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An Analysis of the Effects of Mn²⁺ on Oxidative Phosphorylation in Liver, Brain, and Heart Mitochondria Using State 3 Oxidation Rate Assays

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Abstract

Manganese (Mn) toxicity is partially mediated by reduced ATP production. We have used oxidation rate assays - a measure of ATP production - under rapid phosphorylation conditions to explore sites of Mn²⁺ inhibition of ATP production in isolated liver, brain, and heart mitochondria. This approach has several advantages. First, the target tissue for Mn toxicity in the basal ganglia is energetically active and should be studied under rapid phosphorylation conditions. Second, Mn may inhibit metabolic steps which don't affect ATP production rate. This approach allows identification of inhibitions that decrease this rate. Third, mitochondria from different tissues contain different amounts of the components of the metabolic pathways potentially resulting in different patterns of ATP inhibition. Our results indicate that Mn²⁺ inhibits ATP production with very different patterns in liver, brain, and heart mitochondria. The primary Mn²⁺ inhibition site in liver and heart mitochondria, but not in brain mitochondria, is the F₁F₀ ATP synthase. In mitochondria fueled by either succinate or glutamate + malate, ATP production is much more strongly inhibited in brain than in liver or heart mitochondria; moreover, Mn²⁺ inhibits two independent sites in brain mitochondria. The primary site of Mn-induced inhibition of ATP production in brain mitochondria when succinate is substrate is either fumarase or complex II,

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while the likely site of the primary inhibition when glutamate plus malate are the substrates is either the glutamate/aspartate exchanger or aspartate aminotransferase.

Keywords

² Mn toxicity; oxidative phosphorylation; mitochondrial differences; inhibition of Ca²⁺ activation; TCA cycle

INTRODUCTION

While manganese (Mn) is an essential element necessary for the proper function of important enzymes such as mitochondrial superoxide dismutase, pyruvate carboxylase, glutamine synthase, and arginase (Aschner et al., 2009), chronic or extremely high occupational and environmental exposures to Mn have long been known to lead to a progressive neurological disorder similar to parkinsonism (Aschner et al., 2007; Au et al., 2008). This disorder, known as manganism, is characterized by excessive Mn accumulation in multiple brain regions, primarily in the basal ganglia and related areas associated with control of movement (Aschner et al., 2009; Aschner et al., 2007; Barbeau, 1984; Guilarte et al., 2008; Roth, 2009; Schneider et al., 2006; Sloot and Gramsbergen, 1994). Morphologic changes associated with manganism are neuronal loss and gliosis, primarily in the globus pallidus, substantia nigra pars reticulata, and striatum (Aschner et al., 2009; Olanow, 2004). Although it has been demonstrated that brain Mn levels decrease following the period of exposure (Newland et al., 1989; Roth, 2009), the motor and behavioral effects of manganese toxicity are usually considered irreversible, and there is evidence that they can progress even after chronic exposure has ended (Huang et al., 1993). On the other hand, early signs of manganism are sometimes ameliorated by cessation of exposure (Roth, 2009). These observations suggest that in key regions of the basal ganglia Mn induces initial damage that later evolves to produce the signs and symptoms of manganism. While the exact nature of this initial damage remains speculative, the initial injury probably includes inhibition of mitochondrial energy production, perhaps with a concomitant increase in mitochondrial reactive oxygen species (ROS). This scenario is plausible because the discrete nuclei of the basal ganglia are characterized by complex, interconnected, and synaptically active inhibitory and excitatory pathways, dependent on intricate regulation of pathway activity and a constant supply of energy. Disruption of this communication network could lead to interruption of normal function and eventually to irreversible functional deficits. It is by investigating and delineating the pathways through which Mn-induced damage begins and evolves that we can select appropriate sites of intervention.

A number of reports have associated Mn accumulation with deficits in energy production (Brouillet et al., 1993; Du et al., 1997; Galvani et al., 1995; Gavin et al., 1992; Malecki, 2001; Malthankar et al., 2004; Roth et al., 2000; Roth et al., 2002; Zwingmann et al., 2003) and some have directly shown that Mn inhibits ATP production by oxidative phosphorylation (Gavin et al., 1992; Roth et al., 2000; Zwingmann et al., 2003). The metabolic pathways leading to ATP production by oxidative phosphorylation are complex, with potential inhibition by Mn at multiple sites. Since Mn²⁺ readily binds to Ca²⁺ binding sites, we have hypothesized in the past that Mn²⁺ could inhibit oxidative phosphorylation by binding to the intramitochondrial sites at which Ca²⁺ binds and activates this process; namely, PDH, ICDH, α KGDH, and the F₁F₀ ATP synthase (Gunter et al., 2006). This hypothesis will be partially supported by some of the results discussed below. However, in the work described here we developed a novel approach that allowed us to identify the likely sites at which Mn inhibition affects overall ATP production.

The vast majority of the ATP made in a typical mammalian cell metabolizing glucose is made by oxidative phosphorylation in mitochondria. Oxidative phosphorylation represents the culmination of the processes through which sugars, carbohydrates, fats, proteins, etc. are utilized for energy production. Mitochondria sequester pyruvate, di- and tricarboxylic acids, amino acids, acyl carnitines from fat metabolism, etc. from the cytosol and use these to make ATP. Inside the mitochondria, important steps of the metabolic pathways lie in the TCA cycle, the electron transport chain (ETC), and the F_1F_0 ATP synthase as shown in Fig. 1. Different metabolic substrates utilize different parts of these mitochondrial metabolic pathways to produce two substrates of the ETC, NADH and $FADH_2$. These substrates enter the ETC at complexes I and II, respectively, and two of their electrons are passed sequentially to redox pairs in the ETC containing increasingly stronger oxidants and finally to molecular oxygen at complex IV. Substrate oxidation and the sequential transfer of electrons within the ETC provide the energy needed at complexes I, III, and IV to pump protons from the matrix into the intermembrane space, setting up the electrochemical proton gradient, which provides the energy used to phosphorylate ADP at the F_1F_0 ATP synthase. The rates of oxidation and ATP production are tightly coupled (Chance and Williams, 1955; Chance and Williams, 1956; Voet and Voet, 2004).

Mn^{2+} may inhibit components of the metabolic pathways without inhibiting the rate of ATP production. Enzymes and transporters, representing steps in the metabolic pathways, may be present in excess in some types of mitochondria and the ones present in excess and the extent to which they are in excess can vary between mitochondria from different tissues. Mn^{2+} inhibition at such steps must become rate limiting for the overall process before Mn^{2+} inhibition of ATP production is seen. This can lead to tissue differences in the sites of Mn^{2+} inhibition. Furthermore, different schemes of energization utilize different portions of the metabolic pathways. If Mn^{2+} inhibits a site which is not utilized under a given set of conditions, this inhibition will not affect ATP production under those conditions. Therefore, in order to determine the physiologically relevant primary sites at which Mn^{2+} inhibits ATP production, we developed a novel approach based on analysis of oxidation rates measured under a variety of conditions. Experiments were conducted using State 3 oxidation rates.

