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Review

The function and the role of the mitochondrial glycerol-3-phosphate dehydrogenase in mammalian tissues

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ABSTRACT

Mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) is not included in the traditional textbook schemes of the respiratory chain, reflecting the fact that it is a non-standard, tissue-specific component of mammalian mitochondria. But despite its very simple structure, mGPDH is a very important enzyme of intermediary metabolism and as a component of glycerophosphate shuttle it functions at the crossroads of glycolysis, oxidative phosphorylation and fatty acid metabolism. In this review we summarize the present knowledge on the structure and regulation of mGPDH and discuss its metabolic functions, reactive oxygen species production and tissue and organ specific roles in mammalian mitochondria at physiological and pathological conditions.

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1. Introduction

It is hard to believe that the research on the mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) started already 75 years ago [1] and yet there are still many questions to be answered, both concerning the structure/function relationship and regulation of this enzyme. This becomes even more striking when we consider its simple structure. With only one 74 kDa subunit, mGPDH is the simplest component of the mammalian respiratory chain.

mGPDH is an integral component of the mammalian respiratory chain and glycerophosphate (GP)-shuttle connects mitochondrial and cytosolic processes and plays an important role in cell bioenergetics, both under physiological and pathological conditions. GP-shuttle function depends on a highly variable expression of mGPDH in various mammalian tissues. It can be regulated by two mechanisms — by the content of mGPDH protein, because cytosolic cGPDH is not rate limiting for GP-shuttle function and by allosteric regulation of mGPDH activity by several metabolites or ions. However, it is still not completely understood why mGPDH expression is so highly suppressed in most mammalian tissues and what is the real reason for extremely high mGPDH activity in others.

Three possible metabolic roles for high mGPDH/GP-shuttle function have so far been suggested: (i) reoxidation of cytosolic NADH in glycolytic cells; (ii) bypassing complex I during cytosolic NADH oxidation with possible implications in thermogenesis and/or metabolic efficiency; and (iii) regulation of cytosolic glycerol-3-phosphate (G3P) as metabolite connecting glycolysis, lipogenesis and oxidative phosphorylation (OXPHOS).

Another important feature we will focus on is the reactive oxygen species (ROS) generation by mGPDH that is not sufficiently protected against electron leak and could also facilitate ROS production at other sites of the respiratory chain. This may also be one of the reasons why in most tissues its expression is suppressed.

Finally we will discuss tissue or organ-specific aspects of mGPDH metabolic involvement at physiological and pathological states which enables us to define the specific roles of this enzyme in mammalian organism.

2. Simple, yet unclear structure and reaction mechanism of mGPDH

Mitochondrial glycerol-3-phosphate dehydrogenase is a flavin-linked respiratory chain dehydrogenase that oxidizes glycerol-3-phosphate (G3P) to dihydroxyacetone phosphate (DAP) (redox potential −190 mV) with concurrent reduction of flavin adenine dinucleotide (FAD) to $FADH_2$ and transfers electrons to coenzyme Q (CoQ).

Mammalian mGPDH is encoded by a single *GPD2* gene located on chromosome 2 in human and chromosome 3 in rat. It is a conserved

Abbreviations: mGPDH, mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase; cGPDH, cytosolic NADH-dependent glycerol-3-phosphate dehydrogenase; CoQ, coenzyme Q; GP-shuttle, glycerophosphate shuttle; G3P, glycerol-3-phosphate; DAP, dihydroxyacetone phosphate; BAT, brown adipose tissue; FFA, free fatty acid; RET, reverse electron transport; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SDH, succinate dehydrogenase; T_3, 3,5,3′-tri-iodo-L-thyronine; $\Delta\Psi$, mitochondrial membrane potential

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and only slowly evolving gene with mammalian mGPDH showing a high degree of homology with yeast, insect and bacterial flavin-linked G3P dehydrogenases. On the other hand, there is no homology with the cytoplasmic NAD-linked glycerol-3-phosphate dehydrogenase (cGPDH) encoded by *GPD1* gene, the soluble dehydrogenase that reduces DAP to G3P while oxidizing cytosolic NADH (Fig. 1).

Rat mGPDH is synthesized as 79 kDa precursor that is processed to ~74 kDa mature protein upon removal of the N-terminal (~5 kDa) presequence, after the import into mitochondria [2–4]. Following the early attempts to isolate mGPDH [5,6], mammalian enzyme was isolated from several tissues of rat, pig or rabbit [2,7–9] yielding in best cases very pure mGPDH protein with varying content of noncovalently bound FAD, non-heme Fe and acid-labile sulfide that ranged from substoichiometric amounts to approximately one molecule of cofactor and acid-labile sulfide per mol of enzyme [2,7,10]. This is a uniquely low content of prosthetic groups (cofactors) among mitochondrial electron transport enzymes that mirrors the simple structure of mGPDH comparable with rubredoxin oxidase of the strict anaerobe *Desulfovibrio gigas* [11,12]. Despite the early studies indicating the presence of non-heme iron, there is no FeS center present in the crystal of bacterial enzyme and neither there is consensus CxxCxxC motif for 4Fe4S center coordination in the sequence of mammalian protein. Therefore FAD is most likely the only prosthetic group present in mGPDH.

As the crystal structure of mammalian mGPDH is not yet available, the membrane topology can only be deduced from the hydropathy plot of amino acid sequence, biochemical studies and 3-D structure of its bacterial homologue GlpD [13] or closely related bacterial G3P oxidase (GlpO) [14].

