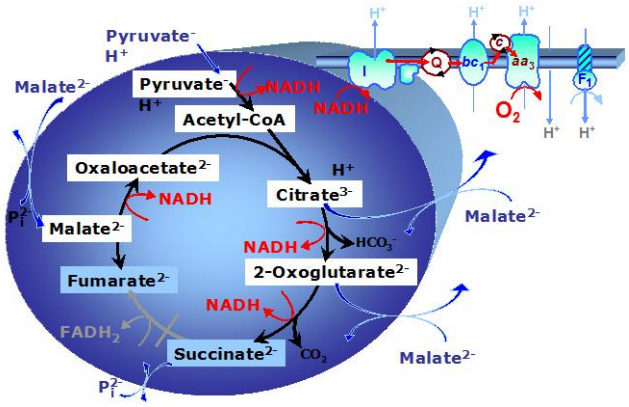
OROBOROS INSTRUMENTS
high-resolution respirometry

O2k-Protocols

Mitochondrial Physiology Network 11.04: 1-9 (2011)

Version 6: 2011-12-11

Mitochondrial Pathways to Complex I: Respiration with Pyruvate, Glutamate and Malate



Erich Gnaiger

Medical University of Innsbruck
D. Swarovski Research Laboratory
A-6020 Innsbruck, Austria
OROBOROS INSTRUMENTS Corp, high-resolution respirometry
Schöpfstr 18, A-6020 Innsbruck, Austria
erich.gnaiger@oroboros.at; www.oroboros.at

Section

Page

Introduction

Mitochondrial respiration depends on a continuous flow of substrates across the mitochondrial membranes into the matrix space. Glutamate and malate are anions which cannot permeate through the lipid bilayer of membranes and hence require carriers, which is also true for pyruvate. Various anion carriers in the inner mitochondrial membrane are involved in the transport of mitochondrial metabolites. Their distribution across the mitochondrial membrane varies mainly with ΔpH and not $\Delta\psi$, since most carriers (but not the glutamate-aspartate carrier) operate non-electrogenic by anion exchange or co-transport of protons. Depending on the concentration gradients, these carriers also allow for the transport of mitochondrial metabolites from the mitochondria into the cytosol, or for the loss of intermediary metabolites into the incubation medium. Export of intermediates of the tricarboxylic acid (TCA) cycle plays an important metabolic role in the intact cell. This must be considered when interpreting

the effect on respiration of specific substrates used in studies of mitochondrial preparations (Gnaiger 2009). Substrate combinations of pyruvate+malate (PM) and glutamate+malate (GM) activate dehydrogenases with reduction of nicotinamide adenine dinucleotide (NADH), then feeding electrons into Complex I (NADH-UQ oxidoreductase) and down the thermodynamic cascade through the Q-cycle and Complex III of the electron transfer system to Complex IV and O_2.

