*Manuscript

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

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35 **Abstract**

36 Peroxisomes are single-membrane cellular organelles, present in most eukaryotic cells 37 and organisms from human to yeast, fulfilling essential metabolic functions in lipid 38 metabolism, free radical detoxification, differentiation, development, morphogenesis, etc. 39 Interestingly, the protozoan parasite species *Trypanosoma* contains peroxisome-like 40 organelles named glycosomes, which lack hallmark peroxisomal pathways and enzymes, such 41 as catalase. Glycosomes are the only peroxisome-like organelles containing most enzymatic 42 steps of the glycolytic pathway as well as enzymes of pyrimidine biosynthesis, purine salvage 43 and biosynthesis of nucleotide sugars. We present here an overview of the glycosomal 44 metabolic peculiarities together with the current view of the raison d'être of this unique 45 metabolic peroxisomal sequestration.

46

47 **1. Introduction**

48 Glycosomes are specialized peroxisomes found in organisms belonging to the protist 49 group of Kinetoplastea and Diplonemida from the eukaryotic supergroup Excavata (for a 50 recent review see Gualdron-Lopez et al., 2012). This includes trypanosomatid parasites of the 51 *Trypanosoma* and *Leishmania* species, which are responsible for a number of diseases 52 affecting humans and livestock. *Trypanosoma brucei*, under the scope of this review, is 53 responsible for sleeping sickness in Africa with two developmental stages available *in vitro*, 54 the bloodstream (BSF) and procyclic (PCF) forms found in the blood of mammalian hosts and 55 the digestive tract of the insect vector (tsetse fly), respectively. After their initial discovery 56 glycosomes were not right away classified as peroxisome or peroxisome-like organelles, as it 57 lacks one of the key peroxisomal activities, catalase (Penketh and Klein, 1986). The later 58 location of enzymes of the ether-linked lipid pathway (Opperdoes, 1984) led to the 59 classification as a peroxisome-like structure, although the identification of several glycolytic 60 enzymes within this organelle (Opperdoes and Borst, 1977) initially was puzzling. Glycolytic 61 enzymes are not the only particularity rendering the glycosome such a special organelle. 62 Indeed, additional pathways have been specifically identified within this peroxisome-like 63 organelle, *i.e.* succinic fermentation, the oxidative branch of the pentose phosphate pathway 64 (PPP), purine salvage, pyrimidine biosynthesis and sugar nucleotide biosynthesis.

65 Exchange of metabolites between the glycosomal and the cytosolic compartments is 66 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