Crystal structures of bacterial GlpD from *E. coli* [13] and GlpO from *Streptococcus* sp. [14] suggest anchoring to the membrane without transmembrane helices and the presence of dimeric form of enzyme that interacts through the hydrophobic regions, supporting the view that mGPDH is strongly associated with the inner membrane but rather as a peripheral, than an integral protein. Although this fold is rather unusual it is shared with another dehydrogenase communicating with the CoQ pool — electron transfer flavoprotein (ETF):Q dehydrogenase [15,16] and several other enzymes utilizing hydrophobic ligands. All these proteins share structural motifs that run parallel to the membrane surface and form apolar plateaus that are hypothesized to bury into one leaflet of the membrane only [17]. This view is also supported by the fact, that yeast mGPDH is extracted from the membrane by carbonate but not by high salt treatment implying that the enzyme is peripheral and holds in the membrane by hydrophobic interactions [18].

Nevertheless detailed structural information for the mammalian enzyme is still missing. Given the relatively good sequence conservation between mammalian and bacterial enzyme (30% identity and 48% similarity), it has most likely monotypic structure as well. The C terminal Ca^{2+} binding domain is not present in the bacterial enzyme and its structure is unclear. It should be noted that early studies of MacDonald and Brown [19], predicted three transmembrane segments in the N terminal third of the protein with the long C-terminal region exposed to the intermembrane space. However, this fold which was based on von Heine's algorithm [20] is rather unlikely, as parts of the predicted transmembrane helices comprise FAD binding site which is very well conserved from the bacterial enzyme and presumably has the same fold not protruding the membrane. Some of the hydrophobic parts,

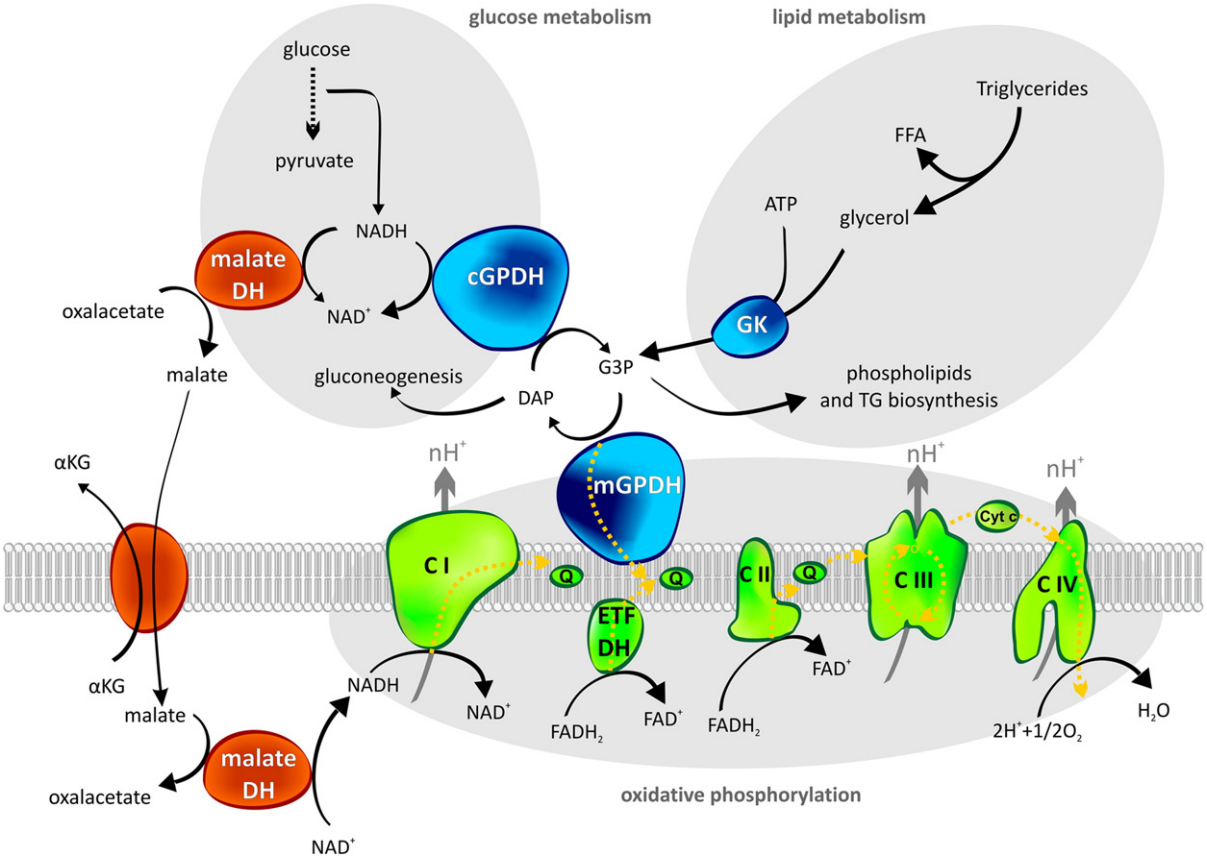

Fig. 1. mGPDH and its place in intermediary metabolism. Enzymes constituting GP-shuttle are depicted in blue as well as glycerol kinase (GK) which phosphorylates glycerol coming from lipolysis. Part of the malate/aspartate-shuttle as an alternative pathway of reducing equivalents transport is depicted in orange. Complexes of the mitochondrial respiratory chain are in green with yellow arrows indicating flow of electrons through the OXPHOS. Q — Coenzyme Q, CI — complex I, CII — complex II, CIII — complex III, CIV — complex IV, ETF DH — ETF:Q dehydrogenase, FFA — free fatty acids, G3P — glycerol-3-phosphate, DAP — dihydroxyacetone phosphate.

predicted to be transmembrane regions according to the hydropathy plot are most likely present on the surface of the protein and represent structural prerequisite for protein dimerization [13].