State 3 oxidation rates have traditionally been used in mitochondrial work as a measure of ATP production (Chance and Williams, 1955; Chance and Williams, 1956; Voet and Voet, 2004). State 3 conditions are conditions in which rapid phosphorylation in coupled mitochondria is rate limited only by electron transport in the ETC and the activity of the ATP synthase and not by substrate transport or the concentration of ADP or inorganic phosphate (Chance and Williams, 1955; Chance and Williams, 1956). All of the components necessary for oxidative phosphorylation are present at saturation concentrations, meaning that increasing the concentration of any of them doesn't increase the overall oxidation rate. State 3 conditions are especially appropriate for these studies, because the basal ganglia is very active neuronal tissue which requires a considerable amount of ATP to carry out its function. Analysis of State 3 oxidation rates using a variety of energizing metabolic substrates enabled us to identify steps in the process of oxidative phosphorylation which are rate limiting in the presence of Mn^{2+} and a given set of substrates in both coupled and uncoupled mitochondria.

In the experiments described below, oxidation rates were measured in liver, heart, and brain mitochondria under both coupled and uncoupled conditions after the uptake of 0 to 30 nmoles Mn^{2+} /mg protein, using substrates whose transport was shown to not be excessively rate limiting. Among other results we show that Mn^{2+} inhibition of oxidative phosphorylation is indeed different in liver, brain, and heart mitochondria, and that Mn^{2+} inhibition of α -ketoglutarate dehydrogenase activation by Ca^{2+} does not appear to inhibit the overall process of oxidative phosphorylation. These results put Mn^{2+} inhibition of steps

within the metabolic pathways into the context of the overall process of oxidative phosphorylation, making this approach directly relevant to the actual effects of Mn^{2+} on energy production and Mn^{2+} toxicity.

The techniques employed here can be used to study the effects of any agent which inhibits oxidative phosphorylation and should be particularly useful in studies of agents, such as metal ions, which could inhibit at multiple sites.

MATERIALS AND METHODS

Mitochondrial Preparations and Experimental Conditions

Reagents were purchased from Sigma Chemical Co (St. Louis, MO). All mitochondrial preparations were made from female Sprague-Dawley pathogen-free rats weighing between 180 and 200 g. The media used in mitochondrial preparations and oxidation rate experiments were in mM (except for BSA which was in mg/ml): Experimental medium: 120 KCl, 10 K HEPES (pH 7.2), 5 KPi, 5 $MgCl_2$, 25 sucrose, plus the mitochondrial substrate used in each experiment. Note: KPi is a mixture of KH_2PO_4 and K_2HPO_4 in proportions that vary with the pH. Buffer A: 200 mannitol, 70 sucrose, 2 K HEPES (pH 7.2), 0.05 K EGTA, 0.01 $MgCl_2$, 0.5 BSA. Buffer B : 195 mannitol, 65 sucrose, 6 K HEPES (pH 7.2), 1 BSA. Isolated rat liver mitochondria were prepared as described (Wingrove and Gunter, 1986). Rat heart mitochondria were prepared using a protocol (Gunter et al., 2004) based on that developed by Hoppel, which is a modification of the technique of Palmer et al. (Palmer et al., 1977) as described in Gunter et al. (Gunter et al., 2004). Rat brain mitochondria were prepared using a modification of the technique described in Gunter et al. (Gunter et al., 2004) and also are described in more detail in the Supplementary Material. Isolated mitochondria were tested for viability by measuring RCR's and by assessing their ability to hold 80 nmoles Ca^{2+} /mg protein for 2 hours using a Ca^{2+} electrode. For liver mitochondria, RCR's were generally required to be greater than 6 and for heart and brain mitochondria greater than 4 using glutamate plus malate as substrates. After preparation, mitochondria were initially stored on ice at 30 mg/ml in medium containing buffer B plus 0.5 mM substrate in experiments where one type of substrate was used for the entire experiment or in buffer B plus 1 mM ATP when more than one type of substrate (e.g. succinate and also glu + mal) was used in the experiment.

There are three commonly used sets of mitochondrial substrates that are usually transported into mitochondria fast enough and used in oxidative phosphorylation so that electron transport or ADP phosphorylation is the rate limiting step. These are succinate, glu + mal, and pyr + mal. Since these sets of substrates utilize different portions of the TCA cycle and to a lesser extent the ETC, their use has the advantage of distinguishing different locations of Mn^{2+} inhibition within the metabolic pathways. But because mitochondria from different tissues contain different amounts of the enzymes and transporters making up the steps of the metabolic pathways, substrates that are not rate limiting in one tissue may be rate limiting in another. For example, as our oxidation rate measurements show, transport of pyruvate in liver mitochondria and succinate in brain mitochondria slows the rate of oxidative phosphorylation. For our Mn^{2+} inhibition studies, we used only those substrates whose transport into mitochondria was not excessively rate limiting for each tissue.

Water jacketed and magnetically stirred chambers of two different sizes were used. A glass 2 ml chamber was used for most of the liver mitochondrial experiments for which large quantities of isolated mitochondria were available. A smaller plastic 0.2 ml chamber was used for the heart and brain mitochondrial experiments and a few liver mitochondrial experiments for which only limited amounts of mitochondria were available. Each chamber was sealed with a specially fitted plug containing a capillary through which additions were

made using gel loading pipetter tips. Prior to each oxidation rate experiment, concentrated mitochondria were suspended in buffer B containing the appropriate amount of substrate and pre incubated at 20° C for 4 minutes to bring them to temperature. Then varying concentrations of Mn^{2+} were added, bringing the mitochondrial concentration to 25 mg protein per ml for the large chamber experiments and 10 mg protein per ml for the small chamber experiments. This suspension was incubated for 16 minutes to allow time for complete Mn^{2+} uptake. These “incubated mitochondria” were then stored on ice and used in experiments as quickly as possible. Generally about 98% of the added Mn^{2+} is sequestered by the mitochondria under these conditions (Gunter et al., 1978; Gunter et al., 1975). In these experiments, the final concentration of suspended mitochondria was ~ 1 mg/ml in both the large and small chambers. A larger concentration of ADP was used in the small chamber to extend the linear portion of the oxygen use curve, allowing averaging of more data points and hence better signal to noise. Since ADP concentration is “saturated” and therefore not rate limiting in these experiments, the higher concentration does not affect the measured oxidation rates. Details of the additions made to the different chambers in the oxidation rate experiments to the different chambers are given in the Supplementary Material.

For experiments in the 2.0 ml chamber, the O_2 concentration was measured using a Yellow Springs Instruments Co. YSI 5331 O_2 electrode; the corresponding O_2 electrode for experiments in the 0.2 ml chamber was a Microelectrodes MI-730. The measurement system also contained a Microelectrodes ADPT pre-amp, a DATAQ DI-710 analog to digital converter and computer interface feeding the information into an Apple notebook computer for analysis. The oxygen electrode was calibrated at atmospheric oxygen concentration and zero oxygen concentration. Computer software was used to fit lines to the curves of O_2 concentration vs time, and the rates of O_2 consumption were obtained from the slopes of these fitted lines. In all cases, experiments were performed under both coupled and uncoupled conditions and the relative oxidation rates were plotted against the final Mn^{2+} concentration. The addition of an uncoupler such as DNP or CCCP allows protons which have been pumped out of the mitochondrial matrix to leak rapidly back across the inner membrane, bypassing the F_1F_0 ATP synthase and therefore providing information only on the steps prior to the ATP synthase. Comparing Mn^{2+} inhibition under coupled versus uncoupled conditions thus enabled us to determine immediately whether the ATP synthase was the Mn^{2+} inhibition site. Although the fall in membrane potential induced by an uncoupler would eventually cause Mn^{2+} efflux from the mitochondrial matrix, our past work has shown that Mn^{2+} efflux would take around 10 minutes, while the oxidation rate measurements only require 20 to 30 seconds so that most of the Mn^{2+} is still inside the mitochondria under our measurement conditions (Gunter et al., 1978; Gunter and Puskin, 1975; Gunter et al., 1975; Puskin et al., 1976).