1. Malate

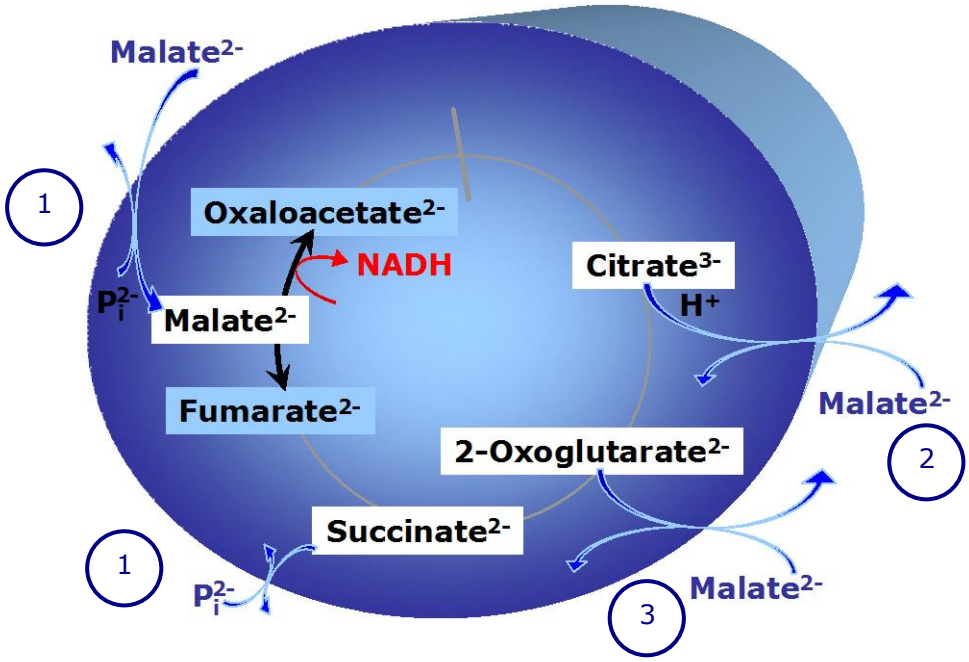


Figure 1. Malate alone cannot support respiration of isolated mitochondria or permeabilized tissue and cells. Oxaloacetate cannot be metabolized further in the absence of a source of acetyl-CoA. Oxaloacetate cannot permeate the inner mitochondrial membrane, and accumulates. Mitochondridal citrate and 2-oxoglutarate are depleted by antiport with malate. Succinate is lost from the mitochondria through the dicarboxylate carrier.

Carriers for malate

1. The dicarboxylate carrier catalyses the electroneutral exchange of malate^{2-} or succinate^{2-} for HPO_4^{2-}. It is more active in liver than heart mitochondria (Nicholls and Ferguson 2002), although respiration in rat heart mitochondria is as high with succinate+rotenone as with pyruvate+malate (Kuznetsov et al 2004).
2. The tricarboxylate carrier exchanges malate^{2-} for citrate^{3-} or isocitrate^{3-} (with co-transport of H^+). It is highly active in liver, but low in heart mitochondria.
3. The 2-oxoglutarate carrier exchanges malate^{2-} for 2-oxoglutarate^{2-}.

A significant respiratory flux with malate alone is not possible in mitochondria after depletion of endogenous substrates (Fig. 1). Depletion of endogenous substrates is required for evaluation of the effect of exogenously added substrates on mitochondrial respiration, and is aided by a small initial addition of ADP (Chance and Williams 1955). Low concentrations of isolated mitochondria or small amounts of permeabilized cells or tissue may facilitate depletion of endogenous substrates. With malate alone and saturating ADP, isolated rat skeletal muscle mitochondria respire at only 1.3% of OXPHOS capacity (*P*; State 3) with pyruvate+malate (Messer et al 2004; see Note 6.1).

