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*Manuscript

Glycosomes: a comprehensive view on the metabolic role in *T. brucei*

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Abstract

Peroxisomes are single-membrane cellular organelles, present in most eukaryotic cells and organisms from human to yeast, fulfilling essential metabolic functions in lipid metabolism, free radical detoxification, differentiation, development, morphogenesis, etc. Interestingly, the protozoan parasite species *Trypanosoma* contains peroxisome-like organelles named glycosomes, which lack hallmark peroxisomal pathways and enzymes, such as catalase. Glycosomes are the only peroxisome-like organelles containing most enzymatic steps of the glycolytic pathway as well as enzymes of pyrimidine biosynthesis, purine salvage and biosynthesis of nucleotide sugars. We present here an overview of the glycosomal metabolic peculiarities together with the current view of the raison d'être of this unique metabolic peroxisomal sequestration.

1. Introduction

Glycosomes are specialized peroxisomes found in organisms belonging to the protist group of Kinetoplastea and Diplonemida from the eukaryotic supergroup Excavata (for a recent review see Gualdron-Lopez et al., 2012). This includes trypanosomatid parasites of the *Trypanosoma* and *Leishmania* species, which are responsible for a number of diseases affecting humans and livestock. *Trypanosoma brucei*, under the scope of this review, is responsible for sleeping sickness in Africa with two developmental stages available *in vitro*, the bloodstream (BSF) and procyclic (PCF) forms found in the blood of mammalian hosts and the digestive tract of the insect vector (tsetse fly), respectively. After their initial discovery glycosomes were not right away classified as peroxisome or peroxisome-like organelles, as it lacks one of the key peroxisomal activities, catalase (Penketh and Klein, 1986). The later location of enzymes of the ether-linked lipid pathway (Opperdoes, 1984) led to the classification as a peroxisome-like structure, although the identification of several glycolytic enzymes within this organelle (Opperdoes and Borst, 1977) initially was puzzling. Glycolytic enzymes are not the only particularity rendering the glycosome such a special organelle. Indeed, additional pathways have been specifically identified within this peroxisome-like organelle, *i.e.* succinic fermentation, the oxidative branch of the pentose phosphate pathway (PPP), purine salvage, pyrimidine biosynthesis and sugar nucleotide biosynthesis.

Exchange of metabolites between the glycosomal and the cytosolic compartments is another important aspect of the organelle biology. Glycosomes, as well as human and yeast

peroxisomes, contain channel-forming proteins involved in the selective transfer of metabolites across the single membrane surrounding the organelles (Gualdron-Lopez et al., 2012). By analogy with peroxisomal pores, one may consider that these glycosomal channels allow transmembrane diffusion of solutes with a molecular mass up to 300–400 Da but are unable to transfer bulky metabolites such as ATP and some cofactors whose molecular mass exceeds 500 Da, although the size-discrimination of the glycosomal pore has not been investigated. In addition, trypanosomes express a few glycosomal integral membrane proteins (GAT1, GAT2, GAT3, GIM5A and GIM5B) probably required to exchange bulky metabolites between the glycosomal and cytosolic compartments (Voncken et al., 2003; Igoillo-Esteve et al., 2011).

2. Organelle functions

Glycolysis and the maintenance of the energy and redox balance

The most prominent metabolic pathway within glycosomes is the eponymous glycolysis. In BSF of *T. brucei* the first seven enzymes, from hexokinase (HK, step 1) to phosphoglycerate kinase (PGK, step 8a) are sequestered in the glycosomes and can make up to 95% of the protein content (Misset et al., 1986), whereas in PCF the first six steps until glyceraldehyde-3-phosphate dehydrogenase (GAPDH, step 7) can comprise up to 50% of glycosomal protein content (Hart et al., 1984). This has a huge impact on how the cells maintain the glycosomal ATP/ADP and NAD^+/NADH balances, since as mentioned above these bulky glycosomal metabolites (430-660 Da) are not, or only poorly, exchanged with the cytosol (Haanstra et al., 2008). In contrast, all glycolytic intermediates (170-260 Da) can presumably be exchanged through the glycosomal pore, if similar to the peroxisomal one.

