and compare the maximum rates of superoxide/H\(_2\)O\(_2\) production by each complex.

**Complex I Flavin (Site I\(_p\))—**Complex I (NADH-ubiquinone oxidoreductase) oxidizes NADH to NAD\(^{+}\) and reduces ubiquinone (Q) to ubiquinol (QH\(_2\)). During this process two electrons are transferred through multiple redox centers, and four protons are pumped from the matrix to the intermembrane space. The enzyme is fully reversible, either oxidizing NADH and reducing Q (and pumping protons) in the forward reaction or oxidizing QH\(_2\) and reducing NAD\(^{+}\) in the reverse reaction driven by protonmotive force (34).

mm Hapes/KOH (pH 7.4), 0.2 mm EDTA, bovine serum albumin (1 mg·ml\(^{-1}\)), 2.5 mm MgCl\(_2\), and alamethicin (40 μg·ml\(^{-1}\)). The suspension was incubated for 5 min, diluted 2.5-fold with the same buffer but ice-cold and lacking MgCl\(_2\) and alamethicin, then centrifuged at 30,000 g for 15 min. The permeabilized mitochondria were suspended in 0.25 M sucrose, 10 mM HEPES (pH 7.4), 0.2 mm EDTA, and 10 μg·ml\(^{-1}\) bovine serum albumin and stored on ice.

**Complex I Activity—**NADH:quinone oxidoreductase activity was assayed at 30 °C as a decrease of A\(_{340}\) with 100 μM NADH as substrate and 100 μM ubiquinone-1 (Q\(_{1}\)) as acceptor in the presence of 1 mm KCN (33) in medium comprising 0.25 M sucrose, 10 mM HEPES (pH 7.4), 0.2 mm potassium EDTA, and permeabilized mitochondria (0.125 mg of protein·ml\(^{-1}\)). A\(_{340}\) was measured in an Olis DW2 dual-beam spectrophotometer in split beam mode. The rotenone-sensitive linear rates of NADH oxidation over 60 s were converted to molar units with ε = 6.22 mm·cm\(^{-1}\)·mol\(^{-1}\).

**Western Blot—**To determine the amount of complex I, 0.4 μg of mitochondrial protein was boiled in NU-PAGE loading buffer. Proteins were separated by 4–12% NU-PAGE gradient gel using 1× MES buffer (Invitrogen) and transferred to a nitrocellulose membrane. Anti-complex I 75-kDa subunit (NDUFS1) (Santa Cruz: sc-271510) was used at a 1:1000 dilution. Secondary antibody was horseradish peroxidase-conjugated goat anti-mouse (Bio-Rad) at 1:50,000 dilution. Chemiluminescence was generated with SuperSignal West Pico (Thermo Scientific) and quantified with Image J software (National Institutes of Health).
Complex I produces superoxide at two different internal sites: the NADH-oxidizing flavin site, IF, and the ubiquinone-reducing site, IQ (11, 36–40), although site IQ activity has yet to be measured in the reconstituted complex (41). The production of superoxide from site IF occurs when electrons leak to O2 from the fully reduced flavin, FMNH2 (30). As electrons can only be transferred to oxygen when the flavin is reduced, it is likely that the steady-state concentration of reduced FMNH2 is an important determinant of the rate of superoxide production from this site. Similarly, the steady-state reduction level of FMN depends on the steady-state redox state of its reductant, NADH (i.e., the NADH/NAD+ ratio). Therefore, the steady-state reduction level of NADH/NAD+ in situ predicts the rate of superoxide production from site IF (27). Indeed, as the NAD(P)H/NAD(P)+ ratio increases, the rate of superoxide production attributed to site IF in complex I increases steeply (26, 27, 32).

