Biogenesis and metabolic homeostasis of trypanosomatid glycosomes

REVIEW ARTICLE

(to be published in the special issue 'Organelles in parasitic protists' of the Journal of Eukaryotic Microbiology)

Biogenesis and metabolic homeostasis of trypanosomatid glycosomes: new insights and new questions

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Running head title: Glycosomes of trypanosomatids

ABSTRACT

Kinetoplastea and Diplonemea possess peroxisome-related organelles that, uniquely, contain most of the enzymes of the glycolytic pathway and are hence called glycosomes. Enzymes of several other core metabolic pathways have also been located in glycosomes, in addition to some characteristic peroxisomal systems such as pathways of lipid metabolism. A considerable amount of research has been performed on glycosomes of trypanosomes since their discovery four decades ago. Not only the role of the glycosomal enzyme systems in the overall cell metabolism appeared to be unique, but the organelles display also remarkable features regarding their biogenesis and structural properties. These features are similar to those of the well-studied peroxisomes of mammalian and plant cells and yeasts yet exhibit also differences reflecting the large evolutionary distance between these protists and the representatives of other major eukaryotic lineages. Despite all research performed, many questions remain about various properties and the biological roles of glycosomes and peroxisomes. Here we review the current knowledge about glycosomes, often comparing it with information about peroxisomes. Furthermore, we highlight particularly many questions that remain about the biogenesis, and the heterogeneity in structure and content of these enigmatic organelles, and the properties of their boundary membrane.

Keywords
Biogenesis; enzyme activity regulation; gluconeogenesis; glycolysis; heterogeneity; membrane permeability; multienzyme complex; peroxin; peroxisome; pore

INTRODUCTION

Trypanosomes, like all organisms belonging to the classes Kinetoplastea and Diplonemea, possess organelles called glycosomes because they contain most of the enzymes of the glycolytic and gluconeogenic pathways (Fig. 1) (Borst & Opperdoes 1977; Morales et al. 2016). Glycosomes are derived from peroxisomes that, in a common ancestor of the kinetoplastids and diplonemids, after separation from the Euglenida lineage, have acquired these enzymes of carbohydrate metabolism (Gualdrón-López et al. 2012a; Gabaldón et al. 2016). In addition, enzymes of several other metabolic processes have been detected in glycosomes of *Trypanosoma brucei*. In common with peroxisomes from mammals, plants, and fungi, glycosomes may contain, dependent on the trypanosome's life-cycle stage, enzymes involved in ether-lipid biosynthesis and fatty-acid β-oxidation. Other metabolic systems, that are generally not or only rarely present in peroxisomes, were also found fully or in part associated with glycosomes. These include some enzymes of pyrimidine synthesis, purine salvage and squalene biosynthesis, as well as additional enzymes of carbohydrate metabolism such as those involved in the pentose-phosphate pathway (PPP), succinic fermentation that branches from glycolysis at the level of phosphoenolpyruvate (Fig. 1) and nucleotide-sugar biosynthesis. Similar glycosomal content has been experimentally detected for the other 'TriTryps species': *Trypanosoma cruzi* and *Leishmania* spp. (Colasante et al. 2006; Vertommen et al. 2008; Güther et al. 2014; Jardim et al. 2018; Acosta et al. 2019). By bioinformatics analysis of genome sequence data, using potential peroxisome-targeting signal (PTS) motifs as queries, the (partial) glycosomal localization of most of these metabolic processes has also been predicted for representatives of all other major kinetoplastid lineages (Butenko et al. 2020; Durrani et al. 2020). Remarkably, glycosomes of the TriTryps do not contain typical peroxisomal enzymes such as catalase, H_2O_2-producing D-amino-acid oxidase and α-hydroxy-acid oxidase, and glyoxylate cycle enzymes (Opperdoes 1987).

The identity of glycosomes as peroxisome-related organelles has not only been established by morphological resemblance and the common presence of enzymes of ether-lipid biosynthesis and fatty-acid β-oxidation, but even more convincingly by the similar system of biogenesis, involving homologous proteins called peroxins (or PEX-proteins) and PTS motifs in proteins to be routed to the glycosomal matrix after their synthesis in the cytosol (Galland & Michels 2010; Gualdrón-López et al. 2013a).

Glycosomes have been intensively studied since their discovery in 1977, because the organelles are intriguing and offer also possible targets for drugs to be developed against the diseases caused by trypanosomatids. Moreover, such research was feasible because *T. brucei* is highly amenable for biochemical and molecular biological research. In recent articles, we and others have reviewed different aspects of glycosomes of trypanosomes: their proteome and metabolic activities during different life-cycle stages (Allman & Bringaud 2017; Quiñones et al. 2020; Michels et al. 2021), the permeability properties of their surrounding membrane (Quiñones et al. 2020), their biogenesis and heterogeneity (Bauer & Morris 2017; Crowe & Morris 2021) and the possible origin and evolution of the organelles (Gualdrón-López et al. 2012a; Gabaldón et al. 2016). Glycosomes, like the other members of the large peroxisome family that are found in representatives of all major eukaryotic lineages, are in several respects still enigmatic organelles with properties that are very different from other organelles involved in major metabolic processes such as mitochondria and chloroplasts. In this paper, we will address several of the many questions that remain about these organelles despite the large amount of information already collected in previous decades. The use of methodologies developed in recent years in molecular, cellular, and structural biology, together with the powerful methods nowadays available for genetic manipulation of trypanosomes is expected to help solving these open questions and the enigma about the function(s) of the organelles.

PROPERTIES OF THE GLYCOSOMAL MEMBRANE

Peroxisomes and glycosomes are surrounded by a single phospholipid bilayer membrane. The permeability properties of this membrane have been a matter of debate for over 30 years. However, in the early 2000s strong evidence was provided that the membrane of the organelles in several organisms contain proteins with the capacity to form pores (reviewed by Antonenkov & Hiltunen 2012). Also, for glycosomes of *T. brucei* three pore-forming activities with different electrophysiological characteristics were detected when solubilized glycosomal membrane proteins were reconstituted into planar lipid bilayers (Gualdrón-López et al. 2012b). The identity of two peroxisomal pore-forming proteins has been established. In mammalian peroxisomal membranes, PXMP2 has been shown to form pores allowing compounds with Mr up to 300 Da to pass freely. The permeation of larger molecules, from 300 to 500–600 Da is partially restricted, while larger metabolites do not enter the pores (reviewed by Antonenkov and Hiltunen 2012; Quiñones et al. 2020; Chornyi et al. 2021). PXPM2 is part of a small family of integral membrane proteins, with four members detected in mammals and two in *Saccharomyces cerevisiae*. Several of these proteins have been shown to form channels. However, PXMP2 has only been found in mammals, and it is the only family member associated with the peroxisomal membrane; the other ones are in the inner mitochondrial membrane. Moreover, PXMP2's channel function seems different from that of its mitochondrial homologs. No PXMP2 homologs have so far been identified in trypanosomatids. The second protein is PEX11, a peroxin generally present in peroxisomes where it is involved in the organelle's proliferation and determination of the curvature of the organelle's membrane. In yeast, PEX11 has been shown to also possess the capacity to form pores conducting solutes with molecular mass below 300-400 Da (Minthoff et al. 2016). The identity of the pore-forming proteins of *T. brucei* glycosomes and the molecular selectivity of the pores remain to be determined. It should be noted that in trypanosomatids a PEX11 has been identified (Lorenz et al. 1998), as well as two homologs called GIM5A and GIM5B, predominant glycosomal membrane proteins whose depletion in *T. brucei* caused a pleiomorphic phenotype, including effects suggesting that these proteins may also be involved in metabolite transport (Maier et al. 2001; Voncken et al. 2003).

The presence of such pores implies that many common metabolites, such as glycolytic intermediates can readily equilibrate across the membrane, but larger molecules such as proteins, cofactors (e.g. NADH, ATP and CoA) and large substrates and metabolites (e.g. fatty acids/acyl-CoAs and nucleotide sugars) cannot and will require transporters. Indeed, ABC transporters for fatty acids/acyl-CoAs have been identified in peroxisomal membranes from different organisms (reviewed by Chornyi et al. 2021), also in glycosomes of *T. brucei* (Fig. 2) (Igoillo-Esteve et al. 2011). Candidate transporters for different cofactors (AMP, NAD^+, FAD, CoA) have been found in peroxisomes (Chornyi et al. 2021), although proof is still lacking, but little information is as yet available for any such transporters in glycosomal membranes. Alternatively, cofactors may be imported bound to their protein, since peroxisomal and glycosomal matrix proteins can be imported, after their synthesis in the cytosol, in folded and even oligomeric form (Walton et al. 1995; Häusler et al. 1996).