BSF convert glucose into pyruvate, the main excreted end product from glycolysis. During this process, they replenish their glycosomal ATP pool by the reaction of the PGK, restoring the ATP/ADP balance, which has been affected by the phosphorylating activities of HK and phosphofructokinase (PFK, step 3) upstream. Per molecule of glucose the GAPDH reduces two equivalents of NAD^+ to NADH. In order to maintain the glycosomal redox balance, *T. brucei* BSF utilize the so-called glycerol 3-phosphate/dihydroxyacetone phosphate (gly3P/DHAP) shuttle (Guerra et al., 2006). DHAP produced from fructose 1,6-bisphosphate (F1,6BP) is reduced by the glycosomal glycerol-3-phosphate dehydrogenase (G3PDH, step 16) to gly3P, which is oxidized back by a mitochondrial FAD-dependent G3PDH (step 18) to DHAP, being converted in the glycosomes to glyceraldehyde 3-phosphate (G3P), the substrate of GAPDH. By this detour of the glycolytic flux, relying on oxygen as electron

acceptor within the mitochondrion, BSF trypanosomes avoid depletion of NAD^+ within the glycosomes. In the absence of oxygen, an alternative way of maintaining the glycosomal redox balance is the production of equal amounts of glycerol and pyruvate from glucose to reoxidize NADH *via* the G3PDH reaction (Hammond and Bowman, 1980). However, this hampers cell growth with a two-fold reduction of net ATP production per glucose consumed.

Another way of maintaining these equilibriums can be observed in the PCF of *T. brucei*, which uses the so-called succinate branch within the glycosomes to maintain both the ATP/ADP and the NAD^+/NADH balances (Besteiro et al., 2002). This 4-step glycosomal pathway redistributes part of the produced cytosolic phosphoenolpyruvate (PEP) back into the glycosomes to produce succinate (steps 12-15). This succinic fermentation pathway contains two NADH-dependent dehydrogenases, malate dehydrogenase (MDH, step 13) and fumarate reductase (FRDg, step 15), which restore the NAD^+/NADH balance within the glycosomes. This pathway is also involved in the maintenance of the ATP/ADP balance through the PEP carboxykinase (PEPCK, step 12), together with the glycosomal pyruvate phosphate dikinase (PPDK, step 11), which produces pyruvate from PEP. These two glycosomal kinases compensate for the cytosolic localization of the PGK (step 8b) in *T. brucei* PCF. These two different strategies developed by BSF and PCF can be clearly seen when looking at the excreted end products from glucose metabolism, which is mainly pyruvate compared to acetate and succinate, respectively (Bringaud et al., 2006).

The pentose phosphate pathway: NADPH production and phosphate leakage

The pentose phosphate pathway (PPP) is a metabolic pathway competing with glycolysis for glucose 6-phosphate, which is converted into pentose phosphates, such as ribose 5-phosphate (steps 20-22), used as a precursor for the synthesis of nucleotides. The oxidative branch of the PPP, which produces NADPH and pentose phosphates is expressed in both the BSF and PCF, while the non-oxidative branch involved in rearranging the pentose carbon scaffolds into various intermediates like F6P or GAP is specific to PCF (Kovarova and Barrett, 2016). Most, if not all, of the PPP enzymes display a dual cytosolic and glycosomal localisation, with most of the enzyme/activity being within the cytosol (Colasante et al., 2006; Vertommen et al., 2008; Guther et al., 2014). One of the reasons supporting glycosomal PPP flux is the finding of some trypanothione reductase (TR, step 31) within the glycosomes (Guther et al., 2014). This enzyme is essential for the regeneration of trypanothione $T(SH)_2$ (732 Da), the parasites main mechanism of H_2O_2 detoxification, which compensates for the absence of catalase (Penketh and Klein, 1986). In addition, the *T. brucei* glycosomal isocitrate

dehydrogenase (IDHg, step 23) could produce glycosomal NADPH by converting isocitrate into α-ketoglutarate (<200 Da). This is not the case for all trypanosomatids, since in *T. cruzi* this IDH isoform localizes to the cytosol (Leroux et al., 2011).