The relationship between superoxide/H2O2 production from site IF and the NAD(P)H redox state can be revealed by titrating mitochondria with a substrate that reduces NAD(P)+ in the presence of rotenone to prevent its rapid reoxidation (squares, Fig. 2D). Malate was chosen here because malate dehydrogenase is thought to reduce NAD(P)+ directly without generating superoxide or H2O2 (35) or providing sufficient Krebs cycle intermediates to support superoxide/H2O2 production from other sites. To test the robustness of the relationship between superoxide produced from site IF and NAD(P)H reduction state, we added physiologically relevant components such as amino acids and nucleotides to the medium. In general, this did not change the established relationship between the observed rate of H2O2 production and the NAD(P)H reduction level (squares, Fig. 2D). However, the addition of two biologically relevant compounds, ATP and aspartate, decreased the observed rate of mitochondrial H2O2 production (Fig. 2A) without changing the reduction state of NAD(P)H (Fig. 2D) or the NADH:Q oxidoreductase activity of complex I (Fig. 2B).

ATP and aspartate may affect other matrix enzymes, for example the OGDH complex (Fig. 2C). ATP is a negative regulator of many Krebs cycle enzymes, including OGDH (42, 43). It may also product-inhibit succinate thiokinase, which generates
succinate and ATP from succinyl-CoA (44); thus, ATP will lower free CoA, a substrate for the OGDH complex, and raise succinyl-CoA, a potent product inhibitor of the complex (45). Aspartate will drive the aspartate aminotransferase reaction, aspartate + 2-oxoglutarate ↔ oxaloacetate + glutamate, to remove 2-oxoglutarate. To check this, we inhibited aminotransferases using aminoxyacetate, which eliminated the effect of aspartate addition (not shown). Thus, the effects of aspartate and ATP on mitochondrial H$_2$O$_2$ production point to the effect of aspartate addition (not shown). Therefore, the lower set of points in Fig. 2D can be used to predict the rate from site I$_F$ at any measured reduction state of NAD(P)H in situ.

The maximum observed rates of H$_2$O$_2$ production from site I$_F$ in muscle mitochondria when NAD$^+$ is highly reduced occur when the size of the NADH$^+$-NAD$^+$ pool and the concentrations of other effectors are set by the system. These rates may be very different from the maximum rates achievable with the isolated enzyme (30) or in submitochondrial particles (46, 47), where the concentrations of effectors can be independently optimized. The same argument applies to the other sites analyzed below. When comparing the capacities of different sites in situ, the maximum rates in intact mitochondria are more relevant.

Based on the logic outlined above, we propose that the effects of ATP and aspartate on the rate of H$_2$O$_2$ production with malate as substrate were mediated by effects on the OGDH complex, not direct effects on complex I. Furthermore, the OGDH complex may often be the predominant source of superoxide/H$_2$O$_2$ when classical complex I substrate mixes are used.

2-Oxocid Dehydrogenase Complexes—The 2-oxoacid dehydrogenase complexes catalyze the oxidation of 2-oxoacids (2-oxoglutarate, branched-chain 2-oxoacids, or pyruvate) to yield the corresponding acyl-CoAs and NADH. Multiple copies of three enzymatic components are organized into complexes (48). The first reaction is the irreversible decarboxylation of the 2-oxoacid, catalyzed by a specific 2-oxoacid dehydrogenase (E1) that requires the cofactor thiamine pyrophosphate. The second reaction is catalyzed by dihydrolipoamide acyltransferase (E2) and requires CoA to generate an acyl-CoA. The third reaction is catalyzed by dihydrolipoamide dehydrogenase (E3) and requires FAD$^+$ and NAD$^+$ to reoxidize the dihydrolipoamide. E1 and E2 are unique to the individual complexes, but E3 is the same gene product in each complex and catalyzes an identical reaction (49); it is also present in the glycine cleavage system. The covalently attached lipoate group in E2 connects the three active sites of E1, E2, and E3 and channels substrates through the complexes. E2 forms the catalytic core and establishes the structural foundation for the complex, as both E1 and E3 bind to it.

2-Oxoglutarate Dehydrogenase Complex—The OGDH complex catalyzes the conversion of 2-oxoglutarate to succinyl-CoA in the Krebs cycle. The mammalian enzyme is regulated by ATP, ADP, calcium, and substrate availability. It is inhibited by its products, succinyl-CoA and NADH (42, 43).