Kinetic properties of isolated mGPDH revealed 2.9 mM K_m for G3P and ~7 μM K_m for FAD in rat liver enzyme [2]. Affinity to G3P was modulated by Ca^{2+} with the maximal activation at [Ca^{2+}] 10^{-4}–10^{-5} M [21]. Interaction of rabbit brain mGPDH with electron acceptors showed high affinity for CoQ_0 and CoQ_1 with K_m ~10–48 μM depending on the stage of purification, while K_m for decylbenzoquinone analog was too low to measure [10].

Mechanistically, 2-hydroxy group of glycerol-3-phosphate is oxidized, forming dihydroxyacetone phosphate. Electron pair is transferred to prosthetic FAD forming $FADH_2$ and these two electrons are subsequently transferred to decylubiquinone forming decylubiquinol in the membrane. Assuming that there is no Fe–S center in mammalian mGPDH (in contrast to succinate dehydrogenase (SDH) or ETF:Q dehydrogenase) the reaction mechanism may be similar to that of the bacterial enzyme, where FAD and CoQ binding sites are closely apposed and electron transfer from $FADH_2$ to CoQ is mediated through protein residues or by a ping–pong interaction of the product DAP and CoQ with the $FADH_2$ [13].

3. Transcriptional regulation, unique tissue specificity and response to thyroid hormone

Mitochondrial content of OXPHOS complexes and mobile electron carriers is known to vary according to different metabolic needs and energy demands specific for individual tissues [22]. Individual OXPHOS subunits show a high degree of coexpression to ensure concerted changes of the OXPHOS machinery as a functional unit and such regulation is achieved through generalized transcription factors such as NRF1, NRF2, and YY1 [23]. On the contrary, mGPDH shows an extremely high variation in tissue expression levels and its tissue content and activity are considerably different from those of other OXPHOS proteins. There are up to two-orders of magnitude differences in mGPDH content between mammalian tissues [24–27]. The highest levels were found in brown adipose tissue (BAT), muscle and brain and the lowest in liver or heart, although heart has similarly high oxidative capacity as BAT. Apart from these extremes, there are also other tissues where mGPDH plays an important role, namely β pancreatic cells [28,29], testis [19,30], placenta [8,31], as well as in various glycolytic cells, *e.g.* fibroblasts [32].

Expression of mGPDH is regulated predominantly at the transcriptional level [26,30,33,34]. *GPD2* gene spans 100 kb and consists of 17 exons. The use of alternate promoters was suggested by the presence of three different first exons that are expressed in a tissue-specific manner. Exon 1a is found primarily in the brain, exon 1b is used in all tissues, and exon 1c is detected predominantly in the testis. Depending on the tissue, different transcript lengths in rat were thus observed: 5.9 kb in all tissues, 3.6 kb in skeletal muscle, and 2.5 kb in testis. The length isoforms are attributable to alternate splicing and polyadenylation site use. Transcription of mGPDH is activated by TR/RXR binding to TRE (thyroid hormone response elements) sequence in promoter region of *GPD2* gene [35]. Thyroid hormone (3,5,3′-tri-iodo-L-thyronine, T_3) and steroid hormone but not the peroxisome proliferator clofibrate or retinoic acid activate the ubiquitous promoter B in a receptor-dependent manner, suggesting that tissue-specific factor(s) contribute to the tissue-restricted responsiveness to thyroid hormone. The other two more tissue-restricted promoters A and C are not inducible by these hormones. Interestingly, human mGPDH promoter B is not regulated by T_3, which is the first example of a differentially T_3-regulated orthologous gene promoter in man and rat [35].

Following the first observation that T_3 activates mGPDH synthesis in the liver but not in other tissues [36], the relationship between hormone dose, receptor occupancy and mGPDH synthesis was studied in the liver of euthyroid and hypothyroid rats [37]. Up to 10-fold difference in mGPDH mRNA levels was observed between hypo- and hyperthyroid states [38] and T_3 induction of mGPDH showed rather fast response in transcript (6–12 h) and protein (24 h) synthesis [38,39] resulting in ~3-fold increase in mGPDH activity and content. Interestingly, mGPDH showed half life of 60 h indicating relatively fast turnover of this dehydrogenase compared with other proteins of mitochondrial inner membrane.

4. Glycerophosphate shuttle — a crossroad of glycolysis, fatty acid metabolism and oxidative phosphorylation

Studies of metabolic function of mGPDH started in 1936 by Green and coworkers [1,40,41] who found that this enzyme is present in variable amount in many mammalian tissues. The highest enzyme activity was found in mammalian BAT [42] and in insect flight muscles [43,44]. It was also the insect flight muscle, where the role of mGPDH was first studied; Zebe and McShan reported a very high activity of mGPDH accompanied by a low activity of lactate dehydrogenase [45] and Kubista observed low production of lactate and high production of G3P during an intensive flight muscle activity [46]. Subsequently two groups independently proposed a new metabolic cycle — glycerophosphate shuttle [47–49].

The GP-shuttle is formed by two glycerol-3-phosphate dehydrogenases, namely cytosolic cGPDH that is soluble and NADH-dependent (E.C.1.1.1.8), and by mitochondrial, membrane-bound, FAD-dependent mGPDH (E.C. 1.1.5.3). They catalyze conversion of DAP and G3P and *vice versa* coupled with oxidation of cytosolic NADH and reduction of FAD in the mitochondrial enzyme (Fig. 1). The main metabolic role of this shuttle is reoxidation of cytosolic NADH produced by glycolysis. This enables sustained rate of cytosolic ATP production without accumulation of an intermediate by-product such as lactic acid. Insect flight muscle can therefore contract without acidification for as long as glycogen is available. This mechanism also leads to transport of reducing equivalents (hydrogen) from cytosol into mitochondria where they can be utilized for aerobic mitochondrial ATP production. GP-shuttle was not considered to be of a general importance because functional activity of the cycle requires equimolar proportions of both components of the cycle — mitochondrial and cytosolic G3P dehydrogenases and in most tissues the content of mGPDH is relatively low in comparison with the cGPDH. GP-shuttle therefore appeared to be only secondary to malate/aspartate-shuttle.