2. Pyruvate+Malate: PM

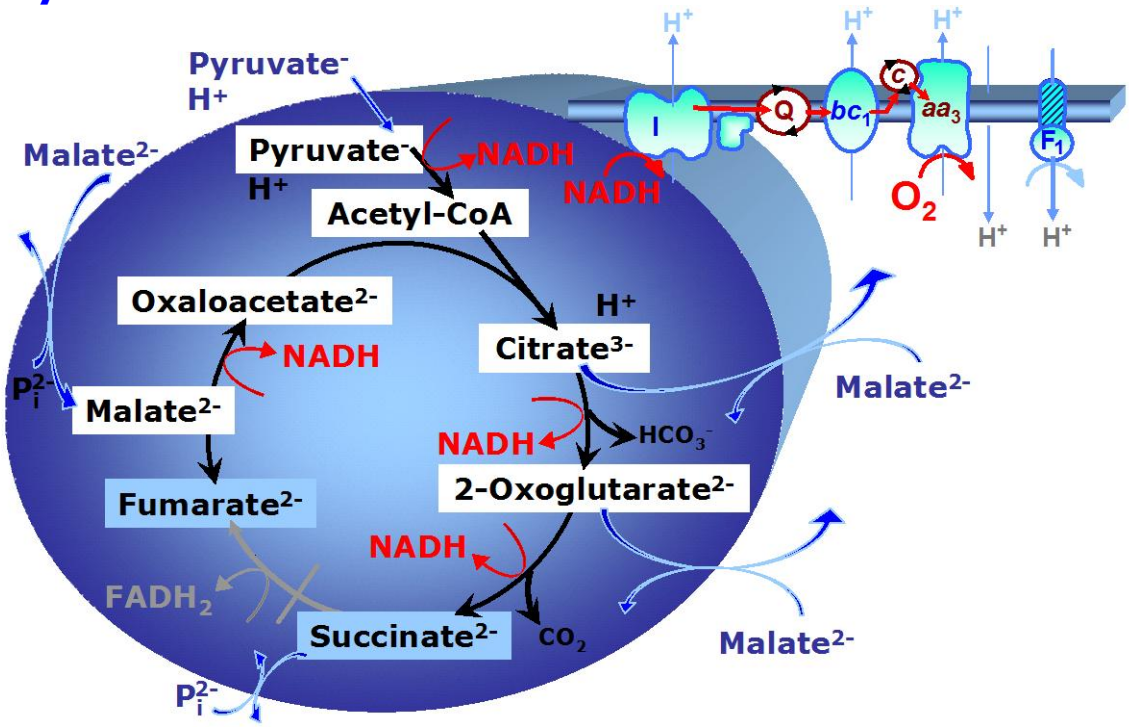


Figure 2. Oxidative decarboxylation of pyruvate is catalyzed by pyruvate dehydrogenase and yields acetyl-CoA. Malate dehydrogenase located in the mitochondrial matrix oxidizes malate to oxaloacetate. Condensation of oxaloacate with acetyl-CoA yields citrate (citrate synthase [MiPNet08.14]). 2-oxoglutarate is formed from isocitrate (isocitrate dehydrogenase).

The pyruvate carrier

The monocarboxylic acid pyruvate$^-$ is exchanged electroneutrally for OH^- by the pyruvate carrier (Hildyard and Halestrap 2003). H^+/anion symport is equivalent to OH^-/anion antiport. Above a pyruvate concentration of 5 mM [MiPNet09.12], pyruvate transport across the membrane is partially noncarrier-mediated. Above 10 mM pyruvate, hydroxycinnamate cannot inhibit respiration from pyruvate.

Complex II is not involved in respiration on pyruvate+malate (PM) in isolated mitochondria (Fig. 2). The malate-fumarate equilibrium is

catalyzed by fumarase with an equilibrium ratio of malate to fumarate at 4.1 in mitochondrial incubation medium (Gnaiger et al 2000a). High added malate concentrations, therefore, equilibrate with fumarate, which inhibits flux from succinate to fumarate, in addition to any inhibition of succinate dehydrogenase by oxaloacetate. This prevents formation of $FADH_2$ (Lemasters 1984) in conjunction with the loss of 2-oxoglutarate and succinate into the medium. Due to the high activity of the tricarboxylate carrier in liver mitochondria, citrate is lost from the mitochondria in exchange for malate, before it can be oxidized. Taken together, these are the arguments of using high malate concentrations (2 mM [MiPNet09.12]), particularly in studies of P/O ratios through Complex I (Note 6.3). Malonate may be added to inhibit the succinate-fumarate reaction, which exerts only a minor effect on liver mitochondrial respiration (Lemasters 1984). Pyruvate alone yields only 2.1% of OXPHOS capacity (State *P*) with PM in skeletal muscle mitochondria (Messer et al 2004).

Uncoupling stimulates coupled OXPHOS respiration, PM_P, by 15% in human skeletal muscle (vastus lateralis; Rasmussen and Rasmussen 2000), and by 14% in rat skeletal muscle (Johnson et al 2006).