The OGDH complex is an important source of reactive oxygen species in the matrix (19–21, 50, 51). It is a major source of superoxide/H$_2$O$_2$ in isolated brain mitochondria at high NADH/NAD$^+$ (20, 21, 52) and of superoxide in neurons during glutamate excitotoxicity (51). The E3 component of the purified enzyme contains the redox-active flavin that reduces oxygen (53, 54) and generates substantial superoxide/H$_2$O$_2$ when NAD$^+$ is limiting (19–21). E3 is abundant in skeletal muscle mitochondria (55), and the midpoint potential of the flavin is sufficiently negative (~−280 mV) to make it a good electron donor to oxygen (56).

When E3 functions within the OGDH or PDH complexes, the reduced lipoyl residue may equilibrate with the FADH$^*$ semiquinone formed by 1e$^-$ oxidation of FADH$_2$, by O$_2$. Because of this, formation of superoxide by E3 is associated with generation of lipoate thyl radicals (19, 50) (Fig. 3). This reaction may occur when FAD is reduced in either the forward reaction (from 2-oxoglutarate oxidation in the presence of CoA leading to reduction of the dihydrolipoyl residue) or the reverse reaction (from NADH oxidation). Efficient addition of O$_2$ to thyl radicals has not been observed (57), but thyl radicals are very reactive species, which in the case of the OGDH and PDH complexes, are more damaging than superoxide/H$_2$O$_2$ (19). Intrinsic thyl radical formation underlies an important regulatory mechanism in the OGDH and PDH complexes, irreversibly inactivating the corresponding E1s when NAD$^+$ is lacking (19, 50). Inactivation is favored by particular matrix environments, e.g., the reduced state of NADH, and
depends on thioredoxin and endogenous thiol-disulfide pools. The isolated E1 components of the OGDH and PDH complexes provide still other intermediates to react with oxygen, supposedly generating either \( \text{H}_2\text{O}_2 \) or peracids (58), but occurrence of this side reaction in the native complexes has not been shown.

In our experiments (Fig. 4, A and B), the observed \( \text{H}_2\text{O}_2 \) production with 2-oxoglutarate in skeletal muscle mitochondria was strongly stimulated by ADP. This probably occurred by two mechanisms, (a) lowering the \( K_d \) of OGDH for its substrate and (b) providing substrate for succinate thiokinase, which regenerates CoA, the substrate of the OGDH complex, and simultaneously removes the potent inhibitor succinyl-CoA (42, 43, 45).

To test whether the observed \( \text{H}_2\text{O}_2 \) production with 2-oxoglutarate was downstream of the OGDH complex, we added malonate, a competitive inhibitor of succinate dehydrogenase (59). However, malonate also inhibits OGDH by acting at the regulatory site responsible for activation by 2-oxoglutarate (60, 61) and may affect 2-oxoglutarate distribution across the mitochondrial inner membrane. Malonate completely inhibits \( \text{H}_2\text{O}_2 \) production rates in intact and alamethicin-permeabilized mitochondria. In the permeabilized mitochondria, malonate, a competitive inhibitor of succinate dehydrogenase, is downstream of the OGDH complex, we added malonate, the substrate of the OGDH complex, and simultaneously generates either \( \text{H}_2\text{O}_2 \) or peracids (58), but occurrence of this side reaction in the native complexes has not been shown.

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To test whether the observed \( \text{H}_2\text{O}_2 \) production with 2-oxoglutarate was downstream of the OGDH complex, we added malonate, a competitive inhibitor of succinate dehydrogenase (59). However, malonate also inhibits OGDH by acting at the regulatory site responsible for activation by 2-oxoglutarate (60, 61) and may affect 2-oxoglutarate distribution across the mitochondrial inner membrane. Malonate completely inhibits \( \text{H}_2\text{O}_2 \) production rates in intact and alamethicin-permeabilized mitochondria. In the permeabilized mitochondria, malonate, a competitive inhibitor of succinate dehydrogenase, is downstream of the OGDH complex, we added malonate, the substrate of the OGDH complex, and simultaneously generates either \( \text{H}_2\text{O}_2 \) or peracids (58), but occurrence of this side reaction in the native complexes has not been shown.
the reversibility of the complex I-catalyzed reaction implies that NADH/NAD⁺ should be close to equilibrium with the superoxide-producing flavin center of complex I. However, the increased matrix NADH/NAD⁺ ratio should also increase the rate of superoxide/H₂O₂ production from the OGDH complex because this side reaction is stimulated by the lack of the terminal substrate NAD⁺ (19, 50).

