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# ROS generation and multiple forms of mammalian mitochondrial glycerol-3-phosphate dehydrogenase

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ABSTRACT

Overproduction of reactive oxygen species (ROS) has been implicated in a range of pathologies. Mitochondrial flavin dehydrogenases glycerol-3-phosphate dehydrogenase (mGPDH) and succinate dehydrogenase (SDH) represent important ROS source, but the mechanism of electron leak is still poorly understood. To investigate the ROS production by the isolated dehydrogenases, we used brown adipose tissue mitochondria solubilized by digitonin as a model. Enzyme activity measurements and hydrogen peroxide production studies by Amplex Red fluorescence, and luminol luminescence in combination with oxygraphy revealed flavin as the most likely source of electron leak in SDH under in vivo conditions, while we propose coenzyme Q as the site of ROS production in the case of mGPDH. Distinct mechanism of ROS production by the two dehydrogenases is also apparent from induction of ROS generation by ferricyanide which is unique for mGPDH. Furthermore, using native electrophoretic systems, we demonstrated that mGPDH associates into homooligomers as well as high molecular weight supercomplexes, which represent native forms of mGPDH in the membrane. By this approach, we also directly demonstrated that isolated mGPDH itself as well as its supramolecular assemblies are all capable of ROS production.



## 1. Introduction

Reactive oxygen species (ROS) are produced by all eukaryotic cells and the predominant source in most of them is mitochondrial respiration [1]. ROS have been implicated to be instrumental in many pathological processes, ranging from oxidative phosphorylation (OXPHOS) dysfunction to chronic neurodegenerative diseases and cancer. In addition and partially in contrary to this detrimental role, ROS have also been proposed to function as signaling and regulatory factors in various metabolic processes [2].

Mitochondrial respiratory chain contains many components that may leak electrons. Since the pivotal experiments of Britton Chance [3,4], two major superoxide producing sites in mitochondria have been established: respiratory chain complex I (NADH:ubiquinone oxidoreductase) [5] and complex III (ubiquinol:cytochrome *c* oxidoreductase) [6]. In addition, several other components of mitochondrial respiratory chain have been proposed as potential sources of ROS. To date, at least four additional sites of superoxide production in mammalian mitochondria have been described. These sites include dihydrolipoamide dehydrogenase (component of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase) [7,8], electron transferring flavoprotein (ETF):Q oxidoreductase [9,10], succinate dehydrogenase (SDH, complex II) [11], and mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) [12,13].

All of these enzymes are flavin dependent dehydrogenases functioning either in tricarboxylic acid metabolism or supplying electrons to coenzyme Q (CoQ) in the respiratory chain. mGPDH and SDH seem to play prominent roles in ROS production. Several studies have shown mGPDH to be a potent ROS producer both in mammalian and insect mitochondria [12,14]. Levels of ROS production from mGPDH can be very high, even comparable with the levels of ROS from $Q_o$ site of complex III when inhibited with antimycin A (AA), i.e. the most potent ROS source in mitochondria [15]. Furthermore, a significant glycerol-3-phosphate (GP)-dependent ROS production has been found even in mitochondria from tissues with low mGPDH content. Here the amount of ROS produced per unit of mGPDH enzyme activity tends to be extremely high [16], although a significant portion of these ROS originates from flavin site of complex II [17]. mGPDH can therefore be a potentially important ROS source even in typically aerobic tissues with negligible enzyme content such as the heart [16].