3. Glutamate

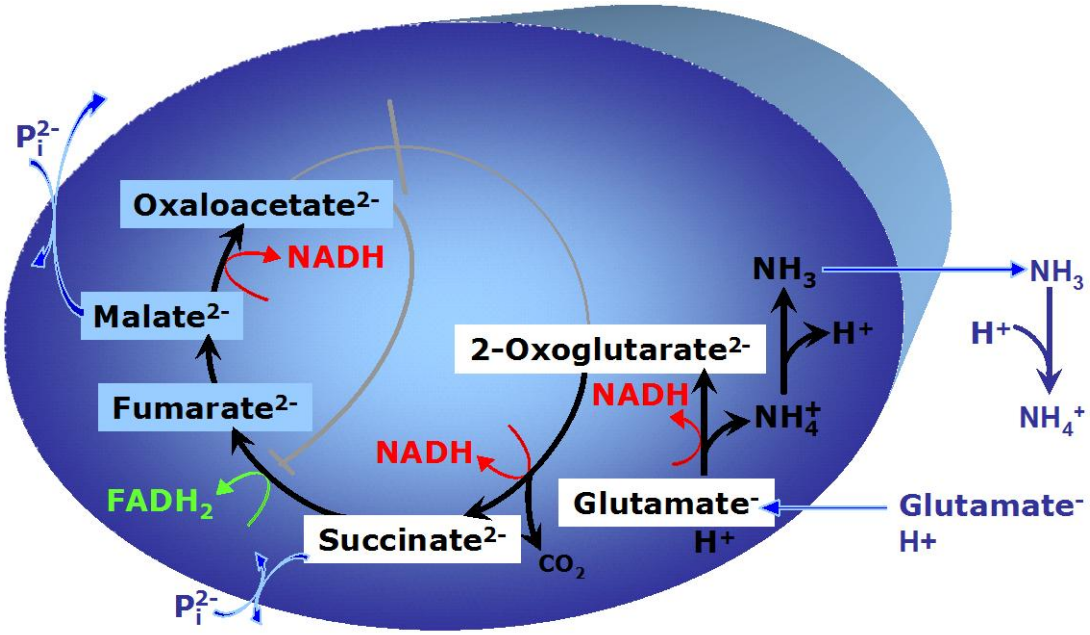


Figure 3. Glutamate as the sole substrate is transported by the electroneutral glutamate$^-$/OH^- exchanger (Fig. 4), and is oxidized via glutamate dehydrogenase in the mitochondrial matrix. Ammonia can pass freely through the mitochondrial membrane.

In human skeletal muscle mitochondria, OXPHOS respiration with glutamate alone (Fig. 3) is 50% to 85% of respiration with glutamate+malate (Puchowicz et al 2004; Rasmussen and Rasmussen 2000). Accumulation of fumarate inhibits succinate dehydrogenase and

glutamate dehydrogenase (Caughey et al 1957; Dervartanian and Veeger 1964; *see* Puchowicz et al 2004).

Glutamate derived from hydrolyzation of glutamine is a very important aerobic substrate in cultured cells (Kemp et al 1994). Mitochondrial glutamate dehydrogenase is particularly active in astrocytes, preventing glutamate induced neurotoxicity (Duchen 2004).