Fig. 5, A and B, shows the dependence of the rate of H₂O₂ production and level of NAD(P)H reduction on 2-oxoglutarate concentration. Combining these data revealed a steep relationship between the H₂O₂ production rate and the reduction state of NAD(P)H (squares, Fig. 5C). Importantly, with malate as substrate (and ATP and aspartate present to inhibit superoxide/H₂O₂ production by the OGDH complex and reveal that from site Iₒ), there was a different relationship between H₂O₂ production and NAD(P)H reduction (circles, Fig. 5C). This indicated that (a) as NAD(P)H became >80% reduced, the rate of superoxide/H₂O₂ production increased steeply from both the OGDH complex and site Iₒ, but with very different slopes, and (b) the OGDH complex produced superoxide/H₂O₂ much more slowly using electrons from NADH than in the forward reaction from 2-oxoglutarate. As mentioned above, the isolated E3 component of the OGDH complex readily oxidizes NADH and reduces the flavin to generate superoxide/H₂O₂ at relatively high rates (20, 21). However, within the native complex, the 2-oxoglutarate-dependent rate of superoxide/H₂O₂ production is faster than the NADH-dependent rate (68). In our experiments when the NADH pool was reduced in the absence of 2-oxoglutarate (circles, Fig. 5C), superoxide/H₂O₂ was produced at a much lower rate than with 2-oxoglutarate (squares, Fig. 5C), indicating that superoxide/H₂O₂ production by the OGDH complex in isolated mitochondria occurred at high rates only in the forward reaction in the presence of 2-oxoglutarate.

**Branched-chain 2-Oxooacid Dehydrogenase Complex**—The BCKDH complex has strong control over oxidation of the branched-chain amino acids valine, leucine, and isoleucine. These amino acids are converted by specific branched-chain amino acid aminotransferases to their respective branched-chain 2-oxoacids, 3-methyl-2-oxobutanoate (KIV), 4-methyl-2-oxopentanoate (KIC), and KMV, then oxidized by the BCKDH complex to an acyl-CoA, CO₂, and NADH. The complex is phosphorylated and inactivated by BCKDH kinase and dephosphorylated and activated by BCKDH phosphatase, both bound to the E2 core. The kinase belongs to the same family as pyruvate dehydrogenase kinase (69) and is regulated allosterically by inhibitors such as 3-methyl-2-oxobutanoate (KIV) (70) and at the expression level (71).

Fig. 6 shows H₂O₂ production during oxidation of branched-chain 2-oxoacids. The addition of 3-methyl-2-oxopentanoate in the presence of antimycin A stimulated rapid H₂O₂ production (Fig. 6A). Under these conditions, several potential sites of superoxide/H₂O₂ production may be active, including complexes I, II, and III. The rate was decreased by myxothiazol (inhibits site Qₒ of complex III, green bar), indicating that site IIIQₒ was recruited in this condition. It was further decreased by atpenin A5 (red bar), an inhibitor of the quinone-binding site of complex II, indicating that 3-methyl-2-oxopentanoate induces superoxide/H₂O₂ production from complex II in the reverse reaction. Both of these effects are driven by reduction of the ubiquinone (Q) pool. A surrogate measure of this is the steady-state reduction level of cytochrome b₅₆₆ (14, 27). Fig. 6B shows that the addition of 3-methyl-2-oxopentanoate in the presence of antimycin reduced cytochrome b₅₆₆ and the Q pool. The addition of rotenone prevented the flow of electrons from 3-methyl-2-oxopentanoate into the Q pool (Fig. 6B), eliminating the contributions from site IIIQₒ and site IIₒ and revealing the maximum rate of superoxide/H₂O₂ production caused by oxidation of 3-methyl-2-oxopentanoate at the level of the NADH isopotential group (Fig. 6A, white and blue bars), i.e. by the BCKDH complex plus site Iₒ.