101 acceptor within the mitochondrion, BSF trypanosomes avoid depletion of NAD^+ within the
102 glycosomes. In the absence of oxygen, an alternative way of maintaining the glycosomal
103 redox balance is the production of equal amounts of glycerol and pyruvate from glucose to
104 reoxidize NADH *via* the G3PDH reaction (Hammond and Bowman, 1980). However, this
105 hampers cell growth with a two-fold reduction of net ATP production per glucose consumed.
106 Another way of maintaining these equilibriums can be observed in the PCF of *T.*
107 *brucei*, which uses the so-called succinate branch within the glycosomes to maintain both the
108 ATP/ADP and the NAD^+/NADH balances (Besteiro et al., 2002). This 4-step glycosomal
109 pathway redistributes part of the produced cytosolic phosphoenolpyruvate (PEP) back into the
110 glycosomes to produce succinate (steps 12-15). This succinic fermentation pathway contains
111 two NADH-dependent dehydrogenases, malate dehydrogenase (MDH, step 13) and fumarate
112 reductase (FRDg, step 15), which restore the NAD^+/NADH balance within the glycosomes.
113 This pathway is also involved in the maintenance of the ATP/ADP balance through the PEP
114 carboxykinase (PEPCK, step 12), together with the glycosomal pyruvate phosphate dikinase
115 (PPDK, step 11), which produces pyruvate from PEP. These two glycosomal kinases
116 compensate for the cytosolic localization of the PGK (step 8b) in *T. brucei* PCF. These two
117 different strategies developed by BSF and PCF can be clearly seen when looking at the
118 excreted end products from glucose metabolism, which is mainly pyruvate compared to
119 acetate and succinate, respectively (Bringaud et al., 2006).
120
121 <u>*The pentose phosphate pathway: NADPH production and phosphate leakage*</u>
122 The pentose phosphate pathway (PPP) is a metabolic pathway competing with
123 glycolysis for glucose 6-phosphate, which is converted into pentose phosphates, such as
124 ribose 5-phosphate (steps 20-22), used as a precursor for the synthesis of nucleotides. The
125 oxidative branch of the PPP, which produces NADPH and pentose phosphates is expressed in
126 both the BSF and PCF, while the non-oxidative branch involved in rearranging the pentose
127 carbon scaffolds into various intermediates like F6P or GAP is specific to PCF (Kovarova and
128 Barrett, 2016). Most, if not all, of the PPP enzymes display a dual cytosolic and glycosomal
129 localisation, with most of the enzyme/activity being within the cytosol (Colasante et al., 2006;
130 Vertommen et al., 2008; Guther et al., 2014). One of the reasons supporting glycosomal PPP
131 flux is the finding of some trypanothione reductase (TR, step 31) within the glycosomes
132 (Guther et al., 2014). This enzyme is essential for the regeneration of trypanothione $T(SH)_2$
133 (732 Da), the parasites main mechanism of H_2O_2 detoxification, which compensates for the
134 absence of catalase (Penketh and Klein, 1986). In addition, the *T. brucei* glycosomal isocitrate

135 dehydrogenase (IDHg, step 23) could produce glycosomal NADPH by converting isocitrate
136 into α-ketoglutarate (<200 Da). This is not the case for all trypanosomatids, since in *T. cruzi*
137 this IDH isoform localizes to the cytosol (Leroux et al., 2011).
138 The point was raised that, in BSF, glycosomal PPP flux would cause a loss of the
139 glucose 6-phosphate high energy bond, which poses a problem for the maintenance of the
140 glycosomal ATP/ADP balance and thus glycolysis (Kerkhoven et al., 2013). The succinate
141 branch activity recently described in the BSF glycosomes, which represents ~1% of the
142 glycolytic flux, may compensate for this PPP related bound-phosphate leakage (Mazet et al.,
143 2013; Creek et al., 2015). Incidentally, the PCF PPDK/PEPCK null mutant still performs
144 glycolysis, although regeneration of glycosomal ATP is theoretically abolished (Deramchia et
145 al., 2014). One of the proposed solutions is an ATP/ADP antiporter, such as a mitochondrial
146 translocase identified in glycosomal proteomes (Colasante et al., 2013; Guther et al., 2014).
147 However, such an antiporter could lead to an accumulation of sugar phosphates, mimicking
148 the lethal situation of glycosome deficient cells (Haanstra et al., 2008). One cannot exclude a
149 second option implying the recovery of bound phosphates by additional unknown glycosomal
150 reactions.
151
152 *Lipid degradation and biosynthesis*
153 One of the peroxisomal hallmarks is lipid metabolism, especially β-oxidation of fatty
154 acids. Although two β-oxidation related enzymatic activities have been detected within the
155 glycosomes (Wiemer et al., 1996), several lines of evidence suggest that glycosomal β-
156 oxidation might play a minor or no role in PCF, *i.e.* (*i*) the apparent absence of active
157 degradation of lipid droplet stored triacylglycerols (TAG) in glucose-depleted conditions and
158 (*ii*) the only *T. brucei* gene candidate for the α-subunit of the trifunctional enzyme (TFEα,
159 enoyl-CoA isomerase and 3-hydroxyacyl-CoA dehydrogenase activities, steps 30α), which
160 encodes a glycosomal protein, is not responsible for the low, but detectable 3-hydroxyacyl-
161 CoA dehydrogenase activity measured in glycosomal fractions (Wiemer et al., 1996; Allmann
162 et al., 2014). Interestingly, a recent publication showed that BSF trypanosomes populating
163 adipose tissues up-regulate glycosomal enzymes and enzymatic activities possibly involved in
164 β-oxidation compared to blood isolated BSF (Caljon et al., 2016; Capewell et al., 2016;
165 Trindade et al., 2016). This suggests that fatty acids feeding an active β-oxidation is used by
166 BSF evolving in tissue environments, for instance to convert the long-chain fatty acids (C16-
167 C24) abundantly present inside the adipocytes into myristic acid (C14) required for