4. Glutamate+Malate: GM

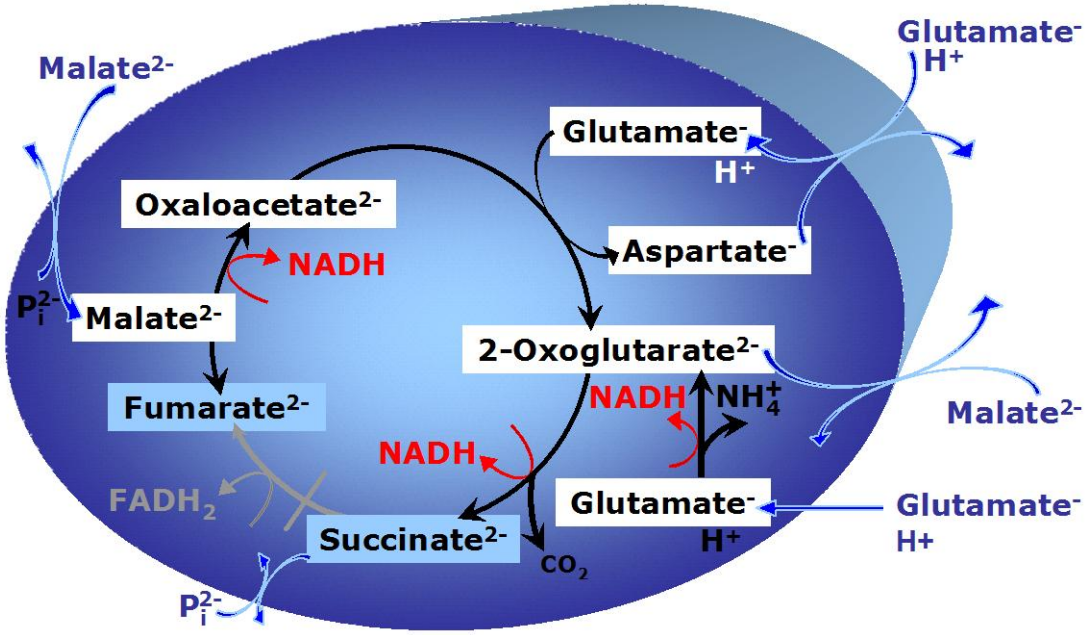


Figure 4. When glutamate+malate are added to isolated mitochondria or permeabilized cells, glutamate and transaminase are responsible for the metabolism of oxaloacetate, comparable to the metabolism with acetyl-CoA and citrate synthase (Fig. 2).

Carriers for glutamate

1. The glutamate-aspartate carrier catalyzes the electrogenic antiport of glutamate$^-$+H$^+$ for aspartate$^-$. It is an important component of the malate-aspartate shuttle in many mitochondria. Due to the symport of glutamate$^-$+H$^+$, the glutamate-aspartate antiport is not electroneutal and may be impaired by uncoupling. Aminooxyacetate is an inhibitor of the glutamate-aspartate carrier.
2. The electroneutral glutamate$^-$/OH$^-$ exchanger is present in liver and kidney mitochondria.

In human skeletal muscle mitochondria, respiration with glutamate+malate (Fig. 4) in the presence of ADP (GM_P) is identical or 10% higher than with pyruvate+malate (PM_P; Puchowicz et al 2004; Rasmussen and Rasmussen 2000; for a review see Gnaiger 2009). These results on isolated mitochondria agree with permeabilized fibres (Kuznetsov et al 1997; Wiedemann et al 1998). In contrast, Thomas et al (2004) and Winkler-Stuck et al (2005) report respiratory capacity for PM_P

at 25% or 16% higher than for GM_P. In fibroblasts, GM_P supports a higher respiratory flux than PM_P (Ouhabi et al 1994).

The PM_P/GM_P flux ratio shifts from <1 in white muscle to >1 in red skeletal muscle fibres from turkey (Opalka et al 2004). In rat heart mitochondria, respiration is 33% higher for GM_P compared to PM_P (Tyler and Gonze 1967), and respiraton with succinate+rotenone is marginally higher than for GM_P (Kuznetsov et al 2004). 2-Oxoglutarate efflux with GM is limited at low malate concentrations, and is half-maximal at 0.36 mM (LaNoue et al 1973). Glutamate+Malate support a higher OXPHOS respiration (GM_P) than PM_P in rat liver mitochondria. This suggests that a critical evaluation is required for interpreting Complex I supported respiration on a particular substrate in terms of limitation by Complex I. The PM_P/GM_P ratio is strongly temperature dependent in permeabilized mouse heart fibres (Lemieux et al 2006).