The rate of superoxide/H₂O₂ production from the BCKDH complex plus site Iₒ was titrated with KMV (Fig. 6C) and 4-methyl-2-oxopentanoate (KIC). The highest rates were obtained with 20 mM 3-methyl-2-oxopentanoate (Fig. 6D). We tested whether they were further stimulated by putative inhibitors of BCKDH kinase. However, the addition of leucine or isoleucine (72) or dichloroacetate plus CaCl₂ did not increase H₂O₂ production nor did carnitine, which should recycle inhibitory acyl-CoA products (73) and regenerate CoA, a required substrate of BCKDH (Fig. 6D).
In the presence of oligomycin, ADP inhibited the rate of superoxide/H$_2$O$_2$ production with 3-methyl-2-oxopentanoate as substrate (Fig. 6D). This is the opposite of its effect with 2-oxoglutarate as substrate (Fig. 4), supporting the hypothesis that the observed H$_2$O$_2$ production with 3-methyl-2-oxopentanoate was largely from the BCKDH complex and not a residual activity of the OGDH complex using 3-methyl-2-oxopentanoate as a weak substrate.

Fig. 7, A and B, show the dependence of the rate of H$_2$O$_2$ production and level of NAD(P)H reduction on 3-methyl-2-oxopentanoate concentration. Fig. 7C shows that there was a steep relationship between superoxide/H$_2$O$_2$ production by the BCKDH complex (plus site I$_F$), and NAD(P)H level as 3-methyl-2-oxopentanoate was varied. NAD(P) was maximally only 40% reduced, perhaps because of kinetic limitation by the activity of the BCKDH complex, which is inhibited by NADH (73). However, the important observation is that there was a different relationship between H$_2$O$_2$ production and NAD(P)H in the presence of 3-methyl-2-oxopentanoate (squares, Fig. 7C) and in the presence of malate plus aspartate and ATP (circles, Fig. 7C), indicating that when 3-methyl-2-oxopentanoate is oxidized a different site of superoxide/H$_2$O$_2$ production is recruited, most likely the BCKDH complex. Fig. 7C shows that the contribution of site I$_F$ to the total observed H$_2$O$_2$ production with 20 mM 3-methyl-2-oxopentanoate in these conditions was <20%. It also indicates that the enzyme only produces superoxide/H$_2$O$_2$
at high rates in the forward reaction (during 3-methyl-2-oxopentanoate oxidation), as the observed rates of H_2O_2 production at the same reduction level of NAD(P)H were always higher in the presence of 3-methyl-2-oxopentanoate.

Pyruvate Dehydrogenase Complex—Mammalian PDH complex is mechanistically similar to the other 2-oxoacid dehydrogenases. Its regulation is similar to that of the BCKDH complex. It is rapidly and reversibly controlled by two mechanisms: end-product inhibition and enzyme phosphorylation/dephosphorylation by specific kinases and phosphatases.

In experiments to assess superoxide/H_2O_2 production by the PDH complex, a primary concern was removal of inhibitory acetyl-CoA, which competes potently with CoA (K_\text{a} \approx 5–10 \mu M) (74). In isolated mitochondria, acetyl-CoA can be removed by (a) the addition of malate to generate oxaloacetate, which will condense with acetyl-CoA to generate citrate through citrate synthase, and (b) the addition of carnitine to convert acetyl-CoA to acetylcarnitine, catalyzed by carnitine acetyltransferase (Fig. 8A). Removal of acetyl-CoA by either pathway should promote flux through the PDH complex, but the downstream carbon flows will be different. The use of malate as co-substrate with pyruvate promotes Krebs cycle carbon flux, as evident from the decrease in H_2O_2 production when ATP and aspartate were added to suppress that flux (Fig. 8B). However, when carnitine was co-substrate with pyruvate, H_2O_2 production was not appreciably affected by ATP and aspartate (Fig. 8B). In this system Krebs cycle carbon flows were likely limited as acetylcarnitine was exported from the matrix in exchange for carnitine on the carnitine-acylcarnitine translocase (75, 76).