Increasing interest in GP-shuttle started when active GP-shuttle was described in mammalian BAT [24]. It had been already known that brown fat mitochondria have unusually high activity of mGPDH [42], but in this study it was demonstrated that both mGPDH and cGPDH are highly active and in equimolar proportions, which is prerequisite for the functional activity of the shuttle. Therefore it was suggested that the shuttle could play an important role in BAT metabolism supporting cytosolic ATP generation by NADH reoxidation. Cytosolic ATP production in BAT is important despite the high rate of fatty acid oxidation as mitochondria contain only tiny amount of ATP synthase [50,51] and all energy generated by OXPHOS is released by the action of uncoupling protein (UCP1) as heat [52]. Simultaneously, similarly to insect muscle cells, GP-shuttle may support thermogenic function of BAT mitochondria by transport of additional reducing equivalents formed in cytosol (NADH). It was also proposed that GP-shuttle may be involved in the regulation of cytosolic G3P availability for triglyceride synthesis. Esterification of G3P to acylglycerolphosphate is the first and rate-limiting step of the lipid synthesis [53]. mGPDH and G3P acyltransferase compete for the same substrate and due to its very high activity in BAT, mGPDH can significantly influence concentrations of the available G3P. Thus the mGPDH has two important roles in BAT: (i) control of cytoplasmic NADH level and (ii) control of triglyceride and phospholipid synthesis through regulation

of cytosolic G3P concentration. The role of mGPDH and GP-shuttle in intermediary metabolism is summarized in Fig. 1.

Oxidation of NADH by GP-shuttle is energetically "inefficient" when compared to NADH oxidation by complex I, as mGPDH has no proton pumping activity. These findings stimulated interest in mGPDH as factor participating in processes of heat dissipation and energy balance regulation of mammalian organism [54]. In this respect, phenotype of Lou/C rat is particularly interesting. This rat shows pronounced resistance to age or diet induced obesity most likely due to increased mGPDH expression in liver caused by high TRα1 expression [55]. Partial dissipation of energy *via* channeling of cytosolic NADH through GP-shuttle instead of malate/aspartate-shuttle may therefore represent an attractive target for obesity treatment.

5. Allosteric regulations of mGPDH

As an important enzyme of intermediary metabolism, mGPDH shows complex regulation by a range of activators and inhibitors. The most important endogenous activator is calcium [19,49,56]. Indeed, mGPDH has a calcium binding site containing the canonical EF hand motif for coordination of Ca^{2+} [57] and binding of Ca^{2+} decreases K_m for substrate. Ca^{2+} binds to the protein moiety exposed to intermembrane space because its effect is not inhibited by the ruthenium red, an inhibitor of calcium transport through the mitochondrial membrane [58]. To a certain extent, activating effect was also observed for other bivalent cations such as Mg^{2+} [8], Mn^{2+} [56] and Cd^{2+} [59].

Competitive inhibitory effect of mGPDH activity was described for various phosphoderivates of glycerol such as L- and D-glyceraldehyde-3-phosphate, D-phosphoglyceric acid and D-glycerol phosphate [31,60,61]. This indicates the importance for a strict regulation of the enzyme standing at the crossroads between glycerol oxidation and its utilization for lipid biosynthesis. However, precise regulatory feedback loops *in vivo* remain unclear.

The regulatory role of free fatty acids (FFA) and their acyl-CoA esters is also very important. While the inhibition by palmitoyl-CoA is of a competitive type [62], FFA display non-competitive inhibition, which is completely reversible by extraction of FFA by serum albumin [63]. Endogenous fatty acids often inhibit mGPDH activity in mitochondrial preparations as they bind to the mitochondria during the isolation process. Such inhibition can be released by FFA oxidation after addition of malate, carnitine and ATP [64]. The inhibitory effect or FFA was localized as inhibition of the transfer of electrons from the enzyme to CoQ as the oxidoreductase activity (acceptor oxygen or cytochrome *c*) was inhibited whereas dehydrogenase activity (acceptor phenazine methosulfate) was insensitive to FFA addition [65]. The regulation of mGPDH activity by FFA is very important especially in cells with high mGPDH content. High activity of GP-shuttle can deplete cytosolic concentrations of G3P and thus inhibit the rate of triglyceride and phospholipid synthesis. Suppressed oxidation of G3P by FFA and acyl-CoA therefore allows their esterification. We may conclude that both FFA and their acyl-CoA esters are strong but reversible inhibitors of mGPDH and can efficiently regulate both GP-shuttle activity and rate of lipid synthesis.