168 biosynthesis of the GPI-anchored variable surface glycoprotein (VSG), which covers the
169 whole surface of the parasite. The identification of a glycosomal ABC transporter (GAT1)
170 involved in glycosomal import of oleoyl-CoA (1030 Da) (Igoillo-Esteve et al., 2011) is
171 consistent with this hypothetical activation of β-oxidation, which still needs to be further
172 experimentally validated. Oleoyl-CoA, as well as other fatty acyl-CoA could serve as carbon
173 backbone for β-oxidation or biosynthesis of ether-linked lipids (see below). Alternatively,
174 fatty acyl molecules (oleic acid, 282 Da) could be exchanged through the glycosomal pores
175 and converted into fatty acyl-CoA by fatty acyl-CoA synthetases identified in glycocomal
176 proteomes (Colasante et al., 2006; Guther et al., 2014). It is noteworthy that the peroxisomal
177 carnitine transfer system expressed in other eukaryotes to exchange activated fatty acids has
178 not been detected in the glycosomes (Colasante et al., 2006; Guther et al., 2014).
179 Another aspect of lipid metabolism is the biosynthesis of ether-linked lipids. These
180 lipids are characterized by the ether bonds that link the fatty acids to the glycerol backbone.
181 This ensures a higher chemical stability against hydrolysis due to high or low pH
182 environments (Shinoda et al., 2004). Two steps of the ether lipid biosynthesis pathway have
183 been identified in *T. brucei* and localized to the glycosomes (steps 25, 26) (Opperdoes, 1984;
184 Zomer et al., 1995, 1999). The study of two integral membrane PEX11-related proteins,
185 GIM5A and GIM5B, revealed their connection to ether-linked phospholipid biosynthesis, as
186 the impaired cell lines showed reduced levels of ether-linked phospholipids, as well as a
187 reduced number of glycosomes and increased cellular fragility (Voncken et al., 2003).
188
189 *Pathways linked to Nucleotide metabolism*
190 Purine catabolism is present in the peroxisomes of most eukaryotes. In contrast,
191 glycosomes miss this pathway, but have specifically acquired three other pathways involved
192 in nucleotide metabolism, *i.e.* the last two steps of the pyrimidine biosynthetic pathway (steps
193 48, 49) (Hammond et al., 1981), four phosphoribosyltransferases and enzymes (steps 50-53 in
194 Figure 2) involved in the purine salvage (Luscher et al., 2014) and most of the enzymatic
195 steps leading to biosynthesis of nucleotide sugars from glucose (Roper et al., 2005; Turnock
196 et al., 2007; Stokes et al., 2008; Marino et al., 2010, 2011; Bandini et al., 2012; Kuettel et al.,
197 2012). The reason for the glycosomal sequestration of these pathways is still unknown. The
198 pyrimidine biosynthetic pathway is essential for *T. brucei* in pyrimidine-depleted medium, but
199 not in the *in vivo* murine model because of the efficient pyrimidine salvage pathway (Ali et
200 al., 2013; Ong et al., 2013). The physiological role of purine salvage enzymes in the
201 glycosomes is unclear, since replacement of glycosomal xanthine phosphoribosyltransferases