Biochemical and proteomic analyses of glycosomal membranes from *T. brucei*, *T. cruzi* and *Leishmania donovani* have yielded several proteins of which the identity and function remain to be determined (Colasante et al. 2013; Güther et al. 2014; Quiñones et al. 2015; Acosta et al. 2019). Several factors complicate the unambiguous identification and functional analysis of peroxisomal and glycosomal transporters. First, peroxisomes and glycosomes have contacts with other organelles such as the endoplasmic reticulum (ER), mitochondria and lipid droplets (see below), with the possibility to also exchange proteins. Second, the difficulty to purify

intact organelles hamper in vitro experiments. Third, analysis of the involvement putative transporters by in vivo transport studies is challenging because the organelles are usually very small and in large numbers – about 60 glycosomes in bloodstream-form (BSF) *T. brucei* (Tetley & Vickerman 1991; Hughes et al. 2017) – but comprise only about 5% of the cell volume. This makes it difficult to determine changes in transmembrane metabolite concentrations and kinetics of solute translocation.

The permeability of glycosomal membranes for many metabolites raises a question whether a glycerol-3-phosphate/dihydroxyacetone phosphate (Gly3P/DHAP) exchanger in the glycosomal membrane actually exists (Fig. 1). Such an antiport system has been invoked to shuttle electrons from NADH, reduced in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzed reaction, to the glycerol-3-phosphate oxidase (GPO) system in the mitochondrion to maintain the redox balance within the glycosomal matrix (Fig. 1) (Opperdoes & Borst 1977; reviewed in Michels et al. 2021). Does such antiport indeed occur by a designated transporter, and if so, how does it function, exchanging Gly3P and DHAP in a strict 1/1 ratio, with the membrane being permeable to these metabolites? Could the shuttle function, and thus sustain glycosomal metabolism, if Gly3P and DHAP merely diffuse in opposite directions through the pores? It should be noted that also for maintaining $NAD(P)^+$/NAD(P)H homeostasis in the matrix of peroxisomes mechanisms have been proposed to shuttle electrons across the membrane by exchange of a variety of metabolites, but likewise no specific transporters have so far been identified (Chornyi et al. 2021).

Support for the permeability of glycosomal membrane for small solutes comes from pulse-labeling BSF *T. brucei* with ^{14}C-glucose (Visser et al. 1981). Two pools of radiolabeled glycolytic intermediates were detected, a small one (20-30%) labeled very quickly, like the secreted metabolic end-product pyruvate, and interpreted as representing glycosomal metabolism and another one (70-80%) not directly involved in glycolysis and considered to represent the cytosol. The labeling of the latter pool seems to result from equilibration of metabolites across the membrane. The labeling of the first pool and pyruvate occurs very fast because of the high activity of glycolysis, whereas that of the second pool is labeled more slowly. Although the result was initially interpreted differently, the labeling of the cytosolic pool is suggestive of easy permeation of metabolites through the membrane. It occurs only 60 times slower than the glycolytic flux, what is fast when considering that it is not subjected to a direct drain caused by enzymatic activity, except for Gly3P that is part of the redox shuttle to the mitochondrial GPO, neither is it likely that important concentration gradients across the membrane are involved.

Another question is related to the presence of long-chain polyphosphate (polyP) molecules within glycosomes (Negreiros et al. 2018). Not only is their function in the organelles still unknown – they interact with several enzymes of carbon metabolism and may act as a negatively charged scaffold in the creation of an assembly of the intraglycosomal enzymes which have generally a high pI or have a role in regulating the activities of the enzymes (Quiñones et al. 2020) – but also how they arrive in the organelles. Inorganic phosphate and pyrophosphate may readily pass through the pores, but it is unknown if polyP is synthesized inside. The polymer is not only present in glycosomes, but also in nucleoli and primarily in acidocalcisomes where polyP-synthesizing activity has been detected. If it is imported into glycosomes, would it occur by a designated transporter, in association with a glycosomal protein, or could it, despite its high Mr but due to its linear form, pass through a pore?

BIOGENESIS OF GLYCOSOMES

The basics of glycosomal biogenesis has been established by research during the last 30 years. It occurs by similar mechanisms as those for peroxisomes in mammals, plants, and yeasts, but with several unique features, and it involves homologous peroxins albeit in most cases with highly divergent sequences, and similar sequence motifs to target proteins to the organelles. At present, a not-redundant set of 37 peroxins have been identified in different eukaryotes (Jansen et al. 2021). They participate in different aspects of the organelles' biogenesis, such as recruitment of lipids for membranes, insertion of proteins into the membranes, matrix protein import, and proliferation of the organelles. To date, 14 peroxin homologs have been found in trypanosomatids. Peroxisome biogenesis can occur by two possible, not mutually exclusive routes, de novo formation and growth followed by fission – with the existence of the de novo route proved for yeast and mammalian peroxisomes but not yet for glycosomes (see below). A detailed description of the current knowledge of glycosome biogenesis and trypanosomatid peroxins can be found in several other reviews (Moyersoen et al. 2004; Galland et al. 2010; Gualdrón-López et al. 2013a; Bauer & Morris 2017; Kalel & Erdmann 2018; Crowe & Morris 2021). Here we focus on some specific aspects of the biogenesis, highlighting some recent findings and address open questions about the process in trypanosomatids.

The matrix protein import cycle

Peroxisomal and glycosomal matrix proteins are synthesized in the cytosol and post-translationally imported into the organelles. The overall process can be understood as a cycle with several distinct steps, each involving a set of peroxins (Fig. 3A). Step I is the recognition of the protein to be imported by one of the two receptors, PEX5 for binding PTS1 and PEX7 for PTS2. It is followed in step II by interaction of the receptor-cargo complex with a membrane-associated docking complex, comprising PEX13 and PEX14. Step III involves the translocation of the PTS-proteins across the membrane, a process so far only molecularly unraveled for *S. cerevisiae* in reconstitution experiments that revealed two different, independently acting pores for import of PTS1 and PTS2 proteins (Fig. 3B) (Meinecke et al. 2010; Montilla-Martinez et al. 2015). The pore for PTS1 proteins comprises PEX14, PEX5 and the PTS1-cargo protein, whereas the pore for PTS2 proteins is made up with PEX14, coreceptor PEX17 that forms a ternary complex with PEX7 and the PTS2 protein, and a yeast-specific additional docking complex factor PEX18. Data suggested that in vivo the interaction of the cargo-loaded receptor with the docking complex triggers the formation of the pore, with as result that the cargo is released in the organelle's matrix. The PTS1 pore, reconstituted in a lipid membrane, opened transiently upon induction by the cargo, whereas the PTS2 pore was constitutively open yet appeared largely impermeable to solutes and ions. Whereas PEX5 is a constituent of the PTS1 pore, the fate of PEX7 is less clear. Initial reports claimed that PEX7 is released in the matrix (Lazarow 2006), whereas later data suggest that, like PEX5, it is retained at the translocation machinery (Rodrigues et al. 2015). In step IV the receptors are recycled from the organelle to the cytosol. For PEX5, it involves first the mono-ubiquitination via a membrane complex of the E3 ubiquitin ligases PEX2, PEX10 and PEX12, and the E2 ubiquitin-conjugating PEX4 at the cytosolic face of the membrane. In step V, a complex of two cytosolic AAA-ATPases, PEX1 and PEX6, also anchored to the cytosolic side of the membrane, extracts the receptor from the organelle in an ATP-dependent manner, so providing the free energy required for the overall process. The receptor is then released in the cytosol where it, after de-ubiquitination, is available for new cycles of import. Whether PEX7 retrieval occurs by the same mechanism remains to be established.

It is noteworthy that this elaborate matrix protein import mechanism shows remarkable similarity with that of the ER-associated degradation (ERAD) system by which damaged or misfolded proteins are retrieved from the ER lumen and transported back to the cytosol for proteasomal degradation (Schliebs et al. 2010). Moreover, the proteins involved in the two

processes are homologous, thus indicating that they evolved from a common ancient system by duplication of its multiple genes followed by the respective specializations.