The point was raised that, in BSF, glycosomal PPP flux would cause a loss of the glucose 6-phosphate high energy bond, which poses a problem for the maintenance of the glycosomal ATP/ADP balance and thus glycolysis (Kerkhoven et al., 2013). The succinate branch activity recently described in the BSF glycosomes, which represents ~1% of the glycolytic flux, may compensate for this PPP related bound-phosphate leakage (Mazet et al., 2013; Creek et al., 2015). Incidentally, the PCF PPDK/PEPCK null mutant still performs glycolysis, although regeneration of glycosomal ATP is theoretically abolished (Deramchia et al., 2014). One of the proposed solutions is an ATP/ADP antiporter, such as a mitochondrial translocase identified in glycosomal proteomes (Colasante et al., 2013; Guther et al., 2014). However, such an antiporter could lead to an accumulation of sugar phosphates, mimicking the lethal situation of glycosome deficient cells (Haanstra et al., 2008). One cannot exclude a second option implying the recovery of bound phosphates by additional unknown glycosomal reactions.

Lipid degradation and biosynthesis

One of the peroxisomal hallmarks is lipid metabolism, especially β-oxidation of fatty acids. Although two β-oxidation related enzymatic activities have been detected within the glycosomes (Wiemer et al., 1996), several lines of evidence suggest that glycosomal β-oxidation might play a minor or no role in PCF, *i.e.* (*i*) the apparent absence of active degradation of lipid droplet stored triacylglycerols (TAG) in glucose-depleted conditions and (*ii*) the only *T. brucei* gene candidate for the α-subunit of the trifunctional enzyme (TFEα, enoyl-CoA isomerase and 3-hydroxyacyl-CoA dehydrogenase activities, steps 30α), which encodes a glycosomal protein, is not responsible for the low, but detectable 3-hydroxyacyl-CoA dehydrogenase activity measured in glycosomal fractions (Wiemer et al., 1996; Allmann et al., 2014). Interestingly, a recent publication showed that BSF trypanosomes populating adipose tissues up-regulate glycosomal enzymes and enzymatic activities possibly involved in β-oxidation compared to blood isolated BSF (Caljon et al., 2016; Capewell et al., 2016; Trindade et al., 2016). This suggests that fatty acids feeding an active β-oxidation is used by BSF evolving in tissue environments, for instance to convert the long-chain fatty acids (C16-C24) abundantly present inside the adipocytes into myristic acid (C14) required for

biosynthesis of the GPI-anchored variable surface glycoprotein (VSG), which covers the whole surface of the parasite. The identification of a glycosomal ABC transporter (GAT1) involved in glycosomal import of oleoyl-CoA (1030 Da) (Igoillo-Esteve et al., 2011) is consistent with this hypothetical activation of β-oxidation, which still needs to be further experimentally validated. Oleoyl-CoA, as well as other fatty acyl-CoA could serve as carbon backbone for β-oxidation or biosynthesis of ether-linked lipids (see below). Alternatively, fatty acyl molecules (oleic acid, 282 Da) could be exchanged through the glycosomal pores and converted into fatty acyl-CoA by fatty acyl-CoA synthetases identified in glycocomal proteomes (Colasante et al., 2006; Guther et al., 2014). It is noteworthy that the peroxisomal carnitine transfer system expressed in other eukaryotes to exchange activated fatty acids has not been detected in the glycosomes (Colasante et al., 2006; Guther et al., 2014).