A second concern was the phosphorylation state of the enzyme (77). Three serines on the α-subunits of E1 may be phosphorylated by four pyruvate dehydrogenase kinases (PDK1–4) (78), inactivating PDH (79). Dephosphorylation is accomplished by two pyruvate dehydrogenase phosphate phosphatases (PDP1 and PDP2). The activities of pyruvate dehydrogenase kinase and dehydrogenase phosphate phosphatase are

FIGURE 8. Experimental design for measuring superoxide/H_2O_2 production from the pyruvate dehydrogenase complex. A, carbon flows with pyruvate plus carnitine or pyruvate plus malate as substrates. MDH, malate dehydrogenase. B, H_2O_2 production in non-phosphorylating medium with 2.5 mM pyruvate plus 5 mM malate or 2.5 mM pyruvate plus 5 mM carnitine as substrate. The contribution from other sites during oxidation of these substrate pairs was assessed by the sensitivity of the rates to 2.5 mM ATP and 1.5 mM aspartate (ASP). 4 μM rotenone was added where indicated. a.u., arbitrary units. C, Amplex UltraRed traces in non-phosphorylating medium show that at a low pyruvate concentration (25 μM) the rates of H_2O_2 production were low. The presence of 450 nM free Ca^{2+} and 1 mM dichloroacetate (DCA) increased the observed rate at low pyruvate concentration. D, rates of H_2O_2 production in non-phosphorylating medium at low (25 μM) and high (2.5 mM) pyruvate concentrations in the presence and absence of 450 nM free Ca^{2+} and 1 mM dichloroacetate. Data are the means ± S.E. (n = 3). Panels B and C show representative traces; numbers indicate mean rates in pmol of H_2O_2/min/mg of protein (n = 3).
highly regulated. Pyruvate dehydrogenase kinase is inhibited by high concentrations of the E1 substrate pyruvate and activated by acetyl-CoA and NADH, products of both the PDH complex and β-oxidation (80). The non-metabolizable pyruvate analog dichloroacetate inhibits the kinase and thereby activates PDH (81). PDP1, the dominant isozyme in muscle, requires Mg\(^{2+}\) and is stimulated by Ca\(^{2+}\) (82) because PDP1 binding to E2 requires Ca\(^{2+}\) (83), and Ca\(^{2+}\) decreases the \(K_m\) of PDP1 for Mg\(^{2+}\) (84).

In skeletal muscle mitochondria, \(H_2O_2\) production after the addition of a low concentration of pyruvate (25 \(\mu\)m) was increased \(>3\)-fold by dichloroacetate and CaCl\(_2\) (Fig. 8, C and D). However, at high pyruvate concentrations (2.5 mm), dichloroacetate and CaCl\(_2\) did not affect the rate (Fig. 8D). These data indicate that at low pyruvate concentrations pyruvate dehydrogenase kinase phosphorylated and inactivated PDH.

Based on these observations, the experimental conditions chosen for assay of superoxide/\(H_2O_2\) production by the PDH complex were a high concentration of pyruvate in the presence of dichloroacetate and CaCl\(_2\). The \(H_2O_2\) production rate (Fig. 9A) and the steady-state reduction level of NAD(P)H (Fig. 9B) were titrated in parallel with carnitine to achieve different steady-state concentrations of inhibitory acetyl-CoA. The data were combined to reveal the relationship between the rate of \(H_2O_2\) production from the PDH complex (plus site I\(_f\)) and NAD(P)H reduction state as PDH activity was altered (Fig. 9C). Similar to the other 2-oxoacid dehydrogenase complexes described above, the rate of superoxide/\(H_2O_2\) production from the PDH complex increased as the NAD(P)H/NAD(P)\(^+\) pool was reduced, and PDH was activated by removal of acetyl CoA. As with the OGDH complex, the increase in PDH complex-dependent superoxide/\(H_2O_2\) production at higher NADH/NAD\(^+\) was ascribed to the lack of the terminal substrate NAD\(^+\), as this lack stimulates superoxide production by both PDH and OGDH complexes (19). In contrast, increasing the reduction state of NAD(P) in the absence of pyruvate did not cause rapid superoxide/\(H_2O_2\) production (circles, Fig. 9C). These observations indicate that superoxide/\(H_2O_2\) production upon oxidation of NADH by the E3 component of the PDH complex was not favored in these intact mitochondria. Thus, superoxide/\(H_2O_2\) production by the PDH complex in isolated mitochondria occurs at high rates only in the forward reaction (during pyruvate oxidation). The E3 subunit is also present in the glycine cleavage system. However, there was no additional \(H_2O_2\) production when glycine was added to rotenone-inhibited mitochondria (<5 pmol of \(H_2O_2\)min\(^{-1}\)-mg of protein\(^{-1}\)) (not shown).