Measurements of the activity of α ketoglutarate dehydrogenase

These assays used 0.2 units/ml isolated, purified α KGDH (Sigma, St Louis, MO), 1 mM β NAD, 0.25 mM CoA, and 2 mM $Mg(NO_3)_2$. NADH was measured by absorption at 340 nm.

Derivation of equation for confirming that use of glu + mal produces NADH and very little $FADH_2$

To identify probable sites of Mn^{2+} inhibition using oxidation rates, it is necessary to know which of the ETC substrates (NADH or $FADH_2$) are produced by each substrate set. When succinate is used as substrate, product inhibition of MDH (see Fig. 1) caused by accumulation of oxaloacetate, which is not transported across the mitochondrial inner membrane, leads to exchange of intramitochondrial malate for external succinate. This causes the $FADH_2$, produced at SDH, to be the only ETC substrate produced in quantity. In

quantitative oxygen jump experiments designed to illuminate the pumping stoichiometries at the sites of mitochondrial proton pumping, Lemasters found that energization with glu + mal produced NADH with very little FADH₂ (Lemasters, 1984). In this process most of the α KG produced by the transamination reaction, in which the amine group from glutamate is transferred to oxaloacetate making aspartate and α KG, was also found to be transported out of the mitochondrial matrix in exchange for malate (Lemasters, 1984). These results tell us that when glu + mal are the substrates, NADH is produced at MDH but that little NADH is produced at α KGDH and even less FADH₂ is produced at SDH, since succinate also exchanges for malate (see Fig. 1). These conclusions, that succinate produces FADH₂ and very little NADH and that glu + mal produce NADH with very little FADH₂ greatly simplify the analysis of the oxidation rate data, and the exchange of α KG for malate helps explain our α KGDH results (see Discussion). We can derive an equation to show that the oxidation rate results found in the current work are consistent with this earlier finding.

There is a general consensus that under ideal conditions, four protons are pumped from the matrix to the intermembrane space at complex I and a total of 6 protons are pumped at complexes III and IV. This would mean that the maximum number of protons pumped between NADH and O₂ would be 10 and the maximum number pumped between FADH₂ and O₂ would be 6 (Brand, 2005). Since the measured electrochemical proton gradient (membrane potential plus pH gradient) is the same whether generated by NADH or by FADH₂, and since fewer protons are pumped per FADH₂ than are pumped per NADH, the system compensates by using FADH₂ (and therefore O₂) at a faster rate than it uses NADH. The increase in oxidation rate with FADH₂ over that for NADH must be proportional to the inverse of the protons pumped for each ETC substrate or 10/6. Therefore, since use of succinate produces FADH₂ and only traces of NADH, if the measured ratio of the saturation oxidation rate using glu + mal over that using succinate is near 0.6, use of glu + mal must produce primarily NADH and only a small amount of FADH₂. We can use these observations to set up an equation to estimate the fraction of FADH₂ produced when glu + mal is used as substrate in our experiments. If F is defined as $F = [FADH_2] / ([FADH_2] + [NADH])$ when glu + mal are substrates, this permits us to set up the simple equation:

$$\{(0.6)(1F) + (1)(F)\} / \{(1)(1)\} = \{\text{sat. ox. rate using glu+mal}\} / \{\text{sat. ox. rate using succinate}\}.$$

This equation simply states that the sum of the relative oxidation rates times the fractions of NADH and FADH₂ produced using glu + mal over that for succinate should equal the measured ratio of saturation oxidation rates. From this we can calculate the value of F, which for consistency should be small and positive or zero. If F were zero, we can see that in the ideal case, the value of this ratio should be 0.6 as the discussion above would require. This derivation is based on an "idealized model" without loss processes while oxidative phosphorylation in the real world also involves loss of proton pumping. Notice that while F is defined as a ratio of concentrations, the concentrations themselves are never used directly but only as a dimensionless ratio. We can, however, compare F with numbers derived from the numbers of protons pumped at the three sites of proton pumping and also discuss how F might be expected to change with time during our oxidation rate experiments. This is discussed further in the Results section and in the Supplementary Material.

RESULTS

Direct assays of Mn²⁺ inhibition of intramitochondrial metabolic enzymes

Mn²⁺ binds to almost every Ca²⁺ binding site, often with a higher affinity than Ca²⁺ itself. Accordingly, we hypothesized that a likely mechanism through which Mn²⁺ could inhibit oxidative phosphorylation would be to bind to those Ca²⁺ sites responsible for activation of

oxidative phosphorylation, either inhibiting or activating less than Ca^{2+} (Gunter et al., 2006). As shown in Fig. 2A, Ca^{2+} activates isolated, purified αKGDH , as reported earlier by Denton and coworkers (Rutter and Denton, 1988; Rutter and Denton, 1989). Fig. 2B shows that if the $[\text{Ca}^{2+}]$ is maintained at $\sim 2 \mu\text{M}$ and Mn^{2+} is added in increasing concentrations, the rate of NADH production falls dramatically. However, the rate does not fall to the level observed at very low $[\text{Ca}^{2+}]$ (Fig. 2A), suggesting some activation of NADH production by bound Mn^{2+} . We conclude that Mn^{2+} inhibits Ca^{2+} activation of αKGDH but probably does not inhibit αKGDH itself. This supports our hypothesis, but as will be shown later, other sites not involving Ca^{2+} activation are also sites of Mn^{2+} inhibition.

Saturation oxidation rates using different mitochondrial substrates

Since the rates of substrate transport vary among different types of mitochondria, experiments should be carried out at substrate concentrations large enough to support the highest attainable rates of substrate transport (known as saturation rates), and substrate transport should not excessively limit the measured oxidation rate. Accordingly, we show in Fig. 3 that for liver mitochondria, the oxidation rate both for succinate and for glutamate transport in the presence of malate saturates at around 5 to 6 mM added substrate. Note that the maximum rate of oxygen use is much faster when succinate is used as a substrate. For liver mitochondria, pyruvate transport was found to be strongly rate limiting at all pyruvate concentrations (data not shown), indicating that there was no value in carrying out oxidation rate experiments using pyr + mal as substrates with liver mitochondria. Similar oxidation rate vs substrate concentration experiments were carried out in heart and brain mitochondria to establish the saturation concentrations of each substrate in these mitochondria (see Supplementary Material and figure captions).