Several stages of this import process have also been investigated for glycosomes of *T. brucei* and *Leishmania* spp. revealing some unique features. Orthologs of all yeast and mammalian peroxins mentioned above are present in the trypanosomatids - except for PEX17 that is specific for some yeasts (Jansen et al. 2021), and PEX4 that in trypanosomes is functionally replaced by a ubiquitin-conjugating homolog (Gualdrón-López et al. 2013b). Information about structures of all these trypanosomatid peroxins, their subcellular localization and interactions, and their essentiality for proper glycosome biogenesis and viability of trypanosomes have been described in various reviews (Galland et al. 2010; Bauer & Morris 2017; Crowe & Morris 2021) and the original publications cited in them. However, much remains to be investigated about their precise functioning in the import cycle. Here follow a few highly intriguing aspects that deserve special interest.

Concerning the docking complex: We previously identified in the TriTryps parasites two PEX13 isoforms, denoted PEX13.1 and PEX13.2, which both share very low sequence identity with the single PEX13 identified in peroxisomes and differ also markedly between each other (Verplaetse et al. 2009; Brennand et al. 2012a; Verplaetse et al. 2012). Most strikingly, PEX13.1 contains at its cytosolically exposed C-terminus a typical tripeptide PTS1 motif, conserved in the TriTryps parasites but not found in any other PEX13, whereas PEX13.2 lacks the SH3 domain that is found in all other PEX13s. Both PEX13 isoforms were located in the docking complex where they interact with each other, and both also with PEX14. RNAi depletion showed that the two isoforms are both essential for glycosomal matrix protein import and are not redundant. Morris and coworkers further studied PEX13.2 and provided strong indications for its role as an accessory protein for import of specifically PTS2 proteins, whereas depletion of PEX13.1 had been shown to affect import of both PTS1 and PTS2 proteins. Moreover, they demonstrated that knockdown of PEX13.2 also resulted in fewer, larger glycosomes. Furthermore, by two-dimensional Blue-Native gel electrophoresis they revealed the existence of three glycosome membrane complexes, differing in size and containing different combinations of PEX13.1, PEX13.2, and PEX14. Additionally, by superresolution microscopy it was shown that PEX13.2 localizes to subdomains of the glycosome periphery, indicating that the membrane is not homogenous (Crowe et al. 2020). Future studies are required to unravel further the intriguing role of the unique PEX13.2 in trypanosomatids, and that of the PTS1 motif at PEX13.1. Additional investigation should also reveal if each of the different PEX13/PEX14 complexes is functional in the import process or if they represent different stages in the assembly of a functional complex.

About the translocation step: Whether two separate transient pores, as found in yeast peroxisomes, are also formed in other organisms is uncertain. While in yeast the PTS1 and PTS2 import pathways are separate, although both involving PEX14, in mammals the two pathways converge at PEX5 when the PTS2-PEX7 complex binds to a long isoform of this PTS1 receptor. Strong indications have been obtained that also in trypanosomes PEX7 binds to PEX5 (Galland et al. 2007), but this remains to be proved and the molecular mechanism of the translocation step in trypanosomes to be determined.

Import signals of matrix proteins

Two kinds of targeting motifs have been recognized in peroxisomal and glycosomal matrix proteins, a C-terminal tripeptide PTS1 and a nonapeptide PTS2 close to the N-terminus. The sequences of the PTS motifs are not conserved, but rather the physicochemical properties of the residues at different positions to allow binding to the respective receptors, PEX5 and PEX7. But the targeting efficiency of different PTS1 motifs differs importantly, as demonstrated by the variable in vivo glycosomal uptake of marker proteins in which all possible substitutions

of each residue of the C-terminal tripeptide had been made (Blattner et al. 1992; Sommer et al. 1992). Moreover, important differences were noticed between the efficiency by which some tripeptides target proteins to glycosomes compared to mammalian and yeast peroxisomes.

Several questions about targeting of proteins to the glycosomal matrix remain to be solved. First, the number of matrix proteins with a PTS1 is considerably larger than that having a PTS2, and strikingly, also many matrix proteins don't possess a consensus targeting motif. In a high-confidence proteome of glycosomes from procyclic (PCF) *T. brucei* pulled down using a GFP-tagged membrane-located PEX13.1, out of 158 proteins identified as glycosomal only 60 possessed a PTS (Güther et al. 2014). Furthermore, a surprising number of 77 PTS-containing proteins were not detected in the anti-GFP pulled-down fraction, and therefore were considered likely 'contaminants'. These results led the authors conclude that use of PTS sequence searches to identify glycosomal proteins of *T. brucei*, while useful, has a sensitivity of only <40% and a specificity of <50%. However, it could not be excluded that at least some of the proteins identified as 'contaminants' are (predominantly) cytosolic in PCF cells but (to some extent) imported into glycosomes in other life-cycle stages. Notably some PPP enzymes were labelled as 'contaminant', although it is known from other studies that part of their activity can be detected inside glycosomes, but the extent of compartmentalization may vary between life-cycle stages (Kovářová & Barrett 2016). Remaining questions are thus: How are proteins without consensus PTS sequence imported and why are many proteins with a PTS not, or only to a limited extent imported into glycosomes? Noteworthy is that a discrepancy between the presence or absence of predicted PTS motifs and the peroxisomal proteome has been reported for many organisms, for example another parasitic protist, *Entamoeba histolytica*, based on a study also involving affinity-purified organelles (Verner et al. 2021).

Peroxisomal and glycosomal import of proteins without consensus PTS motif may occur by different means (reviewed by Van der Klei & Veenhuis 2006). One possibility, shown for peroxisomes but not (yet) for glycosomes, is that such motif is recognized by import receptor PEX5 or PEX7, either at its common ligand-binding site and/or elsewhere at the receptor's surface (Kempiński et al. 2020). Another option is piggybacking on proteins containing a PTS, as has been shown for several peroxisomal proteins (e.g. Gabay-Maskit et al. 2020). This has recently also been demonstrated for a *T. brucei* glycosomal enzyme (Villafraz et al. 2021). UDP-glucose pyrophosphorylase, an enzyme involved in nucleotide-sugar synthesis, is imported due to binding to the phosphoenolpyruvate carboxykinase that possesses a PTS1.

A second issue is that for some enzymes activity is required in both the glycosomes and cytosol, whereas in other cases complete compartmentalization is essential. For example, for several glycolytic enzymes in BSF *T. brucei*, dual localization is toxic for the cell, because mechanisms that control their activity within glycosomes do not operate in the cytosol (Blattner et al. 1998; Haanstra et al. 2008; Haanstra et al. 2014). When dual localization is required, it may be due to expression of different isoenzymes, with and without PTS, like in the case of GAPDH and phosphoglycerate kinase in *T. brucei* (Michels et al. 1991; Osinga et al. 1985), or the partial compartmentalization of enzymes as observed for several PPP enzymes (Kovářová & Barrett 2016). The import efficiency of different PTS sequences (Blattner et al. 1992; Sommer et al. 1992) probably affects if enzymes are fully compartmentalized in glycosomes or have a dual localization. However, the lack of correspondence between the strength of a PTS and the extent of import shows that other factors must be involved as well and deserves to be investigated. Possibilities are (i) residues upstream of a PTS1 modulate the affinity of the protein to be imported for PEX5; analysis of interactions between PEX5 and PTS1-containing proteins from different organisms showed that indeed residues further upstream within the C-terminal dodecamer may accommodate the contact (Brocard and Hartig 2006); (ii) the concentration of enzymes in the cytosol, resulting from their rate of synthesis and turnover;

(iii) additional interaction sites between a PTS-containing protein and its import receptors that affect the efficiency; (iv) involvement of regulatory molecules and posttranslational modifications; (v) import is a rate-limiting step in glycosome biogenesis; and (vi) there exists competition for import. Furthermore, it is unknown if an apparent full uptake is also due to proteasomal degradation of any protein erroneously retained in the cytosol to avoid toxic effects.

A third question concerns the correct formation of pathways within glycosomes with enzymes having different targeting signals. The molar ratios of glycolytic enzymes within glycosomes of BSF *T. brucei* differ, in part reflecting their catalytic constants. The concentration of enzymes with high k_{cat} like triosephosphate isomerase (TIM) is low, enzymes with low k_{cat}, like aldolase and GAPDH, high (Aman et al. 1985; Misset et al. 1986; Bakker et al. 1995). The glycosomal concentration of most glycolytic enzymes is strongly decreased in PCF trypanosomes, although to different extents (reviewed in Szöőr et al. 2014). How are these concentrations established? Some enzymes have a PTS1 (e.g. GAPDH), each with its distinct import efficiency, others a PTS2 (e.g. aldolase) and one doesn't have a consensus PTS (TIM) and enters probably by piggybacking (Galland et al. 2010). One may wonder if, in addition to control on protein expression levels, there is also control exerted on the import of the different enzymes, having their distinct signals and following different routes, so that they become present in a correct molar ratio within the organelles. This question pertains also to other glycosomal metabolic pathways.