Another aspect of lipid metabolism is the biosynthesis of ether-linked lipids. These lipids are characterized by the ether bonds that link the fatty acids to the glycerol backbone. This ensures a higher chemical stability against hydrolysis due to high or low pH environments (Shinoda et al., 2004). Two steps of the ether lipid biosynthesis pathway have been identified in *T. brucei* and localized to the glycosomes (steps 25, 26) (Opperdoes, 1984; Zomer et al., 1995, 1999). The study of two integral membrane PEX11-related proteins, GIM5A and GIM5B, revealed their connection to ether-linked phospholipid biosynthesis, as the impaired cell lines showed reduced levels of ether-linked phospholipids, as well as a reduced number of glycosomes and increased cellular fragility (Voncken et al., 2003).

Pathways linked to Nucleotide metabolism

Purine catabolism is present in the peroxisomes of most eukaryotes. In contrast, glycosomes miss this pathway, but have specifically acquired three other pathways involved in nucleotide metabolism, *i.e.* the last two steps of the pyrimidine biosynthetic pathway (steps 48, 49) (Hammond et al., 1981), four phosphoribosyltransferases and enzymes (steps 50-53 in Figure 2) involved in the purine salvage (Luscher et al., 2014) and most of the enzymatic steps leading to biosynthesis of nucleotide sugars from glucose (Roper et al., 2005; Turnock et al., 2007; Stokes et al., 2008; Marino et al., 2010, 2011; Bandini et al., 2012; Kuettel et al., 2012). The reason for the glycosomal sequestration of these pathways is still unknown. The pyrimidine biosynthetic pathway is essential for *T. brucei* in pyrimidine-depleted medium, but not in the *in vivo* murine model because of the efficient pyrimidine salvage pathway (Ali et al., 2013; Ong et al., 2013). The physiological role of purine salvage enzymes in the glycosomes is unclear, since replacement of glycosomal xanthine phosphoribosyltransferases

(XPRTase, step 52) in *L. donovani* with a truncated cytosolic form did not impair interconversion of the purine nucleotides (Zarella-Boitz et al., 2004). Interestingly, glycosomal enzymes of these last three pathways produce PPi, a substrate for the glycosomal PPDK (step 11), the later would prevent accumulation of PPi known to inhibit the former biosynthetic pathways.

Nucleotide sugars (>560 kDa) need to be exported from glycosomes to feed biosynthetic pathways, which implies the expression of glycosomal transporters, since the glycosomal pore is theoretically too small for molecules of this size. Such transporters have not been identified so far.

3. The advantage of peroxisomal compartmentalization of glycolysis

One of the most discussed topics concerning glycosomes is probably the selective advantage provided by glycolysis sequestered into the peroxisome. Compartmentalized of glycolytic/gluconeogenic enzymes in peroxisomes was recently observed in the free-living diplonemids, suggesting that it was not required for parasitism, since it occurred in a free-living ancestor some 600 million years ago, probably before parasitism emerged in this eukaryotic branch (Morales et al., 2016). However, glycosomes may have facilitated the development of parasitism (Gualdron-Lopez et al., 2012). Initial assumptions suggesting that this compartmentalization ensures a higher glycolytic efficiency were proven unlikely, since the ratio between amounts of glycolytic enzymes *versus* glycolytic flux is the same in the *T. brucei* compartmentalized and *Saccharomyces cerevisiae* cytosolic glycolysis (Bakker et al., 2000). More recent hypotheses are related to a faster adaptation when facing an environmental change and thus a change in carbon sources. Instead of adapting several different metabolic pathways, this could be done by autophagy and replacing glycosome populations and thus the metabolic capacity in a modular way, as observed during differentiation of *T. brucei* and *Leishmania major* (Herman et al., 2006; Cull et al., 2014). In addition, recent publications suggest a role of glycosomes in differentiation of *T. brucei* BSF to PCF form (Szoor et al., 2010), highlighting the multiple facets of the organelles that deserve further investigations.