On the contrary, SDH was considered to be well protected against electron leak and SDH associated ROS production was only linked to pathologies, where mutations in SDH subunits lead to defective

*Abbreviations:* AA, antimycin A; BAT, brown adipose tissue; CoQ, coenzyme Q; DCPIP, 2,6-dichlorophenolindophenol; FeCN, ferricyanide, potassium hexacyanoferrate(III); GP, sn-glycerol-3-phosphate; mGPDH, mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase; HAR, hexaammineruthenium(III) chloride; HQNO, 2-n-heptyl-4-hydroxyquinoline N-oxide; HRP, horseradish peroxidase; MXT, myxothiazol; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SDH, succinate dehydrogenase

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coordination of prosthetic groups and subsequent leak of electrons [18]. However, it has been demonstrated very recently, that also SDH can produce significant amounts of ROS when levels of available succinate are low. Here flavin was implicated as the ROS source — under low succinate concentrations, flavin site is not fully occupied by the substrate and may therefore be accessible to oxygen, allowing electron leak and superoxide formation [11]. In vivo steady state concentrations of succinate have been reported to be approximately 0.5 mM in the tissues [19] or even in the micromolar range for cells in tissue culture [20,21]. Such mode of ROS production by SDH may therefore be a significant contributor to the overall cellular ROS levels.

Despite these recent advances in understanding of flavin dehydrogenases dependent ROS production, detailed molecular mechanism of electron leak is still missing and may differ between individual enzymes. For example the mechanism of ROS production by mGPDH has been shown to be in many respects different from ROS production at other sites of the respiratory chain: (i) mGPDH has a simple structure and is localized on the outer side of the inner mitochondrial membrane but despite that, ROS are produced equally to both sides of the membrane [14]; (ii) it displays unique and specific activation of electron leak by ferricyanide [12,15,22]; (iii) its expression is highly tissue dependent and mGPDH may be a significant contributor to overall ROS production in glycolytic tissues [13]. All this stresses out the importance to further characterize pathways leading to electron leak in flavin dehydrogenases.

Over the last couple of years our understanding of inner mitochondrial membrane organization changed significantly as theory of respiratory chain supercomplexes gained traction. OXPHOS supercomplexes were proposed to play several roles — apart from facilitation of their biogenesis, supercomplex organization should improve substrate channeling between individual complexes and thus reduce the chance of electron leak and ROS production. So far, such type of association has clearly been documented only for complex I, but given the potential for electron leak from SDH and mGPDH their protection by streamlining the electron transport by association into supercomplex would make thermodynamic sense. However, so far there are only limited data on supramolecular organization of these enzymes. For example, in bacteria it is documented that SDH forms trimers, which are the active conformation [23] but no data on association with other OXPHOS complexes are available. In case of mGPDH, in yeast it has been shown that several mitochondrial dehydrogenases including mGPDH analog Gut2p associate into supramolecular complex but again without clear further association with downstream OXPHOS complexes [24].

In this study we focused on mGPDH and SDH and their ability to support ROS formation at different sites of respiratory chain. We used mild detergent digitonin to solubilize mitochondrial membranes into individual complexes and supercomplexes of respiratory chain enzymes as a tool for elucidating their role in ROS production. Mild detergent solubilization also allowed us to study native organization of these dehydrogenases in the inner mitochondrial membrane and formation of higher molecular weight complexes.

## 2. Material and methods

### 2.1. Isolation of mitochondria and solubilization

For experiments we used interscapular brown adipose tissue (BAT) of one to three weeks old Wistar rats kept at room temperature and 12 h/12 h light/dark cycle on a standard diet and water supply ad libitum. All animal works were approved by the institutional ethics committee and were in accordance with the EU Directive 2010/63/EU for animal experiments. Mitochondria were isolated in STE medium (250 mM sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4) supplemented with BSA (10 mg.mL$^{-1}$) by differential centrifugation [25] and frozen at −80 °C. Subsequently frozen–thawed mitochondria were used in experiments. Membrane proteins were solubilized in KCl based medium (120 mM KCl, 3 mM HEPES, 5 mM $KH_2PO_4$, 3 mM $MgSO_4$, 1 mM EGTA, pH 7.2) with varying amount of digitonin (1 to 8 w/w ratio detergent/protein) for 10 min on ice and separated into supernatant and sediment fraction by centrifugation 20 min at 20,000 *g*.