It should be noted that corresponding glycosomal proteins of the different TriTryps parasites may have different permutations of a PTS, or even a typical PTS in one species while a consensus motif is absent in another species.

Membrane biogenesis

Less definite knowledge – and more discrepancy in the current literature – exists about the origin of the membrane of the peroxisomes, the targeting signals of peroxisomal membrane proteins (PMPs), and the process by which these proteins are inserted into the membrane compared to the knowledge about the import matrix proteins; the current information about glycosomal membrane biogenesis is even very limited. Three peroxins have been identified for the insertion of proteins in peroxisomal membranes: the cytosolic PEX19, and two integral membrane proteins, PEX3 and PEX16, although PEX16 is absent from most yeast species where a similar function is exerted by PEX36 despite a very weak sequence similarity.

Peroxisomes can proliferate by growth of pre-existing organelles followed by fission and by de novo synthesis (Fig. 4A). In the growth/fission process, proteins destined for incorporation into the peroxisomal membrane are recognized through their membrane peroxisomal-targeting signal (mPTS) that shows little sequence conservation but is characterized by a cluster of basic residues sometimes mixed with some hydrophobic ones and having one or two adjacent transmembrane segments (Mayerhofer 2016). This mPTS is recognized by PEX19 that also serves as a chaperone to stabilize the membrane protein in the cytosol. The cargo-loaded PEX19 docks at PEX3 in the membrane, followed by the insertion of the PMP. This process occurs without requirement for ATP. In the current model of the de novo process, PEX16 is first co-translationally inserted into special domains of the ER membrane and then recruits PEX3 and a variety of other PMPs, including PEX11 isoforms which are responsible for membrane elongation and, by attracting appropriate factors, indentation and fission (Fig. 4A and 4B, route I). As a result, pre-peroxisomal vesicles are formed that can attract additional PMPs in a PEX3/PEX19-dependent manner as well as import matrix proteins and so mature into peroxisomes. Minor variations of this model have been described, based on data obtained about peroxisomal membrane biogenesis in different organisms (for reviews, see Mayerhofer 2016; Hua & Kim 2016; Akşit & Van der Klei 2018;

Mahalingam et al. 2021). In mammalian cells, PEX3 can also insert directly in peroxisomes in a PEX16-PEX19 dependent manner (4B, route II) (Matsuzaki & Fujiki 2008), but future research should still assess the relative importance of this route. We are not aware of any data indicating that PEX16 would also be able to bypass the ER to arrive in peroxisomes.

The ER may also contribute lipids for the peroxisomal membrane via the pre-peroxisomal vesicles. Recent research revealed the existence of physical and functional contacts between peroxisomes and other organelles besides the ER, notably mitochondria (Fransen et al. 2017; Shai et al. 2018), and an involvement of mitochondria-derived vesicles for de novo peroxisome biogenesis was also demonstrated (Sugiura et al., 2017; Kim 2017). This may also offer an explanation for the mitochondrial membrane proteins regularly found associated with peroxisomes (Chornyi et al. 2021).

Preliminary studies of glycosomal membrane biogenesis have been performed. Trypanosomatid orthologs of PEX19, PEX16 and PEX3 have been identified, sharing only very low overall sequence identity with their peroxisomal counterparts: respectively about 20%, 16%, and in the case of PEX3 even only 7% (Banerjee et al. 2005; Yernaux et al. 2006; Kalel et al. 2015; Banerjee et al. 2019; Kalel et al. 2019). In addition, PEX19 was shown to be predominantly cytosolic and to interact with glycosomal membrane proteins such as ABC transporters, while also interactions between PEX19 and PEX3 and the association of PEX3 and PEX16 with glycosomes were demonstrated. Knockdown of each of these peroxins affected glycosome biogenesis and the viability of *T. brucei*. Remarkably, contrary to PEX19s in other organisms, trypanosomatid PEX19s lack a CaaX motif at the C-terminus. This motif serves for farnesylation that has been shown to be important for structural integrity and PMP recognition of PEX19 (Rucktäschel et al. 2009; Emmanouilidis et al. 2017). The consequences of the farnesylation absence in trypanosomatid PEX19 are still unknown. Future research is required to reveal the molecular mechanisms by which these three peroxins, and probably additional factors, achieve glycosomal membrane biogenesis.

Additional proteins whose involvement in glycosome membrane biogenesis has been demonstrated are PEX11 and the SNARE protein Ykt6. Many organisms have multiple PEX11 family members, resulting from lineage-specific duplications and complex evolution (Schrader et al. 2016; Jansen et al. 2021). The PEX11 family plays a role in regulation of peroxisome size and number. Among the three mammalian PEX11 isoforms, PEX11β functions in peroxisome elongation and initiates constriction and fission by recruiting proteins responsible for these processes. Less is known about the molecular roles of PEX11α and γ in this process; they don't complement loss of PEX11β. The situation in trypanosomes is reminiscent with also three family members: PEX11, GIM5A and GIM5B, with the PEX11 most likely exerting a role similar to that of mammalian PEX11β: its overexpression in *T. brucei* transformed the globular glycosomes into clusters of long tubules, while reduced expression resulted in fewer but larger organelles (Lorenz et al. 1998). In contrast, depletion of GIM5A and GIM5B caused a pleiomorphic phenotype, including cellular fragility, decreased glycosome number, and several metabolic effects some of them suggestive that these proteins may also be involved in metabolite transport (Voncken et al. 2003), reminiscent to the additional pore-forming property of PEX11 later demonstrated in yeast (Minthoff et al. 2016).

Ykt6 belongs to the family of SNARE proteins which are associated with membranes and mediate fusion events in vesicle transport between intracellular membranes. The association with membranes occurs by either a single transmembrane domain or a postranslationally added anchor. Banerjee & Rachubinki (2017), screening the *T. brucei* database for proteins having a C-terminal CaaX sequence, identified a single protein (with the motif CTVM) homologous to Ykt6 in other eukaryotes. In yeast, Ytk6 is involved in vesicle trafficking between Golgi, endosomes and vacuole, while in trypanosomes it was found to localize in part to glycosomes. Knockdown reduced the number and increased the size of

glycosomes, caused mislocalization of matrix proteins and affected viability. Therefore, Ytk6 in trypanosomes is likely involved in fusion of ER- or mitochondrion-derived pre-peroxisomal vesicles with growing glycosomes to provide lipids and membrane proteins. It seems feasible that PEX11 could exert its function in both glycosome proliferation by growth and fission and a de novo process, while the data about Ykt6 are suggestive, but no proof of de novo biogenesis. For both the members of the trypanosomatid PEX11 family and Ytk6 – and other factors involved in membrane fission and fusion still to be identified – further investigation is required to unravel these processes in glycosome biogenesis.

Not only about the molecular aspects of glycosome proliferation remain many questions, but also about the process at the cytological level. So far, no systematic study has been made if it occurs by growth followed by fission and/or by de novo biogenesis. Growth and fission is usually considered the standard route, but the existence of a functional de novo pathway is also supported by several data. First, the presence and essentiality of PEX16 in trypanosomatids, a peroxin usually produced in the ER, although in the yeast *Yarrowia lipolytica* this peroxin has also been shown to be involved in fission by recruiting dynamin-like protein Vps1 to peroxisomes. The finding that knockdown of PEX16 caused a strong reduction of glycosomes in the posterior part of the cell and less so in the anterior part suggests that the exit from the ER was affected. Second, the high rate of glycosome degradation by autophagy during differentiation of trypanosomatids (Herman et al. 2008; Cull et al. 2014) makes it more likely that, at least during developmental transitions, new glycosomes are formed de novo. Third, the involvement of the SNARE protein Ykt6 in glycosome biogenesis also supports this notion. Furthermore, close proximity, possibly interaction, of glycosomes with the ER and mitochondrion has repeatedly been observed in electron microscopy (EM) pictures of *T. brucei* and *T. cruzi* (e.g. in Opperdoes et al. 1984; Quiñones et al. 2020; Zuma et al. 2021). Probably both routes for glycosome biogenesis exist, but this remains to be proved and how their use is regulated to be determined.

Another question specifically related to the growth and division model is if fission results in two similarly sized daughter glycosomes or if it occurs asymmetrically by budding as has been described for yeasts (e.g. Kumar et al. 2018). As stated by Crowe & Morris (2021), there is currently no evidence for asymmetrical glycosome division, but it cannot be excluded by lack of data. A possible relevance of such information will be discussed in the next section.