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Figure legends

Figure 1: Glycosomal metabolic pathways – Part1. Enzymatic reactions are indicated by black arrows. Dashed arrows indicate the absence of experimental proof for the glycosomal activity. Enzymes highlighted in white boxes have no identified glycosomal candidate protein. When applicable, we considered the reversibility of reactions catalysed by enzymes of the glycolytic/gluconeogenic pathway and the succinate branch, since the direction of the metabolic flux changes depending on the growth condition (glucose-rich *versus* glucose-depleted). For all the other pathways the expected direction of the metabolic flow is considered, although reactions catalysed by most enzymes may be reversible. Grey arrows indicate transport or diffusion processes. Excreted metabolites from glucose degradation are

indicated by black boxes. Metabolites <400 kDa can theoretically be exchanged through the glycosomal pore (P), while exchange of larger metabolites would require uncharacterized (?) or unidentified glycosomal integral membrane transporters or exchangers. Colour-code for pathways: blue, β-oxidation; ether-linked lipid synthesis; green, glycolysis/gluconeogenesis; purple, pentose phosphate pathway; brown, trypanothione cascade. Enzymes are: 1, hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, fructose-1,6-bisphosphatase; 5, aldolase; 6, triose-phosphate isomerase; 7, glyceraldehyde-3-phosphate dehydrogenase; 8a glycosomal (in BSF) or 8b cytosolic (in PCF) phosphoglycerate kinase; 9, phosphoglycerate mutase and enolase; 10, pyruvate kinase; 11, pyruvate phosphate dikinase; 12, phosphoenolpyruvate carboxykinase; 13, glycosomal malate dehydrogenase; 14, cytosolic (and glycosomal) fumarase (FHc); 15, glycosomal NADH-dependent fumarate reductase; 16, glycerol-3-phosphate dehydrogenase; 17, glycerol kinase; 18, FAD-dependent glycerol-3-phosphate dehydrogenase ; 19, cytosolic malic enzyme; 20, glucose-6-phosphate dehydrogenase; 21, 6-phosphogluconolactonase; 22, 6-phosphogluconate dehydrogenase; 23, glycosomal isocitrate dehydrogenase; 24, acyl-CoA reductase; 25, DHAP acyltransferase; 26, alkyl-DHAP synthase; 27, acyl/alkyl-DHAP reductase; 28, acyl-CoA dehydrogenase; 29, 2,4-dienoyl reductase; 30α, 2-enoyl-CoA hydratase/3-hydroxyacyl dehydrogenase (encoded by the α-subunit of a trifunctional enzyme); 30β, β-ketothiolase (encoded by the β -subunit of a trifunctional enzyme); 31, trypanothione reductase; 32, tryparedoxin; 33, tryparedoxin peroxidase; 34, acyl-CoA synthetase; 35, adenylate kinase. Abbreviations: αKG, α-ketoglutarate; 1,3BPGA, 1,3-bisphosphoglycerate; 3PGA, 3-phosphoglycerate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; AOX, alternative oxidase; CoA, coenzyme A; DHAP, dihydroxyacetone phosphate; F1,6BP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; Fum, fumarate; G3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; GAT1, glycosomal ABC transporter 1; Gly3P, glycerol 3-phosphate; isocit, isocitrate; Mal, malate; Oxac, oxaloacetate; P, glycosomal pore; PEP, phosphoenolpyruvate; PPi, pyrophosphate; PPP, pentose phosphate pathway; Rib5P, ribulose 5-phosphate; $T[SH]_2$, trypanothione (reduced); TS_2, trypanothione (oxidized); UQ, ubiquinone pool.