Studies of molecular mechanism of FFA inhibitory action demonstrated that FFA can act also indirectly through the modification of the state of the lipid bilayer. These inhibitory effects correlated with changes in membrane microviscosity monitored by the steady-state fluorescence anisotropy of fluorescent probe 1,6-diphenyl-1,3,5-hexatriene [66]. This was further confirmed by experiments with isolated enzyme incorporated into liposomes with different proportions of saturated and unsaturated fatty acids in phospholipids. mGPDH showed maximum activity within a narrow range of the membrane lateral pressure and both decrease and increase of membrane fluidity had an inhibitory effect [67]. mGPDH thus belongs to the group of membrane-bound enzymes with activities modified by the state of lipid membrane phase and also by phospholipid composition-dependent surface charge [68]. In yeast, photolabeling with phosphatidylcholine analog demonstrated close association between mGPDH and phosphatidylcholine [18]. Existence of such association was further demonstrated by the fact that phosphatidylcholine depletion reduced growth on glycerol and increased glycerol excretion. Phosphatidylcholine therefore seems to be necessary for optimal mGPDH functioning *in vivo*. Authors proposed that low phospholipid content downregulates mGPDH and free G3P may then be used for triglyceride and phospholipid synthesis instead of mitochondrial oxidation [69].

When mGPDH was purified from mammalian mitochondria [2,6], it was also found that the enzyme has a number of functionally important SH groups. Its activity can therefore be inhibited by various SH modifying agents such as mercurials and maleimides [70]. Other strong inhibitors are dicarbanonaborates. The inhibition by mercaptodicarbanonaborate is of a competitive type and completely reversible by serum albumin [71]. However, no inhibitor is available yet that would be highly specific for mGPDH and without effect on other respiratory chain enzymes.

6. What are the sites of glycerol-3-phosphate-dependent ROS production?

Although complexes I and III of the respiratory chain are widely considered as primary sources of mitochondrial reactive oxygen species (ROS), an important role of mitochondrial flavin dehydrogenases in ROS production has been established over the recent years [72]. mGPDH was indeed the first of these dehydrogenases to be characterized as ROS producer [73–75], but further studies identified α-ketoglutarate dehydrogenase [76], ETF:Q dehydrogenase [77] and succinate dehydrogenase [78] to contribute to overall ROS production as well. Levels of ROS production from mGPDH are very high and comparable with maximum rates for complex III when inhibited with antimycin A [79–81]. Furthermore, a significant G3P-dependent ROS production has been described even in mitochondria from tissues with very low mGPDH content where the amount of ROS produced relatively to the mGPDH enzyme activity tends to be extremely high. This makes mGPDH a potentially important ROS source in typically aerobic tissues with negligible enzyme content such as the heart [27].

Detailed molecular mechanism of electron leak from mGPDH as well as other flavin dehydrogenases is still missing and there may be considerable differences among individual enzymes. In many cases the observed, substrate-dependent ROS production cannot be even ascribed to the respective dehydrogenase *per se*. In principle three modes of ROS production can be envisaged: (i) ROS production on the other dehydrogenases connected to the Q pool; (ii) production on the respective dehydrogenase; and (iii) production on the complexes downstream of the Q pool (*i.e.* typically at the level of complex III). The key question is whether or not the given ROS production mode can occur *in vivo*. See Fig. 2 for schematic explanations of possible electron transfer pathways and respective places of ROS production in the respiratory chain under all the modes proposed.

6.1. ROS production at complex I and SDH

Under these conditions the real source of ROS may not be dehydrogenase itself but another enzyme communicating with the Q pool. Originally this was described as reverse electron transfer (RET) towards complex I under high levels of mitochondrial membrane potential $\Delta\Psi$ and low flux through the respiratory chain, typical for mitochondria in respiratory state 4. Indeed, reverse flow of electrons is considered to be main source of SDH-dependent ROS at high $\Delta\Psi$ levels [82]. RET seems to play role in G3P-dependent ROS production as well but the extent varies between tissues and experimental models. Significant RET was observed in brain mitochondria respiring on G3P [83], but it was less pronounced in *Drosophila* mitochondria