Glycosome homeostasis is the result of biogenesis and degradation. Preliminary studies established that the organelles, like peroxisomes, are degraded by a selective form of autophagy, called pexophagy (Herman et al. 2008; Brennand et al. 2012b; Cull et al. 2014). The role of some specific autophagy-related proteins (ATG) in glycosome degradation has been demonstrated. However, in pexophagy studies with yeast and mammalian cells many more proteins – other ATGs and several peroxins – have been identified as being involved in successive stages of peroxisome degradation and their regulation (reviewed by Mahalingam et al. 2021). Such information is still almost completely lacking for glycosomes.

HETEROGENEITY OF GLYCOSOMES

Heterogeneity of glycosome morphology

Trypanosomatids possess multiple small glycosomes, with the number and cell volume occupied varying dependent on species and life-cycle stage. For example, BSF *T. brucei* have approximately 60-65 spherical organelles with an average diameter of 0.27 +/□ 0.03 µm, distributed throughout the cell, often in clusters, with the number increasing to ~120 during parasite growth up to cell division and together comprising 4-5% of the cell volume (Opperdoes et al. 1984; Tetley & Vickerman 1991; Hughes et al. 2017). In contrast, PCF *T. brucei* have

fewer glycosomes with a predominantly elongated form (Gualdrón-López et al. 2012a). In *Leishmania* spp. promastigotes and amastigotes respectively 20 and 10 glycosomes were found, heterogeneous in shape and size (Coombs et al. 1986; Cull et al. 2014). Different numbers, forms, occupied cell volume and arrangement within the cell have been reported for glycosomes in other trypanosomatid species, with also growth conditions affecting these properties (reviewed by De Souza, 2002). Interestingly, in trypanosomatids harboring a bacterial endosymbiont, glycosomes with a shape different from those in symbiont-free cells are concentrated around the prokaryote. EM analysis indicated a strong association between the organelles and symbiont, and preliminary biochemical studies also suggested a metabolic interaction (Motta et al. 1997; Loyola-Machado et al. 2017).

In EM pictures of different trypanosomatids, small appendices at glycosomes have often been observed, sometimes connecting two organelles (e.g. Opperdoes 1987; Sanchez-Moreno et al. 1992; Gualdrón-López et al. 2012a). The meaning of these structures, and how frequently they occur, is still unknown, because no EM tomography studies have been done. Could these structures be related to vesicular transport or asymmetrical division? They deserve further investigation.

Heterogeneity of glycosomal matrix content

Previously, different types of experiments provided indications for some heterogeneity of the glycosome population of *T. brucei*, *T. cruzi* and *L. donovani* regarding their content, as reviewed by Crowe and Morris (2021). This involved levels of peroxins associated with the membrane, matrix enzymes and polyP. Also, the use of a fluorescent marker protein (eYFP) with an N-terminally attached PTS2 revealed a mixed culture of unequally fluorescent PCF *T. brucei* subpopulations. The compartmentalization of the marker was dependent on environmental conditions such as glucose availability, cell density and growth stage in batch culture. Further analysis showed that glycosomes with low uptake of the marker had also reduced levels of several glycolytic enzymes and PEX13 (Bauer et al. 2013). Several of our own studies using trypanosomes expressing GFP containing a strong C-terminal PTS1 (-SKL) also revealed heterogeneity, with the signals from antisera against some glycolytic enzymes and GFP not always merging (e.g. Gualdrón-López et al. 2013b).

How heterogeneity is achieved remains to be determined. Crowe and Morris (2021) described two options, (i) by operation of multiple pathways of glycosome formation and proliferation, and (ii) by functional specialization. The first option seems highly probable if glycosomes can proliferate both by growth and fission and by de novo biogenesis involving the ER and the mitochondrion. The composition of the organelles is then dependent on the regulation of the two processes during the life- and/or cell cycle, the expression of the different glycosomal proteins at each stage, and maybe also additional regulatory factors involved in signaling environmental cues. This option does not necessarily exclude the possibility of functional specialization that will also be dependent on additional regulatory factors. However, with the currently available data, this second option is very speculative but deserves further investigation. At what stage of glycosome biogenesis will regulatory factors have to operate to achieve the observed heterogeneity, not only for authentic glycosomal proteins but also for exogenous markers? It is noteworthy that a single case of peroxisome heterogeneity by functional specialization is currently known; in filamentous ascomycetes such as *Neurospora crassa* two types with different composition and function can be found within the same cell at the same time: glyoxysomes – peroxisomes containing enzymes of the glyoxylate cycle – and Woronin bodies – peroxisomes serving as plugs of septal pores. Woronin bodies develop from some of the glyoxysomes in response to hyphal wounding to restrict loss of cytoplasm at the site of injury (Managadze et al. 2007; Liu et al. 2008).

A variation on the first option is the possibility that not all glycosomes are competent for import of matrix proteins. This has been first proposed for yeast peroxisomes by Van der Klei & Veenhuis (1997). They suggested that, during fission, functional protein-import complexes in the peroxisomal membrane are donated to newly created organelles, rendering them import competent, while the old, mature organelles lost them and became import incompetent. This model for creation of a heterogeneous peroxisome population in yeast received recently experimental support, but it requires the asymmetrical segregation of the organelles during cell division (Kumar et al. 2018). As mentioned above, no data are currently available for asymmetrical division of glycosomes. A constitutively expressed fluorescent marker with a PTS would not end up in all glycosomes if there is heterogeneity in import competence. Whether heterogeneity or selective uptake of such a marker could also be achieved by operation of multiple biogenesis routes or functional specialization remains to be determined. However, as we discussed previously, the extent of selective pressure to adapt the glycosomal metabolism when environmental conditions change might also determine if, at least transiently, a heterogeneous population of organelles arises or not (Haanstra et al. 2016).

The hypothesis of the simultaneous formation of subpopulations with different, specialized metabolic roles is interesting, but the notion that this could serve as a means of metabolic regulation should take into account the specific properties of the glycosomal membrane. For example, it seems likely that a possible compartmentalization of glycolysis and gluconeogenesis in distinct glycosomes will, by itself, not be sufficient to prevent a futile cycle caused by simultaneous activity of PFK and fructose-1,6-bisphosphatase (FBPase) when hexose-phosphate substrates and products can freely pass through pores in the membrane. Additional activity regulation of the enzymes, as occurs in other organisms, will be required. While previous research showed that glycosomal enzymes lack most of the activity regulation mechanisms commonly found in other eukaryotes, recent work provided evidence that the trypanosomatid enzymes are regulated (reviewed in Michels et al. 2021). For *Leishmania* it has been shown that AMP is a potent allosteric activator of PFK and inhibitor of FBPase (Fernandes et al. 2020; Yuan et al. 2017). However, AMP has only limited effect on *T. brucei* PFK. Whereas the *Leishmania* PFK structure favors the inactive T-state in the absence of AMP, *Trypanosoma* PFK adopts preferentially the active R-state. Maybe a unique allosteric inhibitor still to be identified operates in vivo that can push PFK into the inactive T-state. It seems probable that the activity of the enzymes is additionally regulated by posttranslational modification. FBPase is highly expressed in BSF *T. brucei*, but its activity could not be detected. Moreover, BSF *T. brucei* can perform gluconeogenesis using glycerol as substrate, but do so in an FBPase independent manner, although the enzyme was detected in the proteome (Kovářová et al. 2018; Pineda et al. 2018). The enzyme was possibly inactivated, and gluconeogenesis mediated by reversal of the PFK reaction, but alternative possibilities can as yet not be discarded.

GLYCOSOMAL MATRIX; STRUCTURAL AND METABOLIC FEATURES

Characteristic of peroxisomes and glycosomes is their high buoyant density and, observed in EM pictures, an electron dense, fine granular matrix with occasionally a crystalloid core suggestive of ordered structure (Opperdoes et al. 1984). Whereas the proteomic composition has been determined for glycosomes of different trypanosomatids, some information about the structural organization of the matrix has only been collected for glycosomes from BSF *T. brucei.* The matrix has a high protein content, with ~90% of it constituted by glycolytic enzymes (Aman et al. 1985; Misset et al. 1986), while in PCF trypanosomes these enzymes make up only 40-50% of the glycosomal protein (Hart et al. 1984). The glycolytic enzymes

seem to be assembled in a complex, as elevated salt concentrations are required to dissolve them after removing the boundary membrane with Triton-X-100. Moreover, close proximity of the enzymes was supported by their cross-linking.