Effect of Decreasing the Amount of Complex I on Superoxide/\(H_2O_2\) Production—The MWFE subunit of complex I encoded by the X-linked \(Ndufa1\) gene is essential for complex I assembly (85). Conversion of the evolutionarily conserved serine 55 to alanine (S55A) decreases complex I assembly/activity in mammalian cells (86). Fig. 10A confirms that mitochondria from mutant mice carrying the S55A substitution in the MWFE protein had lower levels of complex I.

If the assignment of superoxide/\(H_2O_2\) production in the presence of malate, aspartate, and ATP (circles, Fig. 2D) to site I\(_f\) of complex I is correct, this production should decrease proportionally to the decrease in complex I in mitochondria from the mutant mice; Fig. 10B shows that this was indeed the case. Conversely, if we have correctly assigned superoxide/\(H_2O_2\) production with 2-oxoacid substrates (Figs. 5C, 7C, and 9C) to the appropriate 2-oxoacid dehydrogenase complex, this production should not be affected by a decrease in complex I; Fig. 10, C, D, and E, shows that no decrease in \(H_2O_2\) production was observed with these substrates in the mutant (from Fig. 10B the contribution of site I\(_f\) would be only 0.5, 9, and 2% respectively, too small to register). Similarly, we assign superoxide/\(H_2O_2\) production with malate as substrate (squares, Fig. 2D) to the OGDH complex plus site I\(_f\); Fig. 10F shows that no decrease was observed in the mutant (the contribution of site I\(_f\) would be only 6%). These observations strongly support our assignments of superoxide/\(H_2O_2\) production to the specific sites mentioned.

Superoxide production from site I\(_2\) of complex I should also decrease proportionally to the decrease in complex I. We did not observe the predicted decrease when site I\(_2\) was driven by succinate oxidation, but this can be explained by meared compensatory increases in the amount of complex II and the magnitude of the proton-motive force during succinate oxidation (not shown).

Mice lacking apoptosis-inducing factor also have compromised complex I assembly, and superoxide/\(H_2O_2\) generation
from site $I_F$ was decreased in brain mitochondria from these mice (87). However, $H_2O_2$ production driven by glutamate plus malate in the presence of rotenone was not, suggesting that “the major source of superoxide/$H_2O_2$ in this model may lie outside complex I” (see Ref. 87). Our results (Fig. 10) confirm these observations in a different model and support the conclusion that the OGHD complex is the major site of superoxide/$H_2O_2$ generation with glutamate plus malate as substrate in the presence of rotenone.

**Conclusions**—When electron transport through complex I is inhibited by rotenone, the NADH/NAD$^+$ pool becomes reduced, and the enzymes in the isopotential group around this pool, specifically the OGHD, BCKDH, and PDH complexes and site $I_F$, generate more superoxide and/or $H_2O_2$. The contributions of the different sites depend on the conditions. Because site $I_F$ is close to equilibrium with the matrix NADH/NAD$^+$ pool, its rate of superoxide production is a simple function of the redox state of the NADH/NAD$^+$, described by the lower set of points in Fig. 2D.