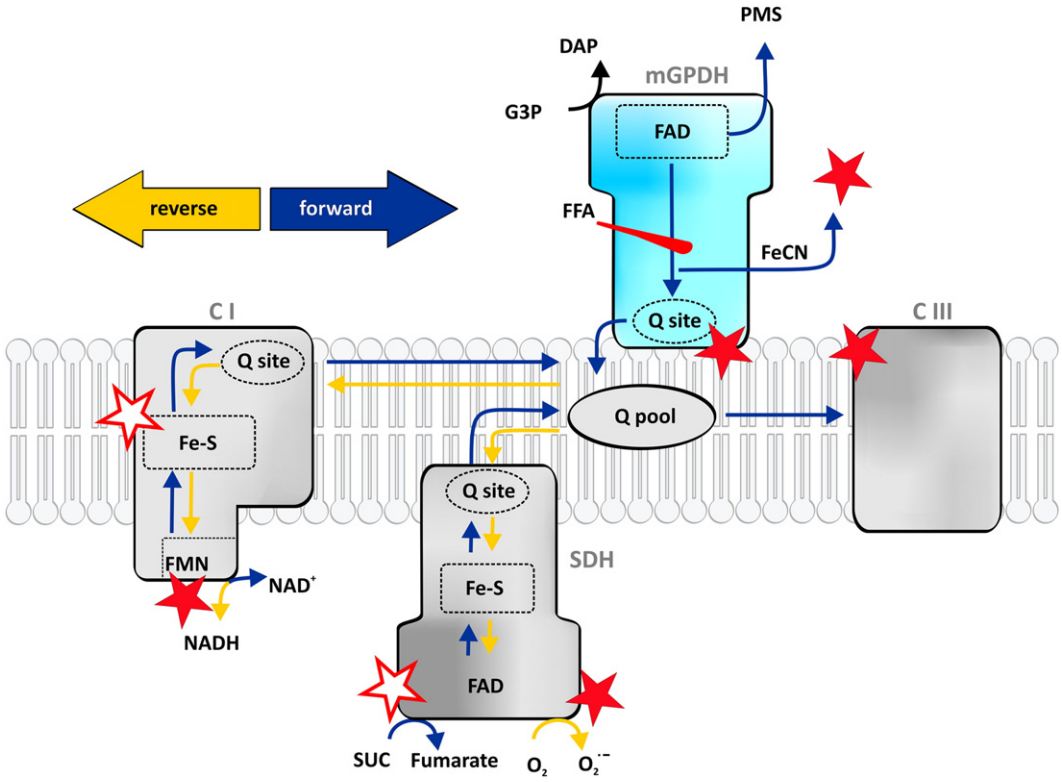

Fig. 2. Possible sites of glycerol-3-phosphate-dependent ROS generation. ROS may be produced on the dehydrogenase itself as well as other sites of OXPHOS enzymes. All sites that may be responsible for G3P-dependent ROS generation are marked with red stars. Other sites of mitochondrial ROS production not associated with mGPDH are indicated with red outlined stars. Traditional way of electron flow through individual complexes is indicated by blue arrows. However, ROS production may be the result of reverse electron transport from mGPDH to CI or SDH as indicated by yellow arrows. Sites of mGPDH interaction with artificial electron acceptors (PMS, FeCN) are also indicated. PMS — phenazine methosulfate, CI — complex I, CIII — complex III, SDH — succinate dehydrogenase, FeCN — ferricyanide, FFA — free fatty acids, G3P — glycerol-3-phosphate, DAP — dihydroxyacetone phosphate, Q — coenzyme Q.

[80], with approximately 1/3 of ROS originating from RET, or in BAT [27,84]. As RET from mGPDH is highly $\Delta\Psi$ dependent, part of the explanation may be loose coupling of BAT mitochondria. This is supported by the observations that ROS production was higher in UCP1$^{-/-}$ animals under both succinate and G3P [85,86]. RET also depends on G3P concentration and is more pronounced at high G3P concentrations (40 mM) [83]. It is however unlikely that these concentrations may occur *in vivo*. Interesting observation supporting existence of at least some G3P-dependent RET is that G3P addition to BAT mitochondria leads to increase in reduction state of NAD(P)H pool which is slowed down by the addition of rotenone [87].

Other example of reverse electron flow was described recently. In skeletal muscle mitochondria, part of mGPDH-dependent ROS production may happen at the level of flavin in SDH, due to reverse electron flow *via* Q pool [78,88]. This mechanism also explains the paradoxical phenomenon, where very high G3P-dependent ROS production was observed in tissues with low mGPDH content [27]. It is most likely that under these conditions the real source of electron leak was flavin in SDH [88]. However, this type of reverse flow was demonstrated when succinate was not present in the solution and it is not clear how forward and reverse electron flow would compete in SDH *in vivo*. The physiological importance of this phenomenon thus remains to be established.

6.2. Electron leak at mGPDH

The principal source of electron leak may be the dehydrogenase itself. Here, flavin, or semiquinone is a possible site for ROS generation by mGPDH. Flavin has been proposed as the source of electron leak in the case of SDH operating in forward mode [78]. This type of leak requires low succinate concentrations and only partial occupancy of flavin site of SDH by succinate. This is in contrast with mGPDH, where ROS production increases linearly with increasing GP concentration [83] and superoxide is produced on both sides of the membrane [79,88]. The most likely source of ROS in mGPDH is therefore the Q site. This is supported by several lines of evidence. The reactive site of mGPDH is located rather superficially and CoQ docking occurs at the planar region facing the lipid bilayer, similar to ETF:Q dehydrogenase, which has also been described as ROS producer [16,77,87]. This is in marked contrast with SDH where the presence of the deep coenzyme Q-binding pocket represents a natural protection by stabilization of ubisemiquinone radical formed during coenzyme Q reduction [89,90]. Of note here is the fact that mutations in CoQ binding site of SDHC subunit lead to significant increase in ROS production [91]. Insufficient stabilization of semiquinone in case of mGPDH thus seems to be the most likely source of electron leak. Interestingly soluble CoQ_1 does have strong antioxidant effect in the case of solubilized mGPDH [75] while it acts pro-oxidatively with SDH. Indeed observed induction of substrate-dependent ROS production by ferricyanide in membrane bound as well as isolated mGPDH, apparently absent in succinate-dependent ROS production, implicates that facilitation of one-electron transfer from mGPDH highly stimulates electron leak. This is also completely inhibited by CoQ_1 [75] or another one electron acceptor hexaammineruthenium chloride, implying that all these compounds compete for the same binding site [92]. In the context of ping–pong reaction mechanism, it is likely, that $FADH_2$ is formed during catalysis. Under normal conditions the transfer of electrons to CoQ is concerted two electron process, while transfer of one electron to ferricyanide leads to formation of flavin semiquinone, which subsequently reacts with molecular oxygen producing superoxide [75].

6.3. ROS production at complex III

The last source may be complex III. This is significant after antimycin A addition but of questionable physiological relevance as blockade of electron transport in respiratory chain alone is not sufficient for ROS production at the level of complex III. G3P-dependent ROS production at the level of complex III seems to be highly tissue dependent and generally inversely correlated to the content of mGPDH. In BAT low decrease of measured ROS production after myxothiazol *versus* antimycin A addition indicates low involvement of complex III [81]. On the contrary complex III component seems to be significant in tissues with low mGPDH content such as the kidney or heart [27,72].