This organization of the glycosomal matrix is reminiscent to that observed for the matrix of different types of plant peroxisomes having also a multienzyme complex surrounded by a membrane that, because of the presence of pores, does not form a permeability barrier for most metabolites. Kinetic analysis of the photorespiratory C_2 cycle activity in leaf peroxisomes provided strong evidence for metabolite channeling in the reaction sequence by the enzymes in the complex, without release of intermediates into the bulk phase (reviewed in Reumann 2000). However, kinetic analysis of glucose catabolism by the glycosomal complex of *T. brucei* suggested absence of channeling; the question if it occurs remains open (Aman & Wang 1986).

The existence of glycolytic enzyme complexes for metabolite channeling is already for years under debate. Interactions between pairs of such enzymes and enzyme complexes – in both cases often transient – have been described for a variety of organisms and tissues, but evidence for a functional role often limited. Channeling would require direct transfer of the metabolites between active sites of sequential enzymes, thus a stoichiometric relationship between the enzymes. Indeed, such relationships have been reported for mammalian tissues (Maughan et al. 2005; Wiśniewski et al. 2015). Interestingly, complexes of different size containing both glycolytic and gluconeogenic enzymes have been detected in human cells, with PFK and FBPase interacting directly with each other, and the complex formation controlled by posttranslational acetylation (Kohnhorst et al. 2017).

No information is yet available about the spatial arrangement of glycolytic enzymes within the glycosomal complex, and the concentration of the different enzymes suggests that their molar ratios may differ importantly (Misset et al. 1986). Future research, for example cross-linking mass spectrometry and cryo-electron tomography (O'Reilly et al. 2020; Lenz et al. 2021) could be used to determine the presence of specific interactions between multiple glycosomal proteins and large-scale organization. It could also establish if glycolytic and gluconeogenic enzymes are separate or assembled together. And how are enzymes of other processes associated with the complex? Furthermore, is the reported acetylation of glycolytic enzymes of trypanosomes related to complex formation (Moretti et al. 2018)?

Another question is how glycolysis and gluconeogenesis within glycosomes are regulated. Kinetic studies of the different enzymes had previously shown that the activity of the trypanosomatid enzymes was not affected by most physiological effectors that operate in other eukaryotes (reviewed in Bakker et al. 1995; Verlinde et al. 2001). Some forms of regulation, such as the need for product inhibition of hexokinase, appeared to be redundant because of the compartmentalization, rendering the enzyme insusceptible to risk of overactivation by the net ATP produced in the cytosol (Bakker et al. 2000; Haanstra et al. 2008). Other regulatory mechanisms were more recently discovered (reviewed in Michels et al. 2021), including the reciprocal allosteric effect of AMP on PFK and FBPase described above. Still the question arises if additional forms of regulation have so far escaped detection. Recent reports of abundant posttranslational modification of glycosomal enzymes, not only by acetylation but also phosphorylation and covalent addition of six other groups (Urbaniak et al. 2013; Zhang et al. 2020) suggest that intraglycosomal enzyme activity regulation may play a much more prominent role than supposed so far. Further research into the effects of these modifications, how they are triggered, where they catalyzed and when undone will be highly interesting.

CONCLUSIONS and PERSPECTIVES

Since their discovery in *T. brucei*, in 1977, glycosomes have been topic of intensive research. Much information has been collected about these organelles which have also been found in all other kinetoplastids and diplonemids studied. The research established glycosomes as peroxisomes that have acquired several unique aspects, most remarkably the sequestering of the major part of the glycolytic pathway. Peroxisomes form a large, highly versatile family of organelles found in representatives of all major eukaryotic lineages diversified from an 'ur-peroxisome' in the last eukaryotic common ancestor (LECA), and the glycosome is just one of the family members (Gabaldón et al. 2016). Nonetheless, still many questions remain. We have discussed several topics related to the biogenesis and morphological, structural and metabolic properties of glycosomes and open questions related to these topics (summarized in Fig. 5). Several other aspects of glycosomes, each one also with open questions, have not been addressed here and readers are referred to other papers: (i) the role of glycosomes in life-cycle differentiation (Szöőr et al. 2014; Szöőr et al. 2019); (ii) the regulation of glycosomal metabolism within the overall metabolic network of the cell (Michels et al. 2021); (iii) variations in glycosomal content and metabolism during the trypanosome's life cycle and between different species (Michels et al. 2021); (iv) the uniqueness of glycosomes – the presence of glycolytic enzymes in peroxisomes outside the lineages of kinetoplastids and diplonemids (Freitag et al. 2012).

A most important other open question is 'Why glycosomes? What has been the selective advantage for the common ancestor of kinetoplastids and diplonemids that peroxisomes acquired enzymes of glycolytic and gluconeogenic and some other pathways?' The hypothesis originally proposed, i.e., increasing the glycolytic flux (Opperdoes 1987) could be invalidated (Bakker et al. 1995; Bakker et al. 2000) and alternatives developed (reviewed in Gualdrón-López et al. 2012a). This topic will be elaborated in a different paper, taking also into account the shared peculiarities of peroxisomes and glycosomes, thus also the permeability properties of the boundary membrane of these organelles, very different from most other biomembranes. The selective advantage of these organelles for the cell should thus – at least for most of their functions – not be sought in compartmentalization of many metabolites, which can readily equilibrate across the membrane through pores, but in that of enzymes and cofactors.

A final aspect with open questions is about glycosomes and glycolytic enzymes as targets for therapeutic treatment of trypanosomatid-borne diseases. Drug target identification and drug discovery have formed a major stimulant for glycosome research during many years (Barros-Álvarez et al. 2014). Recently, breakthroughs have been achieved with the development of PFK inhibitors that cure infected mice from a *T. brucei* infection without apparent toxic effect on the animals (McNae et al. 2021) and compounds that provided proof of concept for glycosomal peroxin interactions as drug targets. Library screenings yielded compounds that interfere with the PEX5-PEX14 and PEX3-PEX19 interaction in *T. brucei* glycosomes, affecting glycosome biogenesis and viability of cultured trypanosomes with no apparent or little effect on cultured human cells (Dawidowski et al. 2017; Banerjee et al. 2021; Li et al. 2021). Will such compounds lead in due time to drugs that can be used for treatment of one or more of the human neglected diseases or veterinary diseases caused by trypanosomatids?

ACKNOWLEDGEMENTS

MG-L is a postdoctoral fellow supported by ISGlobal, Spain. We thank Prof. Michael Ginger (University of Huddersfield, UK) for stimulating discussions.

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FIGURE LEGENDS

Figure 1. Scheme of glycosomal glucose metabolism in BSF *T. brucei*. Most glucose is converted into pyruvate that is excreted from the cell. Minor excreted end products are succinate as well as alanine and acetate, produced respectively in the cytosol and mitochondrion from pyruvate (not shown). Glucose 6-phosphate (G6P) is also used as substrate in the pentose-phosphate pathway (PPP), dually located in glycosomes and cytosol, and in the glycosomal nucleotide-sugar biosynthesis (NSB). The intraglycosomal redox balance is maintained by re-oxidizing the NADH formed in reaction 8 in which the electrons are transferred to the mitochondrion by a shuttle mechanism involving a glycerol 3-phosphate/dihydroxyacetone phosphate (Gly3P/DHAP) exchange across the glycosomal membrane. Enzymes are: 1, hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, fructose-1,6-bisphosphatase; 5, aldolase; 6, triosephosphate isomerase; 7, glycerol-3-phosphate dehydrogenase; 8, glyceraldehyde-3-phosphate dehydrogenase; 9, phosphoglycerate kinase; 10, phosphoglycerate mutase; 11, enolase; 12 pyruvate kinase; 13, phosphoenolpyruvate carboxykinase; 14, malate dehydrogenase; 15, fumarase; 16, fumarate reductase. This figure and all other figures were created with Biorender.com.