Uncoupling stimulates respiration by 25% above OXPHOS in human skeletal muscle mitochondria (the *P/E* ratio with GM is 0.80; Rasmussen and Rasmussen 2000), whereas respiration is not under the control of the phosphorylation system in red fibre type pigeon breast muscle mitochondria (Rasmussen and Rasmussen 1997) and mouse heart fibres (Lemieux et al 2006). Severe limitation was shown of GM_P (OXPHOS) respiration by the phosphorylation system in fibroblasts, since uncoupling exerted a strong stimulation above maximally ADP-stimulated respiration (Naimi et al 2005).

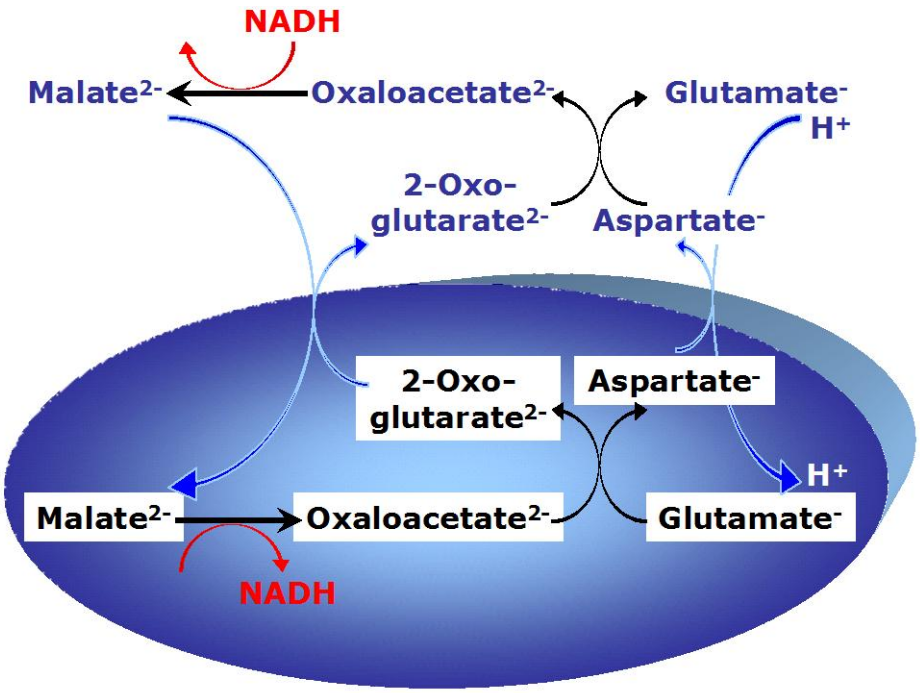


Figure 5. The malate-aspartate shuttle involves the glutamate-aspartate carrier and the 2-oxoglutarate carrier exchanging $malate^{2-}$ for 2-$oxoglutarate^{2-}$. Cytosolic and mitochondrial malate dehydrogenase and transaminase complete the shuttle for the transport of cytosolic NADH into the mitochondrial matrix. It is most important in heart, liver and kidney.

The overall reaction stoichiometry in this pathway is (Quagliariello et al 1965):

$$\text{Malate} + \text{Glutamate} + 0.5\ O_2 \rightarrow \text{Aspartate} + \text{2-Oxoglutarate} + H_2O$$

After transamination in the cytosol (Fig. 6),

$$\text{Aspartate} + \text{2-Oxoglutarate} \rightarrow \text{Glutamate} + \text{Oxaloacetate}$$