Very little FADH_2 is produced when glu + mal are the substrates

When succinate is the substrate, the product of MDH, oxaloacetate, accumulates because it cannot be either transported out of the mitochondria or used to form citrate at citrate synthase because of lack of acetyl coA. This accumulation of product inhibits MDH, allowing malate to accumulate and to exchange for external succinate. Therefore, NADH production at MDH falls to near zero allowing FADH_2 production at SDH with very little NADH production at MDH. FADH_2 production was found to be very small when glu + mal were used as substrates in an earlier report (Lemasters, 1984). In energization experiments with liver and heart mitochondria, the ratio of saturation oxidation rate using glu + mal over that for succinate was near 0.6, suggesting that F (defined as $[\text{FADH}_2]/\{[\text{FADH}_2] + [\text{NADH}]\}$) is small. The ratio of glu + mal to succinate saturation oxidation rates (glu + mal rate/succinate rate), obtained from the same preparation of mitochondria to decrease variability, gave a ratio of 0.657 for liver mitochondria and 0.649 for heart mitochondria, leading to F values of 0.143 and 0.123, respectively. Furthermore, the oxidation rate measurements carried out here were initial rate measurements made at a time when suppression of NADH production by oxaloacetate accumulation may not have been complete, when succinate was used as substrate. As discussed in the Supplementary Material, this suggests that these estimates of F should be adjusted downward probably below 0.1, strengthening the conclusion that very little FADH_2 is produced when glu + mal are the substrates. The small effects of slippage of the proton pumps and production of ROS which take electrons from the ETC are discussed in the Supplementary Material along with an analysis of the error of these measurements. Our experimental results are consistent with the earlier finding that FADH_2 production is small when glu + mal are the substrates (Lemasters, 1984). Knowing that use of succinate produces FADH_2 and only a little NADH and use of glu + mal produces NADH and only a little FADH_2 simplifies the analysis and allows us to identify overlaps and differences between the pathways used by these two sets of substrates, and to identify the number of oxygen atoms reduced per molecule of each.

This information allows us to draw conclusions about likely sites of Mn inhibition (see Discussion).

Mn²⁺ inhibition of oxidation rates in liver mitochondria

Fig. 4A shows Mn²⁺ inhibition of the relative coupled and uncoupled oxidation rates of liver mitochondria with succinate as substrate. Fig. 4B shows the same information using glu + mal. The data were obtained under both coupled and uncoupled conditions. Although both the coupled and the uncoupled data fit straight line dependencies fairly well with each set of substrates, the coupled data show increased inhibition of oxidation rate with increasing [Mn²⁺], while the uncoupled data essentially show no inhibition. These results show that the observed Mn²⁺ inhibition was at the F₁F₀ ATP synthase - another site of Ca²⁺ activation and also a site common to the pathways used by these two sets of substrates. Repeating the coupled experiments shown in Fig. 4A and B using mitochondria from a single preparation to reduce variability (see Fig S4 in the Supplementary Material) confirmed that there is no significant difference between the succinate and glu + mal results. Ca²⁺ also activates the ANT and therefore Mn²⁺ might be expected to bind to the ANT and possibly inhibit it (Hansford, 1985; Wan et al., 1993a; Wan et al., 1993b; Wan et al., 1989). However, the binding site for this activation is on the outer side of the inner membrane where the [Mn²⁺] is lower and effects are less likely as detailed in the Discussion.

Mn²⁺ inhibition of oxidation rates in brain mitochondria

Measurements of oxidation rates in rat brain mitochondria as a function of succinate, glu + mal, or pyr + mal concentrations (see Supplementary Material), similar to those shown for liver mitochondria in Fig. 3, showed rate limitation by succinate transport via the dicarboxylic acid exchanger. This rate limitation was not so strong as to prevent useful data on Mn²⁺ inhibition from being obtained when succinate is used. Rate limitation by succinate transport also affects the shape of the Mn²⁺ inhibition curve (see Fig. 5), since no inhibition by Mn²⁺ can be seen until it overcomes the inhibition by succinate transport. Based on the data shown in Fig. S1, brain mitochondrial experiments were conducted at saturation concentrations of 15 mM succinate, 10 mM glutamate + 5 mM malate, and 5 mM pyruvate + 5 mM malate. Fig. 5A shows the coupled and uncoupled oxidation rates of brain mitochondria as a function of added Mn²⁺ when succinate is used as substrate. Since the coupled and uncoupled data are the same to within the variation in the data, Mn²⁺ inhibition of the F₁F₀ ATP synthase is not rate limiting in brain mitochondria as it was in liver mitochondria. Mn²⁺ inhibition of oxidation rate begins at concentrations over 10 nmoles Mn²⁺/mg protein and becomes very strong above 20 nmoles/mg protein, reaching a level that is about 3 times greater than the level of inhibition in liver mitochondria at a similar Mn²⁺ concentration (Fig. 4 and Fig. 5A). Close examination of Fig. 5A reveals that Mn²⁺ does not appear to inhibit succinate transport itself (i.e. the dicarboxylic acid exchanger). If it did, Mn²⁺ would enhance rate limitation by succinate transport and this would appear as Mn²⁺ inhibition at low Mn concentrations where no Mn²⁺ inhibition is evident.

Fig. 5B shows the coupled and uncoupled oxidation rates of brain mitochondria with glu + mal as substrates. In this case, a relatively large inhibition of oxidative phosphorylation is observed even at low [Mn²⁺], but again there is no significant difference between the coupled and uncoupled rates. The first of these observations is consistent with the earlier observation that transport of glu+ mal is not rate limiting, while the second again corroborates the finding that the primary inhibition is not at the F₁F₀ ATP synthase. As with succinate in Fig. 5A, the inhibition reaches a level almost 3 times larger than in liver mitochondria; however, with glu + mal the maximum rate of Mn²⁺ inhibition (slope of the oxidation rate curve) with increasing [Mn²⁺] is about 2.5 times smaller than with succinate. This, together with the small overlap of pathway components, suggest that these two

inhibitions may be at completely different loci (see Discussion). This would mean that the Mn^{2+} inhibition seen when glu + mal are substrates lies somewhere in the pathway α KGDH, succinyl coA synthase, MDH, aspartate aminotransferase, the glutamate-aspartate exchanger, or complex I. None of these pathway components is shared with the succinate pathway. If the Mn^{2+} inhibition with succinate is completely separate from that with glu + mal, that would eliminate complexes III and IV, which are part of the pathways used by both succinate and glu + mal, as possible sites of Mn^{2+} inhibition.

Fig. 5C shows the coupled and uncoupled oxidation rates for Mn^{2+} inhibition of brain mitochondria when pyruvate and malate are used as substrates, and Fig. S4 compares the maximum coupled Mn^{2+} inhibition rates (slopes) in brain mitochondria for succinate, glu + mal, and pyr + mal showing that these maximum inhibition rates give relative slopes of -13.3 for succinate, -5.3 for glu + mal and -1.0 for pyr + mal. Notably, Mn^{2+} inhibition of oxidation rate with pyr + mal is considerably smaller than that with either succinate or glu + mal (see Discussion).