Although they tend to become more reduced and generate superoxide/$H_2O_2$ at higher rates in the presence of their specific substrates when NADH is high, the redox centers that produce superoxide/$H_2O_2$ in the OGHD, BCKDH, and PDH complexes are not in equilibrium with the NADH/NAD$^+$ pool. This is clear from Figs. 5C, 7C, and 9C, which show that the OGHD, BCKDH, and PDH complexes do not generate superoxide/$H_2O_2$ at high rates in the absence of 2-oxoglutarate, 3-methyl-2-oxopentanoate (D), 2.5 mM pyruvate and 5 mM carnitine (E), and 5 mM malate (F). DH, NADH dehydrogenases. Data in A are expressed as % of paired control from five independent paired skeletal muscle mitochondrial preparations; error bars indicate 95% confidence limits; *, $p < 0.05$ by 95% confidence interval. Data in B–F are the means ± S.E. ($n = 5$); *, $p < 0.05$ by Student’s t-test.

**FIGURE 11.** Maximum rates of superoxide/$H_2O_2$ production by the OGHD complex was ~8 times higher than the maximum rate of superoxide production from site $I_F$; the maximum rate from the PDH complex was more than four times higher, and the maximum rate from the BCKDH complex was almost twice the rate from site $I_F$. Although not as high as the maximal rates from the producers with the greatest capacities, $I_Q$, II$_P$, and III$_{Q_O}$, the rates from the OGHD, PDH, and BCKDH complexes were a much higher proportion of the rate from the NADH/NAD$^+$ isopotential group in the presence of rotenone than we and many others originally envisaged (1–9, 25–27).

**Maximum Capacities of Specific Sites of Superoxide/$H_2O_2$ Production in Intact Skeletal Muscle Mitochondria**—Fig. 11 puts the in situ capacities of the superoxide/$H_2O_2$ producing sites of the NADH/NAD$^+$ isopotential group in context. It shows the maximum rates from the OGHD, BCKDH, and PDH complexes, site $I_F$, site $I_Q$, site $I_P$, mitochondrial glycerol-3-phosphate dehydrogenase, electron transferring flavoprotein/ETF:ubiquinone oxidoreductase, and site III$_{Q_O}$ (all after correction for matrix peroxidase activity using the correction described under “Experimental Procedures”). For this correction, we assumed that 100% of the superoxide/$H_2O_2$ from site $I_F$ and the 2-oxoacid dehydrogenase complexes was superoxide or $H_2O_2$ produced to the matrix, as the rates were insensitive to the addition of exogenous superoxide dismutase (not shown). Of the nine sites examined, site $I_F$ had the lowest maximum capacity in skeletal muscle mitochondria. The maximum rate of superoxide/$H_2O_2$ production by the OGHD complex was ~8 times higher than the maximum rate of superoxide production from site $I_F$; the maximum rate from the PDH complex was more than four times higher, and the maximum rate from the BCKDH complex was almost twice the rate from site $I_F$. Although not as high as the maximal rates from the producers with the greatest capacities, $I_Q$, II$_P$, and III$_{Q_O}$, the rates from the OGHD, PDH, and BCKDH complexes were a much higher proportion of the rate from the NADH/NAD$^+$ isopotential group in the presence of rotenone than we and many others originally envisaged (1–9, 25–27).

**FIGURE 10.** Superoxide/$H_2O_2$ production by mitochondria from complex I-deficient $Ndufa^{S55A/Y}$ mice; *A*, densitometry of Western blots of mitochondria from wild type and mutant mice probed for the 75-kDa NDUFS1 subunit of complex I (inset, representative Western blot). Left, control; right, $Ndufa^{S55A/Y}$.
glutamate (or branched chain 2-oxoacid or pyruvate) and on the activation state of the enzyme.

Under optimal conditions for each system in mitochondria isolated from rat skeletal muscle, the OGDH complex can produce superoxide/$\text{H}_2\text{O}_2$ at about eight times the rate from site IF (Fig. 5C), the PDH complex can produce superoxide/$\text{H}_2\text{O}_2$ at about four times the rate from site IF (Fig. 9C), and the BCKDH complex can produce superoxide/$\text{H}_2\text{O}_2$ at almost twice the rate from site IF (Fig. 7C). Depending on the substrates present and the conditions, the dominant sites of superoxide/H$_2$O$_2$ production at the level of the NADH/NAD$^+$ isopotential pool may be the OGDH and PDH complexes, but in the past their superoxide/H$_2$O$_2$ production may often have been misattributed to complex I.

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