Figure 2: Glycosomal metabolic pathways – Part2. For legend see Figure 1. Colour-code for pathways: blue, synthesis of UDP-galactose; red, synthesis of UDP- N-acetyl-glucosamine; orange, synthesis of GDP-fucose; brown, synthesis of ascorbate; black synthesis of erythroascorbate; green, pyrimidine synthesis; purple, purine salvage pathway. Additional enzymes are: 36, phosphoacetylglucosamine mutase; 37, phosphomannomutase; 38, UDP-

glucose pyrophosphorylase; 39, UDP-galactose 4-epimerase; 40, glucosamine-fructose-6-phosphate aminotransferase; 41, glucosamine-6-phosphate synthase; 42, uridine-acetylglucosamine pyrophosphorylase; 43, phosphomannose isomerase; 44, GDP-mannose pyrophosphorylase; 45, GDP-mannose dehydrogenase; 46, GDP-mannose epimerase/reductase; 47, arabinonolactone oxidase with additional galactonolactone dehydrogenase activity; 48, orotate phosphoribosyltransferase; 49, orotidylate decarboxylase; 50, adenine phosphoribosyltransferase; 51, hypoxanthine-guanine phosphoribosyltransferase; 52, xanthine phosphoribosyltransferase; 53, inosinate dehydrogenase; Additional abbreviations: AraL, arabinonolactone; Asc, ascorbate; EAsc, erythroascorbate; Fuc, fucose; G1P, glucose 1-phosphate; GalL, galactonolactone; Gal, galactose; GDP, guanine diphosphate; GlcN, glucosamine; GlcNAc, N-acetyl-glucosamine; GMP, guanine monophosphate; IMP, inosine monophosphate; M1P, mannose 1-phosphate; M6P, mannose 6-phosphate; Man, mannose; PRPP, phosphoribosylpyrophosphate; UDP, uridine diphosphate; XMP, xanthine monophosphate; UTP, uridine triphosphate; XMP, xanthine monophosphate.