Figure 2. Solute transporters and channels in the glycosomal membrane of *T. brucei*. The membrane contains three half-size ABC transporters called GAT1-3, with a topology

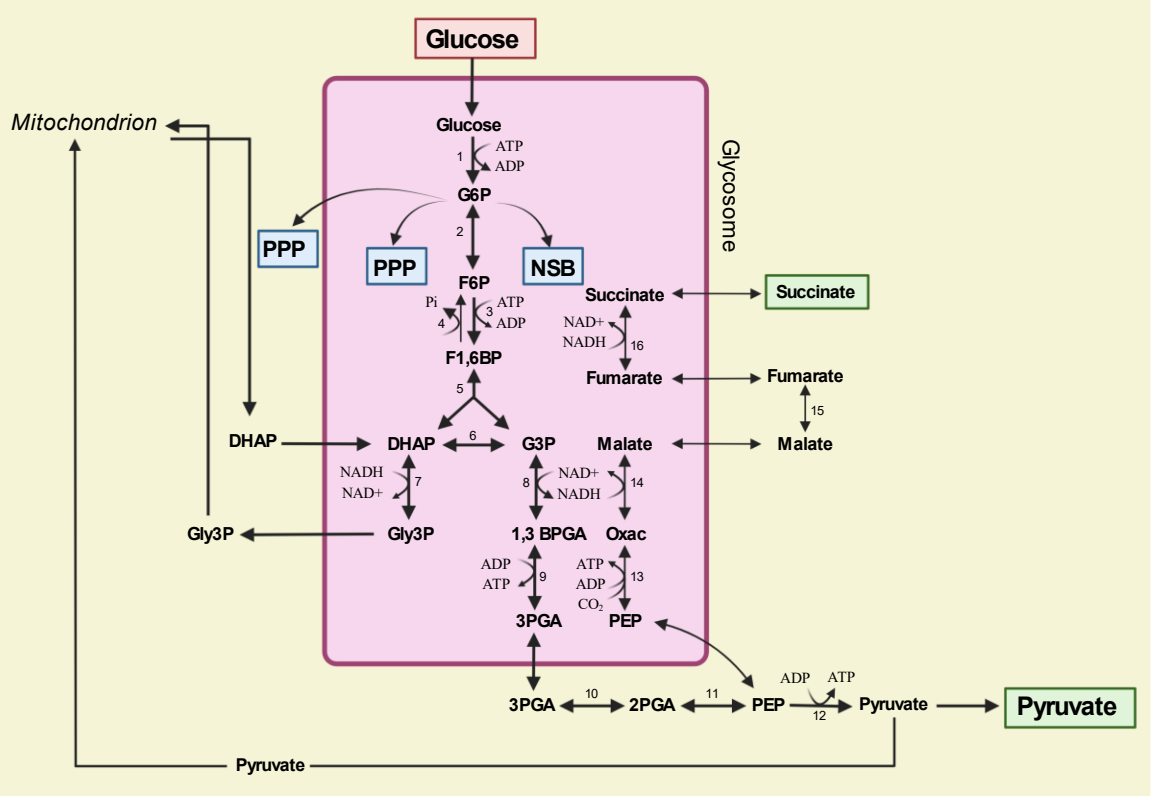
indicating they act as importers. GAT1 can transport oleoyl-CoA, whereas the substrates of GAT2 and GAT3 are still unknown. One or more putative MCF transporters may be involved in exchange of substrates across the membrane. Additionally, solubilized proteins from purified membrane preparations exhibited, upon reconstitution in lipid bilayers, channel-forming activities suggesting the existence of at least three distinct channels with low substrate selectivity. Whereas the channels are thought to allow permeation of non-bulky molecules such as glycolytic intermediates and ions, the ABC and MCF transporters are likely involved in transport of bulky substrates such as cofactors [ATP, ADP, NAD(P), etc.] and acyl-CoAs. Whether PEX11, GIM5A and/or GIM5B act as channels or transporters in the glycosomal membrane remains to be established. Abbreviations: GAT, glycosomal ABC transporter, MCF, mitochondrial carrier family.

Figure 3. The import cycle of peroxisomal matrix proteins. **A.** Five consecutive steps (I – V) can be distinguished in the cycle, each involving a set of interacting peroxins (PEXs) indicated by their Arabic numbers. The PTS1 and PTS2 import pathways are presented together, but note that after docking step (II) import of the PTS1 and PTS2 proteins into (yeast) peroxisomal matrix (step III) occurs through separate pores. Moreover, many questions remain about the retrieval and recycling of the PTS2 receptor PEX7, whereas these processes (steps IV and V) have been well studied for the PTS1 receptor PEX5. **B.** Representation of the pores involved in the transport of PTS1 and PTS2 proteins across the peroxisomal membrane in *Saccharomyces cerevisiae*. Figure modified from Montilla-Martinez et al. (2015). For a detailed explanation of the figures, see main text of the paper. Abbreviation: BD, binding domain.

Figure 4. A. Peroxisomes can proliferate by growth of pre-existing organelles followed by fission and/or de novo synthesis. **B**. Peroxisomal membrane biogenesis. For a detailed explanation of the figures, see main text of the paper.

Figure 5. Summary of open questions about glycosomes.

Figure 1


Glucose
Mitochondrion
Glycosome
Glucose
ATP
ADP
G6P
PPP
PPP
NSB
F6P
Pi
ATP
ADP
F1,6BP
Succinate
Succinate
NAD+
NADH
Fumarate
Fumarate
DHAP
DHAP
G3P
Malate
Malate
NADH
NAD+
NAD+
NADH
Gly3P
Gly3P
1,3 BPGA
Oxac
ADP
ATP
ATP
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CO2
3PGA
PEP
ADP
ATP
3PGA
2PGA
PEP
Pyruvate
Pyruvate
Pyruvate

Figure 2

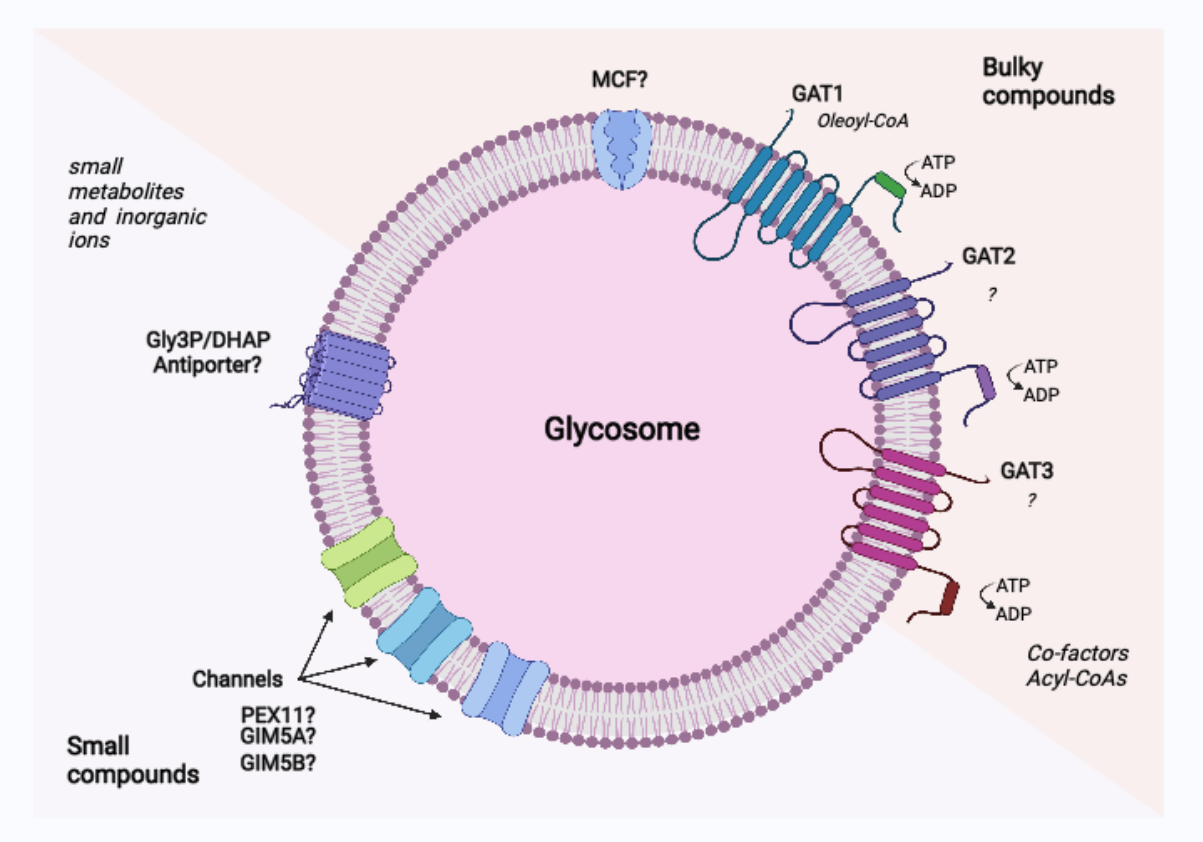

MCF?
GAT1
Oleoyl-CoA
Bulky compounds
small metabolites and inorganic ions
ATP
ADP
GAT2
?
Gly3P/DHAP Antiporter?
Glycosome
ATP
ADP
GAT3
?
ATP
ADP
Co-factors Acyl-CoAs
Channels
PEX11?
GIM5A?
GIM5B?
Small compounds