202 (XPRTase, step 52) in *L. donovani* with a truncated cytosolic form did not impair
203 interconversion of the purine nucleotides (Zarella-Boitz et al., 2004). Interestingly,
204 glycosomal enzymes of these last three pathways produce PPi, a substrate for the glycosomal
205 PPDK (step 11), the later would prevent accumulation of PPi known to inhibit the former
206 biosynthetic pathways.
207 Nucleotide sugars (>560 kDa) need to be exported from glycosomes to feed
208 biosynthetic pathways, which implies the expression of glycosomal transporters, since the
209 glycosomal pore is theoretically too small for molecules of this size. Such transporters have
210 not been identified so far.
211
212 **<u>3. The advantage of peroxisomal compartmentalization of glycolysis</u>**
213 One of the most discussed topics concerning glycosomes is probably the selective
214 advantage provided by glycolysis sequestered into the peroxisome. Compartmentalized of
215 glycolytic/gluconeogenic enzymes in peroxisomes was recently observed in the free-living
216 diplonemids, suggesting that it was not required for parasitism, since it occurred in a free-
217 living ancestor some 600 million years ago, probably before parasitism emerged in this
218 eukaryotic branch (Morales et al., 2016). However, glycosomes may have facilitated the
219 development of parasitism (Gualdron-Lopez et al., 2012). Initial assumptions suggesting that
220 this compartmentalization ensures a higher glycolytic efficiency were proven unlikely, since
221 the ratio between amounts of glycolytic enzymes *versus* glycolytic flux is the same in the *T.*
222 *brucei* compartmentalized and *Saccharomyces cerevisiae* cytosolic glycolysis (Bakker et al.,
223 2000). More recent hypotheses are related to a faster adaptation when facing an
224 environmental change and thus a change in carbon sources. Instead of adapting several
225 different metabolic pathways, this could be done by autophagy and replacing glycosome
226 populations and thus the metabolic capacity in a modular way, as observed during
227 differentiation of *T. brucei* and *Leishmania major* (Herman et al., 2006; Cull et al., 2014). In
228 addition, recent publications suggest a role of glycosomes in differentiation of *T. brucei* BSF
229 to PCF form (Szoor et al., 2010), highlighting the multiple facets of the organelles that
230 deserve further investigations.
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373