7. Can mGPDH form higher structures?

Mitochondrial inner membrane is very rich in proteins and tends to be highly organized and it is tempting to study association of mGPDH into higher order assemblies. Over the last ten years the theory of respiratory chain supercomplexes emerged — it is now widely accepted that complexes of the respiratory chain organize into higher molecular structures [93,94]. Apart from the stabilization of OXPHOS complexes biogenesis, one of the proposed functions for supercomplexes is substrate channeling between individual respiratory chain complexes leading to better protection from electron leak and faster kinetics [95]. However, from the dehydrogenases supplying electrons to the CoQ pool, only complex I is systematically identified in supercomplexes. Kinetic evidence also proposes existence of substrate channeling only for complex I and not for SDH, which seems to communicate with complex III only *via* CoQ pool [95]. Unfortunately, due to the lack of specific inhibitors for mGPDH it is not possible to perform such type of kinetic analysis for mGPDH. On the other hand, there is some support that flavin dehydrogenases may associate into higher structures as well: (i) Isolated supercomplexes were found to be respiratory active when using succinate as a substrate [93]. (ii) Association between fatty acid oxidation enzymes and OXPHOS complexes has been described [96]. (iii) In yeast several mitochondrial dehydrogenases including mGPDH analog Gut2p associate into supramolecular complex [97].

From the early isolation experiments it was obvious that mGPDH forms higher molecular weight aggregates. Under non-denaturing conditions of gel filtration or native electrophoresis, the isolated mGPDH "holoenzyme" migrated at a molecular weight of approximately 250–300 kDa and these complexes were considered to be homooligomeric aggregates of the 74 kDa mGPDH monomers with some bound detergent and phospholipids [10]. Multiple forms of enzyme were also suggested on the basis of bi- or multiphase thermal denaturation profiles [7].

Homooligomers appear to be also present in digitonin solubilizates of BAT mitochondria and may reflect the real *in vivo* organization of the enzyme, as the bacterial GlpD is also active as dimer [13]. It is also of note that in bacteria, SDH forms trimers, which are the active conformation [98]. In addition, supercomplexes may also be formed with proteins other than OXPHOS complexes. Apart from mGPDH homooligomers, in digitonin solubilized BAT mitochondria we have recently observed ~1 MDa big mGPDH containing supercomplexes of yet unknown composition. But since this complex remains intact in cells lacking assembled OXPHOS complexes I, III and IV, they most likely represent an association of mGPDH with other proteins in the inner mitochondrial membrane. Their significance remains to be elucidated [92].

8. Tissue specific (dys) functions of mGPDH

The general role of mGPDH and GP-shuttle in intermediary metabolism is well established, but its importance for different tissues and organs may be connected with highly specific and diverse physiological functions. This is illustrated by pathophysiological studies in BAT, muscle, pancreas, sperm, or placenta as well as transgenic knock-out models.

In contrast to dysfunctions of other respiratory chain dehydrogenases that cause inherited mitochondrial OXPHOS disorders manifesting as progressive neurodegenerative diseases in case of SDH [99] and NADH dehydrogenase [100], or glutaric acidemia type II in case of ETF: Q dehydrogenase [101], no such a severe disorder due to mutation of *GPD2* gene has been reported yet. Numerous studies which focused on potential significance of mGPDH dysfunction indicate pathogenic effects to be associated with tissues where mGPDH is highly active, namely in pancreatic β cells, testis, brain or placenta but altered function of mGPDH may affect metabolism in muscle and adipose tissue as well and indirectly influence metabolism and energy balance of the whole organism.

Animal models of mGPDH dysfunction, knock-outs of *GPD2* gene had generally milder and tissue specific phenotypes rather than devastating consequences [102–104]. mGPDH knockout mice on a C57BL/6J background had normal cold tolerance and normal thyroid thermogenesis but 50% reduced viability and 40% reduction in the weight of white adipose tissue [104]. In another model of mGPDH knockout, mice had a small but distinct reduction in obligatory thermogenesis despite increased plasma T_4 and T_3, which was compensated by increased BAT facultative thermogenesis and by thyroid hormone-dependent mechanisms using other proteins, possibly UCP3 [102]. These animals also had significant alteration of intermediary metabolism primarily in muscle that leads to a thrifty phenotype more pronounced in females [103]. This may reflect the fact that in its main metabolic role, oxidation of cytosolic NADH, GP-shuttle can be substituted by malate/aspartate-shuttle. On the other hand, ablation of both mGPDH and cGPDH resulted in lethal phenotype. Mice were hypoglycemic, had elevated plasma fatty acids, developed ketonuria and died within several days, indicating that in lipid metabolism, mGPDH and cGPDH can substitute for each other but ablation of both leads to its severe disturbances [105].

Despite these functional redundancies, alteration of mGPDH function appears to be associated with several pathological states as apparent from studies on tissue specific roles of this enzyme.

Altered role of brain mGPDH was suggested by a disruption of *GPD2* gene by balanced reciprocal translocation 46,XX,t(2;7)(q24.1;q36.1) that was found in a female patient exhibiting mild nonsyndromic mental retardation. A twofold decrease of *GPD2* transcript and decreased mGPDH activity in patient's lymphoblastoid cell lines indicated that functional defect of mGPDH could be associated with mental retardation. However, it was not found in other patients tested [106].

Much more convincing is the involvement of mGPDH in pathogenesis of polygenic disease, non-insulin dependent diabetes mellitus (NIDDM). In pancreatic islets' β cells, many studies indicate significant participation of mGPDH and GP shuttle in the proximal events that mediate release of insulin in response to increased glucose. Stimulation of insulin secretion by glucose involves a rise in the cytoplasmic concentration of Ca^{2+} [107] caused by the closure of ATP-sensitive K^+ channels in the plasma membrane, membrane depolarization, and influx of Ca^{2+} through voltage-sensitive channels [108,109]. An important effect of glucose is also the amplification of the action of Ca^{2+} on the exocytotic process [110]. Both pathways require glucose metabolism and appear to depend on a rise in the ATP/ADP ratio. Glucose metabolism in β cells essentially occurs through aerobic glycolysis [111]. The β cells have low activity of lactate dehydrogenase and cytosolic NADH formed during glycolysis is re-oxidized by transfer of the reducing equivalents into mitochondria through both GP- and malate/aspartate-shuttles [28].