Mn^{2+} inhibition of oxidation rates in heart mitochondria

In heart mitochondria, none of the three common ways of energizing mitochondria is rate limited by substrate transport under saturation conditions. Fig. 6 shows the relative oxidation rate under both coupled and uncoupled conditions with succinate (A), with glu + mal (B), and with pyr + mal (C). There are significant differences between the results with liver or brain mitochondria and heart mitochondria. First, the total inhibition of oxidative phosphorylation in heart mitochondria energized by succinate or by glu + mal is a little over 1.5 times greater than in liver mitochondria but less than that in brain mitochondria. Second, the inhibition is stronger at low $[Mn^{2+}]$ in heart mitochondria than in liver mitochondria, but it also saturates at a lower $[Mn^{2+}]$. Third, Mn^{2+} inhibition under uncoupled conditions is much less than that under coupled conditions with each of these substrates. When either glu + mal or pyr + mal are the substrates, there is no significant inhibition under uncoupled conditions, and therefore the primary inhibition must be at the F_1F_0 ATP synthase as it was with liver mitochondria. However, when succinate is the substrate, although the primary inhibition seems to be at the F_1F_0 ATP synthase, there also seems to be a small amount of concentration-dependent Mn^{2+} inhibition even under uncoupled conditions. This may represent a small inhibition of the same site inhibited in brain mitochondria when succinate is substrate. This is discussed further in the Discussion section.

DISCUSSION

Interpretation of brain mitochondrial data

The results clearly indicate that the primary site of Mn^{2+} inhibition in liver and heart mitochondria is the F_1F_0 ATP synthase (Fig. 4 and Fig. 6) and that Mn^{2+} does not appear to inhibit the dicarboxylic acid exchanger in brain mitochondria (Fig. 5A); however, interpretation of other data, e.g. most of the brain mitochondrial data, is more complex. Obviously, the site or sites of inhibition seen with each set of substrates must lie on the pathways used by these sets of substrates. To interpret these data, it is necessary to consider the strengths and characteristics of the inhibitions seen with each substrate set in the light of existing knowledge of the pathways used. Since succinate produces $FADH_2$ and very little NADH while glu + mal produce NADH and very little $FADH_2$, we know that the pathways used by these substrates only overlap at the dicarboxylic acid exchanger, complexes III and IV and at the ATP synthase (see Fig. 1 and Table 1 discussed below). As noted in the discussion of Fig. 4 in Results, the differences between the coupled and uncoupled data for brain mitochondria are small and within the variation of the data. Under uncoupled conditions the protons which were pumped out of the mitochondrial matrix at complexes I,

III, and IV leak back into the mitochondria without passing through the ATP synthase as they do under coupled conditions. If Mn^{2+} inhibition were at the ATP synthase, it would be seen under coupled conditions but not under uncoupled conditions. Clearly, this is not the case for brain mitochondria, where the results under coupled and uncoupled conditions are very similar. Therefore, inhibition is not at the ATP synthase. As noted above and in Fig. 5A, inhibition is not at the dicarboxylic acid exchanger. If inhibition were at a site common to both the pathways used when succinate is the substrate and when glu + mal are the substrates, the only sites where that could be true would be complexes III and IV. If the inhibition were at one of these sites the strengths of the inhibitions should be about the same, rather than 2.5 times stronger with succinate as shown in Fig. 5A & B. Therefore, either the Mn^{2+} inhibitions seen with succinate and with glu + mal must be at two completely different (independent) sites, or there is a common inhibition at either complex III or IV which has the strength of that seen when glu + mal are the substrates and an additional stronger inhibition somewhere in the pathway used only by succinate. Since complexes III and IV are also within the pathway used by pyr + mal, pyr + mal results in brain mitochondria can shed further light on whether there is Mn^{2+} inhibition at complexes III or IV. The use of pyr + mal produces 4 NADH's and an $FADH_2$ (see Fig. 1), and its pathway overlaps completely with that of succinate and substantially with that of glu + mal. These characteristics make interpretation of the pyr + mal results more complex, requiring consideration of the amount of oxygen reduced per unit (molecule or molecules) of substrate, but, as will be seen from the discussion that follows, they also indicate that inhibition at complexes III or IV is unlikely.

Only two types of arguments can be invoked to explain why Mn^{2+} inhibition when pyr + mal are used with brain mitochondria is much less than that seen with either succinate or glu + mal: 1) The inhibited site or sites are part of the pathway associated only with a specific substrate such as glu + mal, and therefore no inhibition is seen with pyr + mal, and 2) when pyr + mal are the substrates, each passage through the inhibition site results in the production of many more molecules of NADH and $FADH_2$ (see Fig. 1); therefore, the consumption of much more oxygen per passage through the inhibition site results in a smaller decrease in oxidation rate with pyr + mal. Each of these two possibilities can account for a portion of our results, as discussed below.

The strength of the Mn^{2+} inhibition can be measured by the negative slope in a plot of oxidation rate vs Mn^{2+} concentration (see Fig. S4). Since the strength of an inhibition depends on the details of the interaction between the inhibitor and the inhibition site, there is no general relationship between the strengths of inhibitions at independent sites. However, in the current data, when substrate pathways overlap, the same site of Mn^{2+} inhibition can be encountered in experiments using different sets of substrates. Since oxidation rates are being measured, the strengths of these inhibitions are proportional to the number of passes through the inhibited site divided by the relative amount of oxygen used per molecule or molecules of substrate (e.g. 1 glu + 1 mal), because the larger the number of oxygens used, the smaller the effective inhibition of oxidation rate. (This topic is discussed further in the Supplemental Material.) In order to help keep these factors straight, we have constructed Table 1, which lists the sites of possible Mn^{2+} inhibition in the metabolic pathways associated with each set of substrates. The numbers to the left of the enzyme names indicate the relative strength of Mn^{2+} inhibition; they represent the ratio of the number of times a particular potential inhibition site is traversed divided by the number of $FADH_2$'s and NADH's produced (or number of oxygen atoms reduced) per molecule of all of the components of the substrate. For example, when succinate is used as substrate, the possible sites of inhibition include the dicarboxylic acid exchanger, SDH, fumarase, complex II, complex III, complex IV, and the F_1F_0 ATP synthase (Table 1). It should also be kept in mind that complex II consists of SDH and a pathway for reducing equivalents to reduce

ubiquinone which goes on to reduce complex III, so that listing it separately from SDH is somewhat redundant (Miyadera et al., 2003). The ratio for each of the succinate sites is 1 (i.e., 1 traversal of the inhibited site per molecule of succinate used/1 molecule of FADH_2 produced). One molecule of glu + mal produces either one or two NADH's. If malate exchanges for αKGDH , as was found to be most likely by Lemasters (Lemasters, 1984), one NADH is produced at MDH, while if malate exchanges for succinate, an additional NADH is produced at αKGDH ; therefore, a site on the glu + mal pathway may have 1 where one NADH is produced at MDH or 0.5 where one NADH is produced at MDH and one at αKGDH . Use of pyr + mal produces four NADH's and one FADH_2 (see Fig. 1), so the number for some sites is 1/5 or 0.2. It is important to note that the numbers for complexes III and IV are 1 no matter which set of substrates is used. The underlying reasons for the numbers in Table 1 are discussed further in the Supplemental Material.