Figure 1

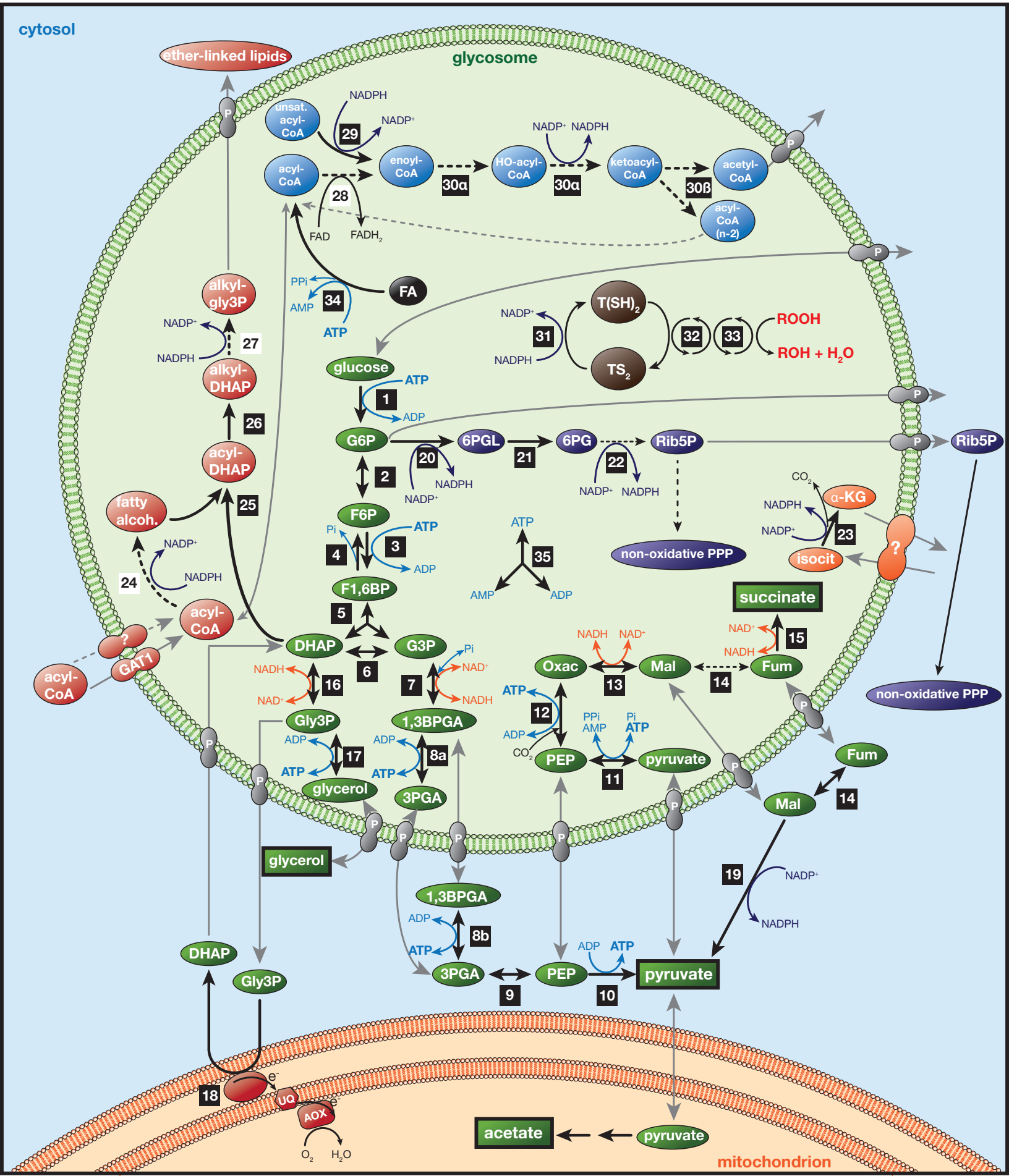
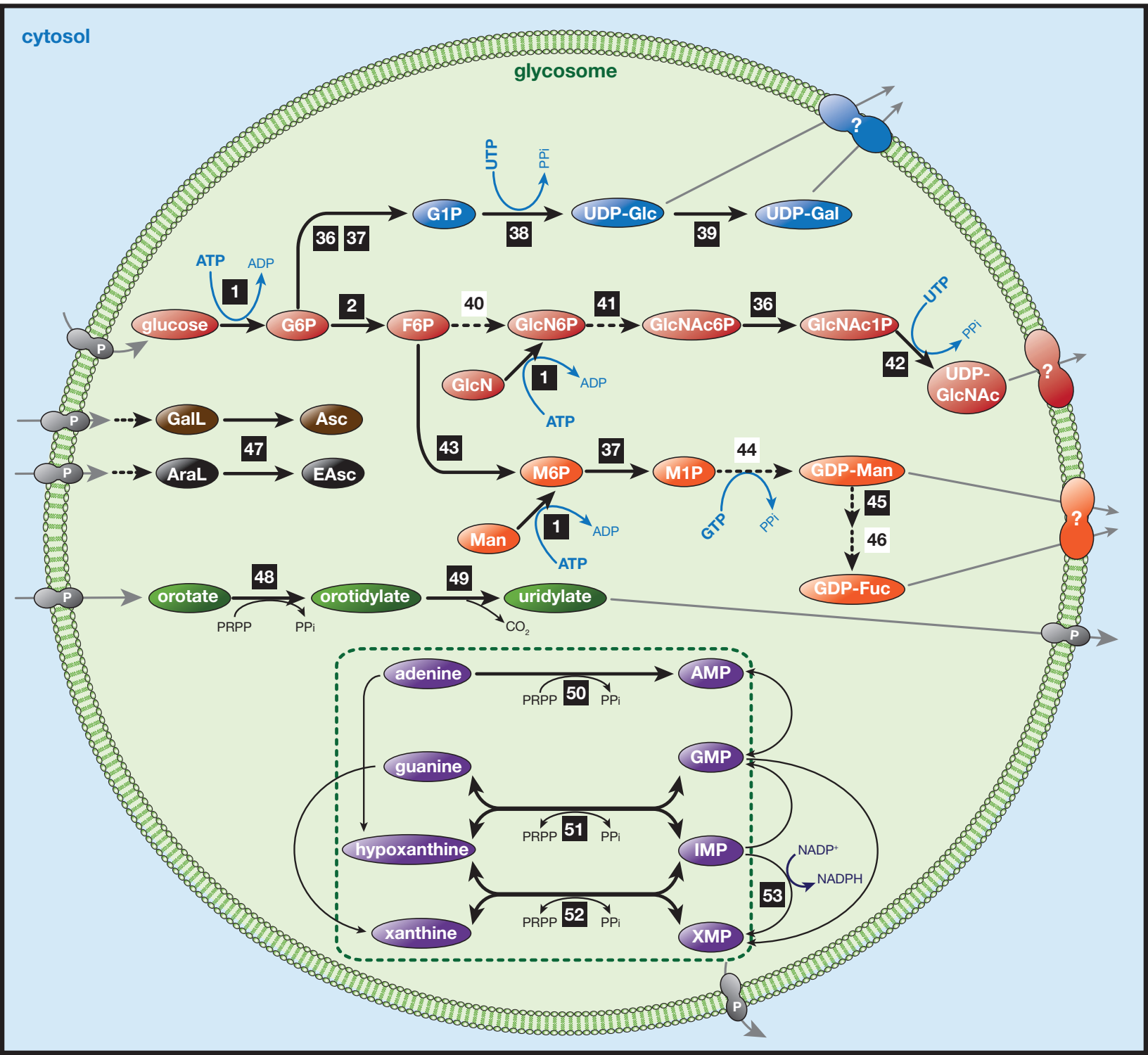