The net reaction is,

$$\text{Malate} + 0.5\ O_2 \rightarrow \text{Oxaloacetate} + H_2O$$

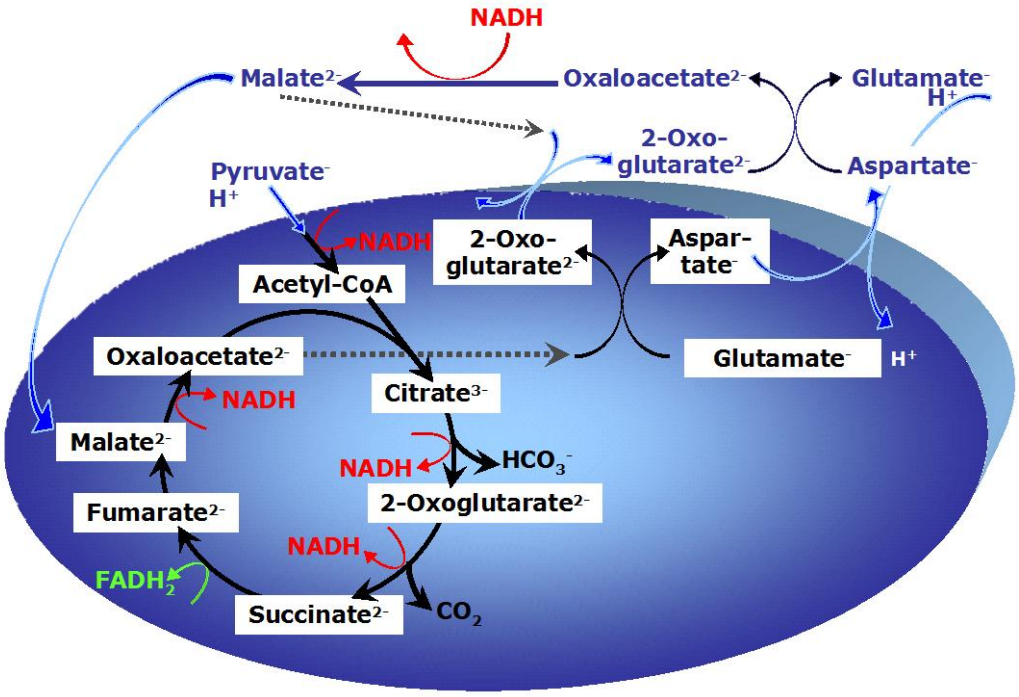


Figure 6. With the malate-aspartate shuttle operating in the intact cell, cytosolic NADH is utilized for respiration at the cost of a proton transported along the electrochemical gradient back into the mitochondrial matrix. This reduces the effective P/O ratio (Lemasters 1984).

At high cardiac workload, the 2-oxoglutarate-malate transporter cannot effectively compete for the same substrate of the 2-oxoglutarate dehydrogenase, thus limiting the activity of the malate-aspartate shuttle and transfer of cytosolic NADH into the mitochondria, reducing the cytosolic glutamate pool, and activating cytosolic reoxidation of NADH through lactate production despite sufficient oxygen availability (O'Donnell et al 2004). Regulation of cytosolic NADH levels by the glutamate-aspartate carrier is implicated in glucose-stimulated insulin secretion in beta-cells (Maechler et al 2006).

5. Boundary Conditions

Substrates feeding into the TCA cycle are generally added at saturating concentrations for measurement of mitochondrial respiratory capacity [MiPNet09.12], providing a buffer against substrate depletion in the course of the experiment. During exercise there is an increase in the concentrations of TCA cycle intermediates (Gibala et al 1998), which are not limiting in contracting skeletal muscle (Dawson et al 2005). An important anaplerotic reaction, replenishing the pools of metabolic intermediates in the TCA cycle, is catalyzed by pyruvate carboxylase in the mitochondrial matrix, which synthesizes oxaloacetate from pyruvate. Balanced anaplerosis and cataplerosis (entry and exit of TCA cycle intermediates) is responsible, particularly in metabolism of amino acids and gluconeogensis (export of malate) and lipogensis (export of citrate) for maintaining TCA cycle intermediates at steady states which shift under changing metabolic conditions of activity and starvation (Owen et al 2002).