As noted above, we have already ruled out the F_1F_0 ATP synthase and the dicarboxylic acid exchanger as possible sites of Mn^{2+} inhibition in brain mitochondria. The relative inhibition numbers in the table tell us that if either complex III or IV is inhibited by Mn^{2+} , the apparent strength of the inhibition will be the same whether succinate, glu + mal, or pyr + mal are used as substrates. That is very inconsistent with the data (see Fig. S5), which show that the maximum relative slopes of the inhibition curves are -13.3 for succinate, -5.3 for glu + mal, and only -1 for pyr + mal, strongly suggesting that complexes III and IV are not primary sites of Mn^{2+} inhibition. Table 1 also indicates that all of the sites which could be inhibited when succinate is substrate must also be inhibited when pyr + mal are the substrates; however, it suggests that since 5 oxygen atoms are reduced for each set of molecules of pyr + mal versus 1 oxygen atom per molecule of succinate, the apparent strength of the inhibition at many of these sites will be 5 times weaker for many sites in the pyr + mal pathway. The actual data shows even less inhibition when pyr + mal is used than these calculations would indicate; however, the scatter in the pyr + mal data is large enough so that the error in the slope of the inhibition line for the pyr + mal data could easily mask a slope which is two to three times larger, making these observations more consistent. Since any inhibition seen when succinate is substrate must also be seen when pyr + mal are the substrates, the strength of the inhibition seen with succinate more than accounts for all of the inhibition seen with pyr + mal. This has two important implications: First, it suggests that the independent inhibition seen with glu + mal is also independent of pyr + mal, because if it occurred at a shared site it should add to the slope of the inhibition seen when pyr + mal are substrates in an amount a bit less than that caused by the inhibition site encountered when succinate is substrate. Our results do not show this much inhibition. Second, it suggests that inhibition at any of the sites, such as pyruvate translocase, pyruvate dehydrogenase or isocitrate dehydrogenase, that are used only by pyr + mal must be very small or zero because we've already accounted for all of the inhibition seen with pyr + mal. Indeed, there could be inhibition at one or more of these sites or at αKGDH , but, if so, this inhibition could be disguised by excess enzymatic capacity in any such inhibited sites.

These considerations greatly reduce the likelihood of inhibition at either complex III or complex IV, sites common to the pathways used by all three substrate sets. Every site on the succinate pathway is also on the pyr + mal pathway and the relative strengths of the inhibitions seen are 1 to 13.3. Therefore, the data would suggest that if there is inhibition at either III or IV, the strength of that inhibition must be less than 1/13.3 of the size of the inhibition seen when succinate is substrate and correspondingly smaller than the inhibition seen when glu + mal are the substrates. In fact, such common inhibition would really have to be much smaller than that because while it is true that sites in the TCA cycle can be found which would show a much stronger inhibition when succinate is the substrate than when pyr + mal are the substrates, we have already accounted for all of the inhibition seen when pyr + mal are the substrates regardless of the site inhibited. While we can never rule out the

possibility of a very small inhibition at a common site, this analysis makes it clear that there must be at least two independent inhibitions seen when succinate is the substrate and when glu + mal are the substrates.

The same two explanations given above based on Table 1 apply to comparisons of the (stronger) glu+ mal and (weaker) pyr + mal inhibition as well: either the enzyme inhibited in the presence of glu + mal is not on the pyr + mal pathway, or the inhibition site is on a shared pathway and the inhibition strength seen with pyr + mal is decreased by much greater oxygen use per unit substrate. This latter case seems less likely, since if the inhibition is on both the glu + mal and pyr + mal pathways, the pyr + mal results should show more inhibition than they do. It seems more likely that the site at which Mn^{2+} inhibits when glu + mal are the substrates is not part of the pyr + mal pathway since this would not require additional inhibition when pyr + mal are substrates. These considerations allow us to narrow the identification of the two likely independent sites encountered with succinate and glu + mal, respectively, as SDH, fumarase, or complex II when succinate is the substrate, and glutamate/aspartate exchange or aspartate aminotransferase when glu + mal are the substrates. Since SDH is a part of complex II (Miyadera et al., 2003), it is fair to say that the Mn^{2+} inhibited site encountered when succinate is substrate is either fumarase or complex II. These results are consistent with the results of Zwingmann et al. (Zwingmann et al., 2003) who studied Mn^{2+} inhibition in astrocytes and neurons using nuclear magnetic resonance spectroscopy. They found that Mn^{2+} caused an accumulation of succinate within the TCA cycle, consistent with inhibition of SDH.

These results might appear to suggest that Mn^{2+} is not an effective inhibitor of oxidative phosphorylation in brain mitochondria when pyr + mal are the substrates. Indeed, since the brain primarily metabolizes sugars, pyruvate might be thought to be the more common substrate. However, glutamate is often used metabolically in the brain in addition to its role as a neurotransmitter (McKenna, 2007; McKenna et al., 1996; McKenna et al., 1994). Furthermore, if the inhibition seen with glu + mal is at either the glutamate aspartate exchanger or at aspartate aminotransferase, the inhibition would limit the complement of amino acids available for synthesis of peptides by mitochondrial DNA -all of which are essential to ADP phosphorylation. While this effect would not show up in experiments such as those described here, it could slowly decrease ATP production in animals exposed to Mn^{2+} over the period of Mn^{2+} exposure *in vivo*.

Two isoforms of glutamate/aspartate exchanger have been found in mitochondria, both of which bind Ca^{2+} (Palmieri et al., 2001). Since Mn^{2+} binds to almost every Ca^{2+} binding site, Mn^{2+} might also be expected to bind to these exchangers. However, only one of these types of glutamate/aspartate exchanger is found in brain (aralar 1), while the other is found in liver (citrin), where we did not see this type of inhibition (del Arco et al., 2002). Thus it may be that aralar 1 but not citrin is inhibited by Mn^{2+} .

Returning to the question of the additional Mn^{2+} inhibition seen in heart mitochondria when succinate is used as substrate over and above that caused by inhibition at the ATP synthase (see Fig. 6A), the most likely explanation is inhibition at the same site seen when succinate is substrate in brain mitochondria, i.e. fumarase or complex II. The same rules apply to interpretation of these heart mitochondrial data as applied to brain mitochondrial data, based on Table 1. In this case the reason that we don't see this inhibition when pyr + mal are the substrates is probably that it's a small inhibition to begin with and that when it is decreased by a factor of 5 as suggested by the table, it's simply lost in the noise.

Possible inhibition of the adenine nucleotide translocase

The ANT is also found in the literature to be a site of Ca^{2+} activation of oxidative phosphorylation; however, this site is activated by the $[\text{Ca}^{2+}]$ on the outside of the inner membrane (Hansford, 1985; Wan et al., 1993a; Wan et al., 1993b; Wan et al., 1989). The reason that this site was not given more emphasis as a possible site of inhibition was because of the differences of $[\text{Mn}^{2+}]$ expected on the inside and outside of the mitochondrial inner membrane. These experiments were carried out at 1 mg mitochondrial protein per ml at concentrations of total added manganese of 0 to 30 nmoles/mg protein. Typically about 98% of the Mn^{2+} was sequestered by the mitochondria (Gunter et al., 1978; Gunter et al., 1975; Puskin et al., 1976). At 30 nmoles/mg added Mn^{2+} , this would mean that the external $[\text{Mn}^{2+}]$ was around 6×10^{-7} M, and at a mitochondrial water volume of 2 μl /mg protein, the total intramitochondrial $[\text{Mn}^{2+}]$ would be around 14.7 mM. Undoubtedly, weak binding of Mn^{2+} to mitochondrial matrix components including the inside of the inner membrane would decrease the free ion concentration (Gunter et al., 1978; Gunter et al., 1975; Puskin et al., 1976). Nevertheless, the gradient of free Mn^{2+} ion concentration across the mitochondrial inner membrane would still be very large. This would greatly favor Mn^{2+} inhibition of components on the inside of the inner mitochondrial membrane.