The importance of mGPDH for insulin secretion has been supported by its high activity in pancreatic islets [28,112] contrasting with decreased mGPDH in islets of GK rats [113], db/db mice [114],

fa/fa rats [115], streptozotocin injected neonatal rats [116] as well as in non-insulin dependent diabetic patients [117]. In some patients, mutations in Ca^{2+} or FAD-binding domains of *GPD2* gene were found which can cause catalytic defect of mGPDH [118]. Furthermore, autoantibodies reactive with pancreatic islets were found to cross react with mGPDH antigen in IDDM but not NIDDM patients [119]. But neither could the studies on mGPDH knock-out demonstrate changes in oscillatory responses of β cells to glucose [120] nor did the overexpression of mGPDH correct impaired insulin secretion in GK rats [121]. However, the strong experimental support for mGPDH importance for β cell function came from studies of Eto et al. [122], where it was clearly demonstrated, that glucose-induced insulin release required oxidation of cytosolic NADH. The sole ablation of mGPDH or the sole inhibition of aspartate aminotransferase by aminooxyacetate had little or no effect, but their combination completely abolished glucose-induced insulin release. Therefore, the defects in shuttling of cytosolic NADH into mitochondria may contribute to impaired insulin secretion in non-insulin dependent diabetes mellitus. Substitutable role of the shuttles may explain why modulation of the mGPDH only was without clear effect on β cell function in some other studies.

A substitutable role of the two shuttles is also demonstrated in citrin deficiency [123]. Citrin, the liver-type aspartate/glutamate carrier participates in urea, protein, and nucleotide biosynthetic pathways by supplying aspartate from mitochondria to the cytosol and also participates in transporting cytosolic NADH equivalents into mitochondria as a component of the malate/aspartate-shuttle. Mutations in *SLC25A13* gene cause citrin deficiency manifesting as both adult-onset type II citrulinemia and neonatal intrahepatic cholestasis. Citrin knock-out mice failed to display features of human citrin deficiency but pathogenic phenotype was induced by double knock out of citrin and *GPD2* gene demonstrating that upregulation of GP shuttle activity was sufficient to maintain to transport NADH equivalents from cytosol to mitochondria in the absence of citrin [124]. Metabolomic studies of double knockout further revealed a marked increase of hepatic G3P, decrease of TCA intermediates, and alterations of amino acid levels related to the urea cycle or lysine catabolism [123].

An important biological role is played by mGPDH in sperm capacitation, in accordance with testis specific regulation of promoter C of the *GPD2* gene. Capacitation confers on the spermatozoa the competence to fertilize the oocyte. The mGPDH activity is very high in caudal epididymal spermatozoa of rat and hamster and mGPDH activity was found to correlate with sperm capacitation [30,125–127]. Immunofluorescence studies demonstrated noncanonical localization in the acrosome and principal piece in human, mouse, rat, and hamster spermatozoa that may imply a role of mGPDH in acrosome reaction and hyperactivation. Using $mGPDH^{-/-}$ mice Kota et al. demonstrated that the absence of mGPDH significantly alters hyperactivation and acrosome reaction and reduces ROS generation in mouse spermatozoa which leads to impairment of sperm capacitation [126].

The significance of mGPDH-dependent ROS production for metabolism of some mammalian tissues and cells is also illustrated by high content of mGPDH in placenta and elevated levels in cancer cells. Oxidative stress in pregnancy, namely hypoxia/reoxygenation in the 1^{st} trimester results in increased oxygen tension that leads to increased ROS production in placenta, associated with oxidative damage of proteins and increase in expression of antioxidative enzymes [128]. Placental mitochondria were found to contain high amounts of mGPDH [8,129] which may contribute to oxidative stress in placenta as analysis of human placental mitochondria demonstrated intensive mGPDH-dependent ROS production [130].

Elevated levels of mGPDH were also found in rapidly growing, undifferentiated tumors, while in undifferentiated slow growing tumors the mGPDH activity was normal or decreased [131–133]. Most malignant cells are highly glycolytic and produce high levels of ROS compared to normal cells. In prostate cancer cell lines (LNCaP, DU145, PC3, and CL1) mGPDH abundance and activity were significantly elevated while cytochrome *c* oxidase activity was down-regulated. Consequently, the glycolytic capacity and G3P-dependent ROS production were increased and antioxidant enzymes SOD1, SOD2 and catalase were up-regulated indicating that mGPDH is involved in maintaining a high rate of glycolysis and is an important site of electron leakage leading to ROS production in prostate cancer cells [134,135].

9. Conclusions

Original interest in mGPDH and its function in intermediary metabolism led to the discovery of GP-shuttle and postulated the key role of mGPDH in glycolysis, metabolism of fatty acids and oxidative phosphorylation. Unique tissue specific expression was an attractive aspect of mGPDH function that stimulated further research on its diverse physiological and organ specific functions. At present, new perspectives can be foreseen in elucidation of the involvement of mGPDH in ROS production and oxidative stress *in vivo*, participation of enzyme dysfunction in different pathological states and possible involvement of mGPDH in supramolecular structures of mitochondrial enzymes and components. It can be anticipated that these lines of future research will benefit from the detailed characterization of mGPDH structure and function at atomic level, as it was the case with other respiratory chain dehydrogenases.

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