The respiration medium MiR06 [MiPNet14.13] (Gnaiger et al 2000b) contains 10 mM inorganic phosphate (P_i), 3 mM Mg^{2+}, and saturating ADP concentrations are added to evaluate OXPHOS capacity (State 3). P_i concentrations <10 mM and [ADP] <0.4 mM limit OXPHOS respiration in isolated heart mitochondria (Mootha et al 1997). In permeabilized muscle fibre bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks et al 1998). This implies that >90% saturation is reached only >5 mM ADP, yet few studies use such high ADP concentrations in permeabilized tissues and cells (Pesta and Gnaiger 2012). Even at saturating concentrations, flux control can be exerted by the capacity of the phosphorylation system (ATP synthase, adenine nucleotide translocase and phosphate carrier), which is indicated by stimulation of OXPHOS respiration after uncoupling (Naimi et al 2005; Puchowicz et al 2004; Rasmussen and Rasmussen 2000).

Oxygen is not limiting for respiration of isolated mitochondria and small cells even at 20 µM (20- to 50-fold above the apparent K_m for dissolved oxygen; Gnaiger et al 1998; 2000a). In permeabilized muscle fibre bundles, however, diffusion restriction increases the sensitivity to oxygen supply 100-fold (rat soleus and rat heart; Kuznetsov et al 1998). It appears, therefore, that most studies carried out below air saturation (about 200 µM O_2) imply oxygen limitation of OXPOHS flux in permeabilized fibres (Gnaiger 2003). It is recommended to apply increased oxygen levels in the range of 500 to >200 µM to studies of respiratory capacity in muscle fibres (Pesta and Gnaiger 2012).

Release of cytochrome *c*, either under pathophyisological conditions of the cell or as a result of sample preparation, may limit active respiration. This specific effect can be separated from other OXPHOS defects by addition of cytochrome *c* (10 µM; Gnaiger and Kuznetsov 2002), which thus provides an essential aspect of quality control of isolated mitochondria or permeabilized tissues and cells (Kuznetsov et al 2004; Puchowicz et al 2004; Rasmussen and Rasmussen 1997).

Ca^{2+} at optimum concentration is an activator of dehydrogenases and oxidative phosphorylation. Free calcium in MiR05 is kept low by 0.5 mM EGTA. A modest increase of free calcium concentration may stimulate respiration (Gueguen et al 2005; Territo et al 2000).

Experimental temperature is best chosen at or near physiological conditions, else care must be taken when extrapolating results obtained at a different temperature (Lemieux et al 2006) [MiPNet12.13].

Short-term preservation of isolated mitochondria on ice in a specific preservation medium increases respiratory capacity in the ADP-activated state when compared to storage in typical isolation medium (Gnaiger et al 2000b), and addition of antioxidants even in the isolation medium has a significant beneficial effect (Brewer et al 2004).

6. Notes - Pitfalls

6.1. Schwerzmann et al (1989) Proc Natl Acad Sci U S A 86: 1583-1587. "Of the substrates used here, pyruvate/malate activates the chain at complex I, glutamate/malate and succinate at complexes II and III, .."

6.2. Ponsot et al (2005) J Cell Physiol 203: 479-486. (a) Respiration (State 3) in permeabilized fibres with malate alone gave 25-50% of the flux with pyruvate+malate. This most likely indicates a large content of endogenous mitochondrial substrates, which interfere to an unknown degree with the kinetics of respiration after addition of exogenous substrates. In such a study, the conventional initial depletion of endogenous substrates would be most important.
(b) Maximal respiration rates in muscle should be evaluated at saturating or high P_i, since at a P_i concentration of 3 mM OXPHOS respiration is phosphate limited.

6.3. Hulbert et al (2006) J Comp Physiol B 176: 93-105. Addition of 'sparking malate concentrations' can probably be derived from the misconception that tricarboxylic acid cycle intermediates are conserved during respiration of isolated mitochondria. 380 µM malate (instead of mM concentrations) in conjunction with 2.4 mM pyruvate were used, which makes a comparison difficult between different tissues and different species: the low malate concentration may limit PM_P flux at various degrees in the different sources of mitochondria, and GM_P may support higher fluxes than PM_P at tissue- and species-specific degrees.

6.4. In the printed Edition 1 (2007), Fig. 4 is to be replaced by the new Fig. 4 of the present edition.

References: Gnaiger_2012_MitoPathways_References.pdf