Mn^{2+} may inhibit additional sites within the metabolic pathways and excess enzymatic capacity may mask these additional sites of inhibition

Clearly, while Mn^{2+} inhibits oxidative phosphorylation in all three types of mitochondria, there are important differences in the loci and strengths of these inhibitions. Furthermore, while NADH production by αKGDH is clearly inhibited by Mn^{2+} , as seen in Fig 2B, there is no sign of this inhibition in the data shown in Figures 4, 5, and 6. It is likely that the reason that it is not seen in mitochondria when glutamate plus malate are the substrates is that, as found by Lemasters (Lemasters, 1984), most of the αKG goes out of the mitochondrial matrix in exchange for malate so that very few αKG molecules pass through the αKGDH step. When pyruvate plus malate are the substrates, the relative effect is much less because of the five oxygens reduced per pyruvate used. Inhibition of αKGDH may also be masked by excess activity of the enzyme, as outlined in the Introduction. This shows how inhibition experiments based on the overall process of oxidative phosphorylation help to differentiate between inhibition of a step in the metabolic reactions and inhibition of the overall process.

A rough comparison of the range of $[\text{Mn}^{2+}]$ used in these studies with those found to induce toxicity in primates

An important question is how the amounts of Mn^{2+} used in the brain mitochondrial experiments compare with amounts shown to induce signs of toxicity in test animals. Since we are interested in effects on humans, the best test animals are nonhuman primates. Suzuki et al. (Suzuki et al., 1975) injected Mn weekly over a period of 3 months using a control (vehicle only) and three doses of MnCl_2 (2.25, 4.5, and 9 g per injection). At the end of the 3 month period, the measured amounts of Mn in the striatum and globus pallidus, respectively, were 20.9 and 35.3 μM in the control; 91.0 and 100.7 μM at 2.25 g, 173.7 and 241.7 μM at 4.5 g, and 264.5 and 334.4 μM at 9 g. Dose-dependent signs of toxicity were observed at all doses. If we assume that 100 μM Mn in the target tissue represents a rough threshold for signs of significant toxicity, we can make very approximate calculations as to how this level in tissue compares to the amounts of Mn in the mitochondria used in the experiments reported here. The brain mitochondria used in the current experiments were isolated from trimmed whole rat brain preparations, excluding the brain stem, and white matter was also trimmed away. The ratio of neuronal to glial mitochondria in the final preparation is not well established, but it has been argued that the preparation selects for neuronal mitochondria (Lee et al., 1993). For the purposes of this calculation, let us assume a 50/50 mixture. The fraction of the volume of the neuron taken up by mitochondria is probably somewhere

around 0.15 while that of glia, which rely more on glycolysis, is probably closer to 0.10, so for these brain mitochondria, let's use 0.125. Intracellular Mn is distributed within the cell between mitochondria, nuclei, and other cellular components. While a number of tissue and cell fractionation studies have been conducted in an effort to determine which cell fraction accumulates the most Mn, they did not generally account for the energization state of the mitochondria so that results ranged from most of the Mn being in the mitochondria to most of the Mn being in the nucleus (Ayotte and Plaa, 1985; Kalia et al., 2008; Lai et al., 1999; Liccione and Maines, 1988; Maynard and Cotzias, 1955; Miller et al., 1975). While we have not carried out fractionation studies of this type, we have published data strongly indicating that fully energized mitochondria compete effectively to sequester intracellular Mn (Gunter et al., 2009). Since we consider it likely that well over half of intracellular Mn is intramitochondrial, we will assume for purposes of this calculation that all of the intracellular Mn is intramitochondrial. With these assumptions, 100 μ M intracellular Mn would represent about 0.8 mM mitochondrial Mn. Measurements of mitochondrial water volumes range from 1 to 3 μ l/mg protein (Jensen et al., 1986). If we use 2 μ l/mg in the calculation, that would indicate that at a cell concentration of 100 μ M, the mitochondria contain about $0.8 \times 2 = 1.6$ nmoles Mn/mg protein. While this is a very rough calculation, it would suggest that the Mn concentration range where signs of toxicity begin to be apparent is around 1.6 to 2 nmoles/mg mitochondrial protein and that the dose range of primary interest for toxicity would be up to about one order of magnitude above that level, say up to around 20 nmoles/mg. This agrees with the range of the data presented here.

In a review article (Gavin et al., 1999), we cited earlier work, covering a greater range of Mn^{2+} concentrations, showing that oxidation rate data in liver mitochondria suggested that the primary Mn^{2+} inhibition of oxidative phosphorylation was at the F_1F_0 ATP synthase (Gavin et al., 1992), as is shown by the liver data reported here. The earlier work (Gavin et al., 1992) did not include measurement of oxidation rates in brain mitochondria because of the small yield of mitochondria from brain. In the earlier work, we saw a difference in Mn^{2+} inhibition of liver mitochondria energized with succinate versus glu + mal at Mn^{2+} concentrations at and above 50 nmoles/mg protein. Although we have since replicated these results, for the current work we chose to focus on the physiologically and pathologically relevant dose range of 0 to 30 nmoles Mn^{2+} /mg protein, as seen in the above calculations. In addition, data in the higher dose region show more variability and interpretations would be much more speculative. The large differences between Mn^{2+} inhibition in liver mitochondria and brain mitochondria only came to light with the current work.

The hypothesis that Mn^{2+} inhibits the Ca^{2+} activated sites

Ca^{2+} is known to activate a number of sites within the metabolic pathways so as to increase the overall metabolic rate by a factor of up to about 3 (Balaban, 2002). Mn^{2+} binding to one of these Ca^{2+} -activated sites could either increase or inhibit the activity of the enzyme. The hypothesis that Mn^{2+} might substitute for Ca^{2+} at those sites through which Ca^{2+} activates oxidative phosphorylation and inhibit this activation (Gunter et al., 2006) is partially supported by these data; however, there are clearly additional sites of Mn^{2+} inhibition. In liver and heart mitochondria Mn^{2+} clearly inhibits the F_1F_0 ATP synthase, which is one of the Ca^{2+} -activated sites. The data shown on isolated α KGDH, another Ca^{2+} -activated site, clearly suggests inhibition of Ca^{2+} activation of α KGDH by Mn^{2+} .

Summary

This study, using oxidation rate as a measure of ATP production, along with differing sets of metabolic substrates with overlapping pathways provides insight into Mn^{2+} inhibition of the complex oxidative phosphorylation system, the basis for understanding why Mn^{2+} inhibition of oxidative phosphorylation in the three types of tissue appear so different, and why the