374 **Figure legends**

375

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

385 indicated by black boxes. Metabolites <400 kDa can theoretically be exchanged through the
386 glycosomal pore (P), while exchange of larger metabolites would require uncharacterized (?)
387 or unidentified glycosomal integral membrane transporters or exchangers. Colour-code for
388 pathways: blue, β-oxidation; ether-linked lipid synthesis; green, glycolysis/gluconeogenesis;
389 purple, pentose phosphate pathway; brown, trypanothione cascade. Enzymes are: 1,
390 hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, fructose-1,6-
391 bisphosphatase; 5, aldolase; 6, triose-phosphate isomerase; 7, glyceraldehyde-3-phosphate
392 dehydrogenase; 8a glycosomal (in BSF) or 8b cytosolic (in PCF) phosphoglycerate kinase; 9,
393 phosphoglycerate mutase and enolase; 10, pyruvate kinase; 11, pyruvate phosphate dikinase;
394 12, phosphoenolpyruvate carboxykinase; 13, glycosomal malate dehydrogenase; 14, cytosolic
395 (and glycosomal) fumarase (FHc); 15, glycosomal NADH-dependent fumarate reductase; 16,
396 glycerol-3-phosphate dehydrogenase; 17, glycerol kinase; 18, FAD-dependent glycerol-3-
397 phosphate dehydrogenase ; 19, cytosolic malic enzyme; 20, glucose-6-phosphate
398 dehydrogenase; 21, 6-phosphogluconolactonase; 22, 6-phosphogluconate dehydrogenase; 23,
399 glycosomal isocitrate dehydrogenase; 24, acyl-CoA reductase; 25, DHAP acyltransferase; 26,
400 alkyl-DHAP synthase; 27, acyl/alkyl-DHAP reductase; 28, acyl-CoA dehydrogenase; 29, 2,4-
401 dienoyl reductase; 30α, 2-enoyl-CoA hydratase/3-hydroxyacyl dehydrogenase (encoded by
402 the α-subunit of a trifunctional enzyme); 30β, β-ketothiolase (encoded by the β -subunit of a
403 trifunctional enzyme); 31, trypanothione reductase; 32, tryparedoxin; 33, tryparedoxin
404 peroxidase; 34, acyl-CoA synthetase; 35, adenylate kinase. Abbreviations: αKG, α-
405 ketoglutarate; 1,3BPGA, 1,3-bisphosphoglycerate; 3PGA, 3-phosphoglycerate; 6PGL, 6-
406 phosphogluconolactone; 6PG, 6-phosphogluconate; AOX, alternative oxidase; CoA,
407 coenzyme A; DHAP, dihydroxyacetone phosphate; F1,6BP, fructose 1,6-bisphosphate; F6P,
408 fructose 6-phosphate; Fum, fumarate; G3P, glyceraldehyde 3-phosphate; G6P, glucose 6-
409 phosphate; GAT1, glycosomal ABC transporter 1; Gly3P, glycerol 3-phosphate; isocit,
410 isocitrate; Mal, malate; Oxac, oxaloacetate; P, glycosomal pore; PEP, phosphoenolpyruvate;
411 PPi, pyrophosphate; PPP, pentose phosphate pathway; Rib5P, ribulose 5-phosphate; $T[SH]_2$,
412 trypanothione (reduced); TS_2, trypanothione (oxidized); UQ, ubiquinone pool.
413
414 Figure 2: Glycosomal metabolic pathways – Part2. For legend see Figure 1. Colour-code for
415 pathways: blue, synthesis of UDP-galactose; red, synthesis of UDP- N-acetyl-glucosamine;
416 orange, synthesis of GDP-fucose; brown, synthesis of ascorbate; black synthesis of
417 erythroascorbate; green, pyrimidine synthesis; purple, purine salvage pathway. Additional
418 enzymes are: 36, phosphoacetylglucosamine mutase; 37, phosphomannomutase; 38, UDP-

419 glucose pyrophosphorylase; 39, UDP-galactose 4-epimerase; 40, glucosamine-fructose-6-
420 phosphate aminotransferase; 41, glucosamine-6-phosphate synthase; 42, uridine-
421 acetylglucosamine pyrophosphorylase; 43, phosphomannose isomerase; 44, GDP-mannose
422 pyrophosphorylase; 45, GDP-mannose dehydrogenase; 46, GDP-mannose
423 epimerase/reductase; 47, arabinonolactone oxidase with additional galactonolactone
424 dehydrogenase activity; 48, orotate phosphoribosyltransferase; 49, orotidylate decarboxylase;
425 50, adenine phosphoribosyltransferase; 51, hypoxanthine-guanine phosphoribosyltransferase;
426 52, xanthine phosphoribosyltransferase; 53, inosinate dehydrogenase; Additional
427 abbreviations: AraL, arabinonolactone; Asc, ascorbate; EAsc, erythroascorbate; Fuc, fucose;
428 G1P, glucose 1-phosphate; GalL, galactonolactone; Gal, galactose; GDP, guanine
429 diphosphate; GlcN, glucosamine; GlcNAc, N-acetyl-glucosamine; GMP, guanine
430 monophosphate; IMP, inosine monophosphate; M1P, mannose 1-phosphate; M6P, mannose
431 6-phosphate; Man, mannose; PRPP, phosphoribosylpyrophosphate; UDP, uridine
432 diphosphate; XMP, xanthine monophosphate; UTP, uridine triphosphate; XMP, xanthine
433 monophosphate.
434
435