cytosol
glycosome
ether-linked lipids
unsat. acyl-CoA
acyl-CoA
enoyl-CoA
HO-acyl-CoA
ketoacyl-CoA
acetyl-CoA
acyl-CoA (n-2)
NADPH
NADP+
29
28
30α
30α
30β
FAD
FADH2
FA
PPi
AMP
ATP
34
alkyl-gly3P
alkyl-DHAP
acyl-DHAP
27
26
25
fatty alcoh.
24
acyl-CoA
GAT1
acyl-CoA
glucose
ATP
ADP
1
G6P
6PGL
6PG
Rib5P
Rib5P
20
21
22
2
F6P
Pi
ATP
3
4
F1,6BP
5
DHAP
G3P
6
7
NADH
NAD+
16
Gly3P
17
glycerol
1,3BPGA
8a
3PGA
T(SH)2
TS2
31
32
33
ROOH
ROH + H2O
35
non-oxidative PPP
CO2
α-KG
23
isocit
?
succinate
15
Oxac
Mal
Fum
13
14
12
PEP
pyruvate
11
Fum
Mal
14
19
glycerol
1,3BPGA
8b
3PGA
PEP
pyruvate
9
10
DHAP
Gly3P
18
e-
UQ
AOX
O2
H2O
acetate
pyruvate
mitochondrion

Figure 2


cytosol
glycosome
?
UTP
PPi
G1P
UDP-Glc
UDP-Gal
36 37
38
39
ATP
ADP
1
glucose
G6P
2
F6P
40
GlcN6P
41
GlcNAc6P
36
GlcNAc1P
UTP
PPi
42
UDP-GlcNAc
GlcN
1
ADP
ATP
GalL
Asc
47
AraL
EAsc
43
M6P
37
M1P
44
GDP-Man
45
46
GDP-Fuc
Man
1
ADP
ATP
GTP
PPi
orotate
48
PRPP
PPi
orotidylate
49
uridylate
CO2
P
adenine
AMP
PRPP
50
PPi
guanine
GMP
PRPP
51
PPi
hypoxanthine
IMP
NADP+
NADPH
53
PRPP
52
PPi
xanthine
XMP