Mn²⁺ inhibition of α KGDH does not show up as inhibition of oxidative phosphorylation. Specifically, we have shown that 1) Mn²⁺ inhibition of oxidative phosphorylation differs in liver, brain, and heart mitochondria, 2) α ketoglutarate dehydrogenase, whose Ca²⁺ activation appears to be inhibited by Mn²⁺, does not appear to inhibit the overall process of oxidative phosphorylation, 3) the primary inhibition of oxidative phosphorylation in liver and heart mitochondria is at the F₁F₀ ATP synthase, 4) there are two separate Mn²⁺ inhibitions of oxidative phosphorylation in brain mitochondria, neither of which is the F₁F₀ ATP synthase, 5) one of the two Mn²⁺ inhibitions in brain mitochondria is either complex II or fumarase, while the other is probably either at the glutamate/aspartate exchanger or at aspartate aminotransferase, and 6) the strength of the inhibitions in brain mitochondria, when either succinate or glutamate plus malate are the substrates, is roughly 3 times stronger than the inhibition of the ATP synthase in liver mitochondria using the same substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PDH	pyruvate dehydrogenase
ICDH	isocitrate dehydrogenase
αKGDH	α ketoglutarate dehydrogenase
TCA	tricarboxylic acid cycle
ETC	electron transport chain
NADH	reduced nicotinic adenine dinucleotide
FADH₂	reduced flavin adenine dinucleotide
BSA	bovine serum albumin
RCR	respiratory control ratio
glu + mal	glutamate plus malate
pyr + mal	pyruvate plus malate
MDH	malate dehydrogenase
SDH	succinate dehydrogenase
αKG	α ketoglutarate
ROS	reactive oxygen species
ANT	adenine nucleotide transporter

Fig. 1. Portions of the intramitochondrial metabolic pathways utilized in the current experiments
 Top - The electron transport chain, the F₁F₀ ATP synthase, transporters and aspartate aminotransferase. Bottom - The tricarboxylic acid (TCA) cycle.

Fig. 2. NADH production by α KGDH

A. NADH production by α KGDH as a function of the $[\text{Ca}^{2+}]$ of the medium. No error bars are given since this is a single set of data points. B. If the above experiment is stopped at a $[\text{Ca}^{2+}]$ of 2 μM, and Mn^{2+} added as indicated, the production of NADH decreases with $[\text{Mn}^{2+}]$ as shown. The points are averages of 3 sets of data and the error bars represent plus and minus one standard error of the mean.

Fig. 3. Oxidation rate as a function of substrate concentration in liver mitochondria

The oxidation rate of isolated liver mitochondria in the presence of 1 mM ADP and 5 mM inorganic phosphate (Pi) as a function of succinate concentration (●). Rotenone (5 μ M) was in the samples when succinate was the substrate. A similar plot of oxidation rate as a function of glutamate concentration in the presence of the same concentrations of ADP and Pi, and a malate concentration 0.5 mM greater than the glutamate concentration (■) except at zero glutamate where the malate concentration was also zero. The error bars show one standard error of the mean for three measurements. This data led to the selection of 6 mM succinate and 6 mM glutamate plus 5 mM malate as “saturation concentrations” of substrate for subsequent oxidation rate experiments using liver mitochondria.

Fig. 4. Normalized oxidation rates as a function of Mn^{2+} concentration in liver mitochondria
 Normalized oxidation rates for both coupled (●) and uncoupled (○) liver mitochondria as a function of Mn^{2+} concentration using A. succinate or B. glu + mal as substrate. The data points show the averages and standard errors of the mean for 3 separate measurements each using 3 independent mitochondrial preparations (9 points for each data point). The rates were normalized to the average rate at 0 Mn^{2+} addition. As described in Materials and Methods, the coupled mitochondria were added to medium containing A. succinate (6 mM) and inorganic phosphate (5 mM) or B. glutamate (6 mM) plus malate (5 mM) plus inorganic phosphate (5 mM). They were incubated with Mn^{2+} as described in Materials and Methods. Aliquots of incubated mitochondria were added to the oxidation chamber and 1 mM ADP was added through the capillary in the top of the oxidation chamber to initiate state 3 oxidation and ATP production. Uncoupled rates were measured similarly except that the uncoupler DNP was added just before measurement of the oxidation rate. The oxidation rate

measurements themselves took 20 to 30 seconds. The lines shown represent lines of least squares fit to the data.

Fig. 5. Normalized oxidation rates as a function of Mn^{2+} concentration in brain mitochondria
 Normalized oxidation rates for coupled (●) and uncoupled (○) rat brain mitochondria as a function of Mn^{2+} concentration using A. succinate (15 mM), B. glutamate (10 mM) plus malate (5 mM), and C. pyruvate (5 mM) plus malate (5 mM) as substrates. 5 mM inorganic phosphate was used in each case. The measurements were carried out similarly to those described in Fig. 4 except that the mitochondria were isolated from rat brain and the smaller oxidation chamber was used. Uncoupled rates were measured similarly except that the uncoupler CCCP was added just before measurement of the oxidation rate. As in Fig. 4, the data points represent an average of three independent measurements for each of three separate preparations of brain mitochondria representing 9 points total for each point plotted. The lines fitted through the data points for the glu + mal and pyr + mal data are least squares

fits, while the curves through the succinate data are fitted to a polynomial. The error bars represent one standard error of the mean.

Fig. 6. Normalized oxidation rates as a function of Mn^{2+} concentration in heart mitochondria
 Normalized oxidation rates for coupled (●) and uncoupled (○) rat heart mitochondria as a function of Mn^{2+} concentration using A. succinate (15 mM), B. glutamate (10 mM) plus malate (5 mM), and C. pyruvate (5 mM) plus malate (5 mM) as substrates. 5 mM inorganic phosphate was used in each case. The measurements were carried out similarly to those in Fig. 4 except that the mitochondria were isolated from rat heart and the smaller oxidation chamber was used. Uncoupled rates were measured similarly except that the uncoupler CCCP was added just before measurement of the oxidation rate. As in Fig. 4, the data points represent an average of three independent measurements for each of three separate preparations of heart mitochondria representing 9 points total for each data point plotted. The straight lines fitted to the uncoupled data represent least squares fitted lines, while the

curved lines through the coupled data are exponential decay curves fitted to the data. Error bars represent plus or minus one standard error of the mean.

TABLE 1
Steps of the metabolic pathways utilized by each set of substrates

Steps utilized in mitochondrial energization when succinate, glu + mal, and pyr + mal are used as substrates are shown in columns 1, 2, and 3, respectively. Where a particular locus is a site of Mn^{2+} inhibition, the numbers to the left of each step name represent the ratio of the number of times that step is traversed per substrate set used, divided by the number of NADH's plus $FADH_2$'s produced by the TCA cycle per use of that same substrate set. The numbers on the left in the second column relate to one NADH being produced at MDH while the numbers on their right relate to two NADH's being produced at MDH and at α KGDH by the use of one molecule of each component of substrate. Each NADH or $FADH_2$ contributes two electrons to the ETC to reduce one atom of oxygen to H_2O

Succinate	Glutamate + Malate	Pyruvate + Malate
1 Dicarboxylic Ex.	1 – 0.5 Dicarboxylic Ex.	0.2 Pyruvate Transloc.
1 SDH	1 – 0.5 MDH	0.2 PDH
1 Fumerase	1 – 0.5 Asp Amino	0.2 Citrate Syn.
1 Complex II	1 – 0.5 Glut/asp Ex.	0.2 Aconitase
1 Complex III	0 – 0.5 α -KGDH	0.2 ICDH
1 Complex IV	0 – 0.5 Succ CoA Syn.	0.2 α -KGDH
1 ATP Synthase	1 Complex I	0.2 Succ CoA Syn.
	1 Complex III	0.2 SDH
	1 Complex IV	0.2 Fumerase
	1 ATP Synthase	0.2 MDH
		0.8 Complex I
		0.2 Complex II
		1 Complex III
		1 Complex IV
		1 ATP Synthase
1 H_2O	1 to 2 H_2O 's	5 H_2O 's