Figure 1

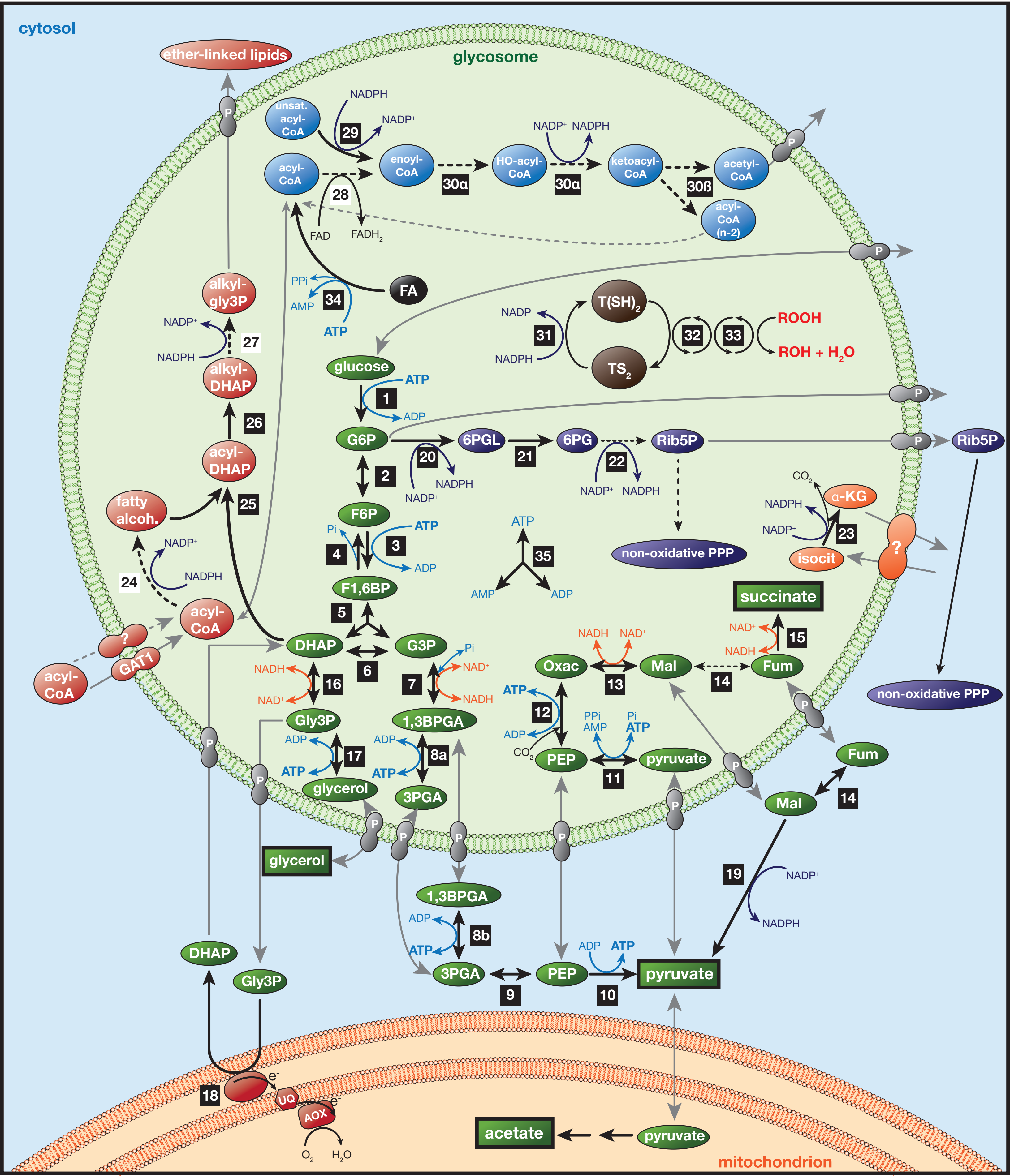

Figure 2