### 2.2. Enzyme activity assays

Activities of SDH and mGPDH were determined spectrophotometrically either as $CoQ_1$ (monitored at 275 nm, $\varepsilon_{275} = 13.6$ mM$^{-1}$.cm$^{-1}$), 2,6-dichlorophenolindophenol (DCPIP, monitored at 610 nm, $\varepsilon_{610} = 20.1$ mM$^{-1}$.cm$^{-1}$) or cytochrome *c* oxidoreductases (monitored at 550 nm, $\varepsilon_{550} = 19.6$ mM$^{-1}$.cm$^{-1}$). The assay medium contained 50 mM KCl, 10 mM Tris–HCl, 1 mM EDTA, 1 mg.mL$^{-1}$ BSA, 1 mM KCN, pH 7.4 and 25 μM $CoQ_1$, 10 mM 2,6-dichlorophenolindophenol (DCPIP) or 50 μM cytochrome *c* respectively. The reaction was started by adding 10 mM sn-glycerol -3-phosphate (GP) or succinate and changes of absorbance were monitored at 30 °C. Enzyme activities were expressed as nmol.min$^{-1}$.mg$^{-1}$ protein.

### 2.3. Fluorometric detection of hydrogen peroxide production

Hydrogen peroxide production was determined fluorometrically by measuring oxidation of Amplex Red coupled to the enzymatic reduction of $H_2O_2$ by horseradish peroxidase (HRP). Fluorescence of the Amplex Red oxidation product was measured at 37 °C using Tecan Infinite M200 multiwell fluorometer. Excitation/emission wavelengths were 544 nm (bandwidth 15 nm)/590 nm (bandwidth 30 nm). The assay was performed with 15 μg of mitochondrial protein per mL in KCl based medium (120 mM KCl, 3 mM HEPES, 5 mM $KH_2PO_4$, 3 mM $MgSO_4$, 1 mM EGTA, pH 7.2) supplemented either with 10 mM succinate or 10 mM GP. Amplex Red was used at the final concentration of 50 μM with HRP at 1 U.mL$^{-1}$. Where indicated, 1 μg.mL$^{-1}$ antimycin A (AA) or 12 μM $CoQ_1$ was added. Fluorescence signal from the well containing all substrates and inhibitors, but not mitochondria, was subtracted as background for every experimental condition used. Thus any non-enzymatic effect of inhibitors on apparent ROS production was eliminated. Signal was calibrated using $H_2O_2$ at the final concentration of 0–5 μM and $H_2O_2$ stock concentration was routinely checked by measuring its absorption at 240 nm.

### 2.4. ROS production in gel slices

Proteins in solubilizates were separated by hrCN3-PAGE [26] on 4–13% gradient gels. Individual lanes were excised and washed 3 × 10 min in KCl based medium (120 mM KCl, 3 mM HEPES, 5 mM $KH_2PO_4$, 3 mM $MgSO_4$, 1 mM EGTA, pH 7.2) to remove salts used in electrophoresis buffers. Subsequently, each lane was cut to 1 mm long slices by custom made cutter. Individual slices (circa 1 × 1 × 6 mm) were transferred into separate wells of 96 well plate and ROS production was detected by Amplex Red dye using the same conditions as for solubilized mitochondria (see above). Four measurements were done for each well (Tecan reader always uses only part of the well area for fluorescence detection) to ensure that presence of gel slice did not cause inhomogeneity and average was used in calculations. In-well SD was within 10%, which was the same as with mitochondria or solubilizates. As we do not know exact protein content in each slice, values were only expressed as pmol $H_2O_2$.min$^{-1}$.

### 2.5. Luminescence detection of hydrogen peroxide production

ROS production was also measured as luminescence, principally as described earlier [27]. Tecan Infinite M200 in luminescence mode was used to detect signal. Measurements were performed at 37 °C in 0.1 mL of KCl based medium, as in Amplex Red assay (120 mM KCl, 3 mM HEPES, 5 mM $KH_2PO_4$, 3 mM $MgSO_4$, 1 mM EGTA, pH 7.2) containing 1 μM myxothiazol (MXT), 1 mM luminol (5-amino-2,3-dihydro-1,4-