Figure 3

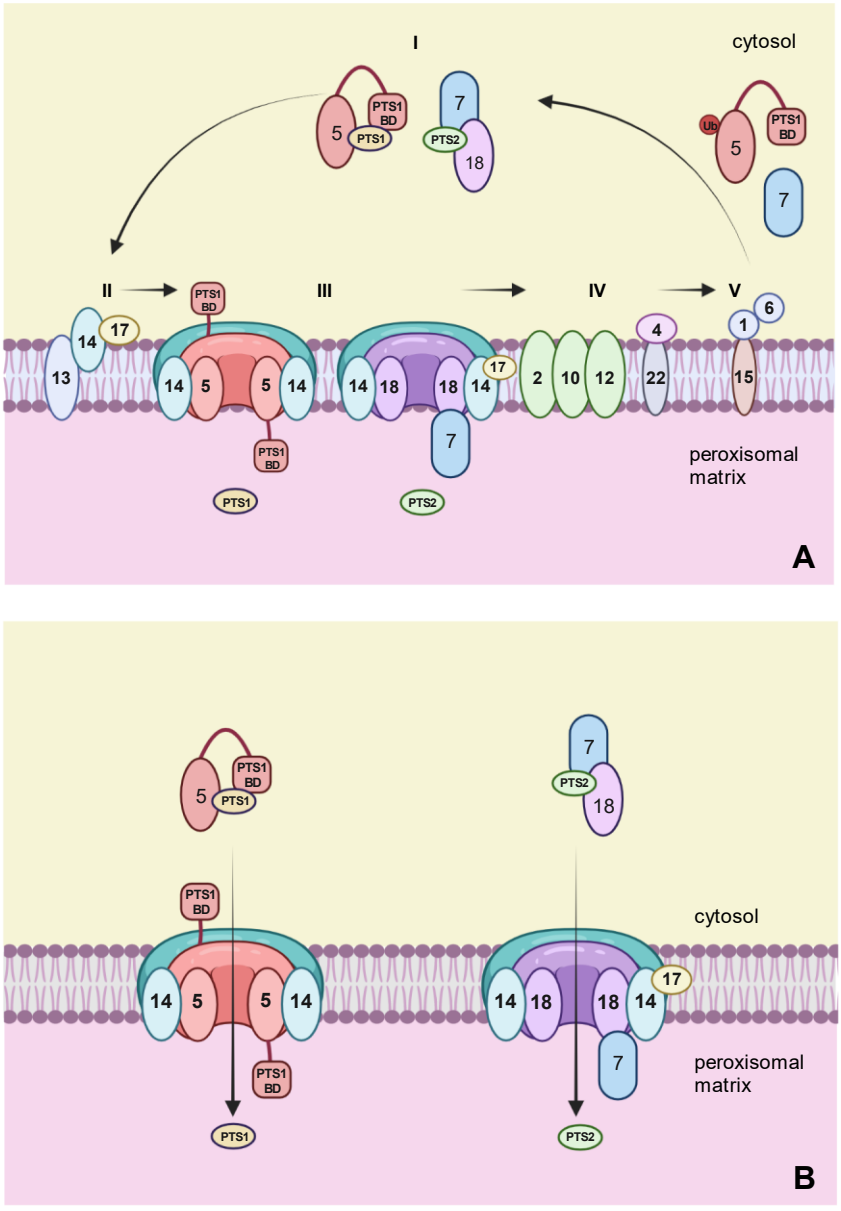
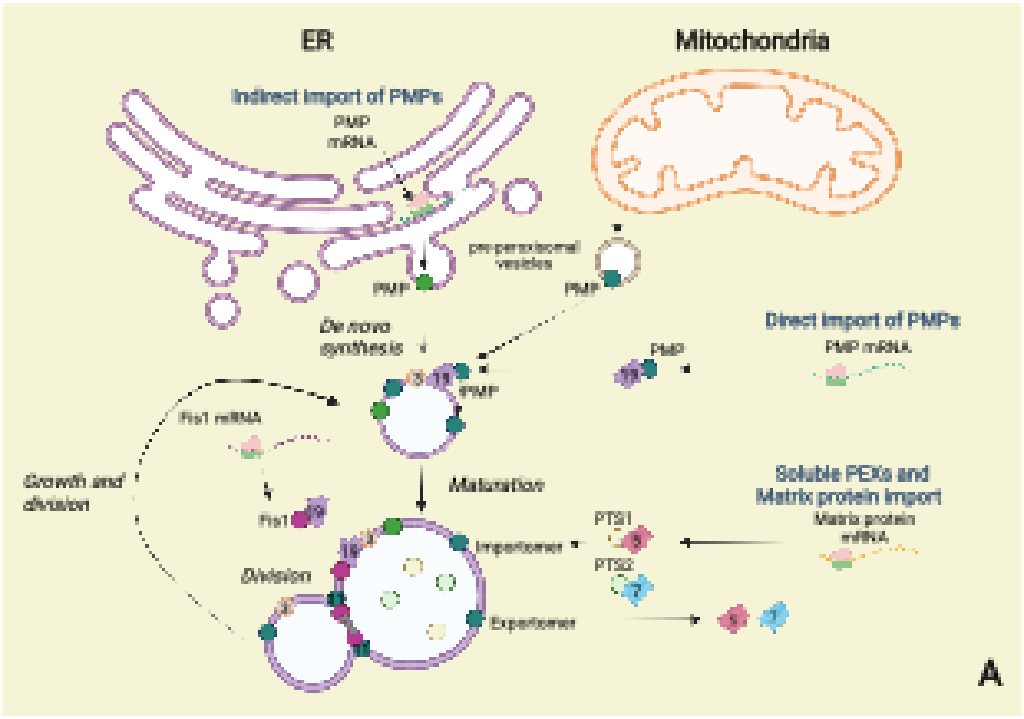

I
cytosol
PTS1 BD
5
PTS1
7
PTS2
18
Ub
II
III
IV
V
17
14
13
6
1
4
2
10
12
22
15
peroxisomal matrix
A
cytosol
peroxisomal matrix
B

Figure 4


ER
Mitochondria
Indirect import of PMPs
PMP
mRNA
pre-peroxisomal vesicles
PMP
PMP
De novo synthesis
PMP
Direct import of PMPs
PMP mRNA
Pex1 mRNA
Growth and division
Maturation
Soluble PEXs and Matrix protein import
Matrix protein mRNA
Pex1
PTS1
Importomer
PTS2
Division
Exportomer
A

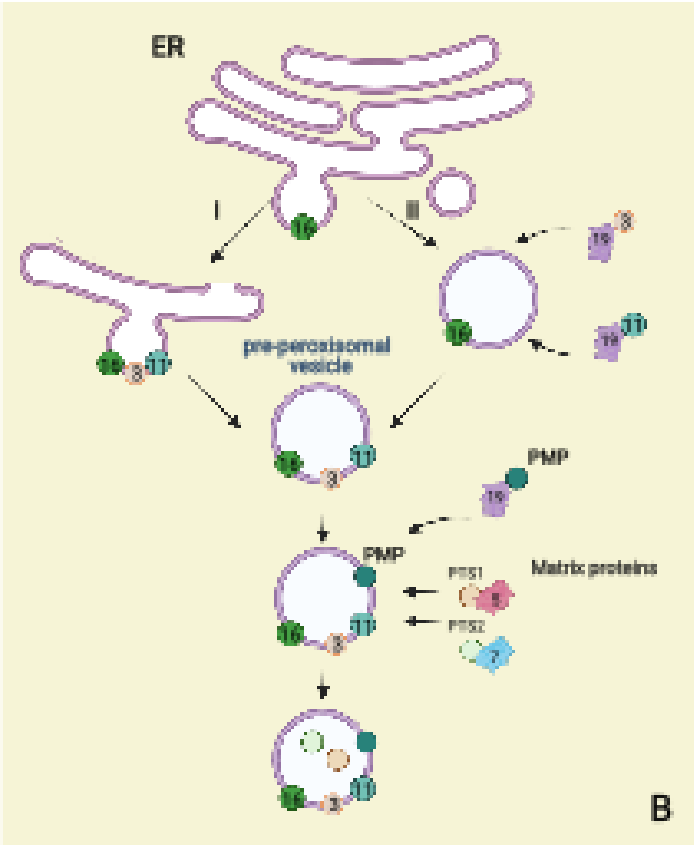
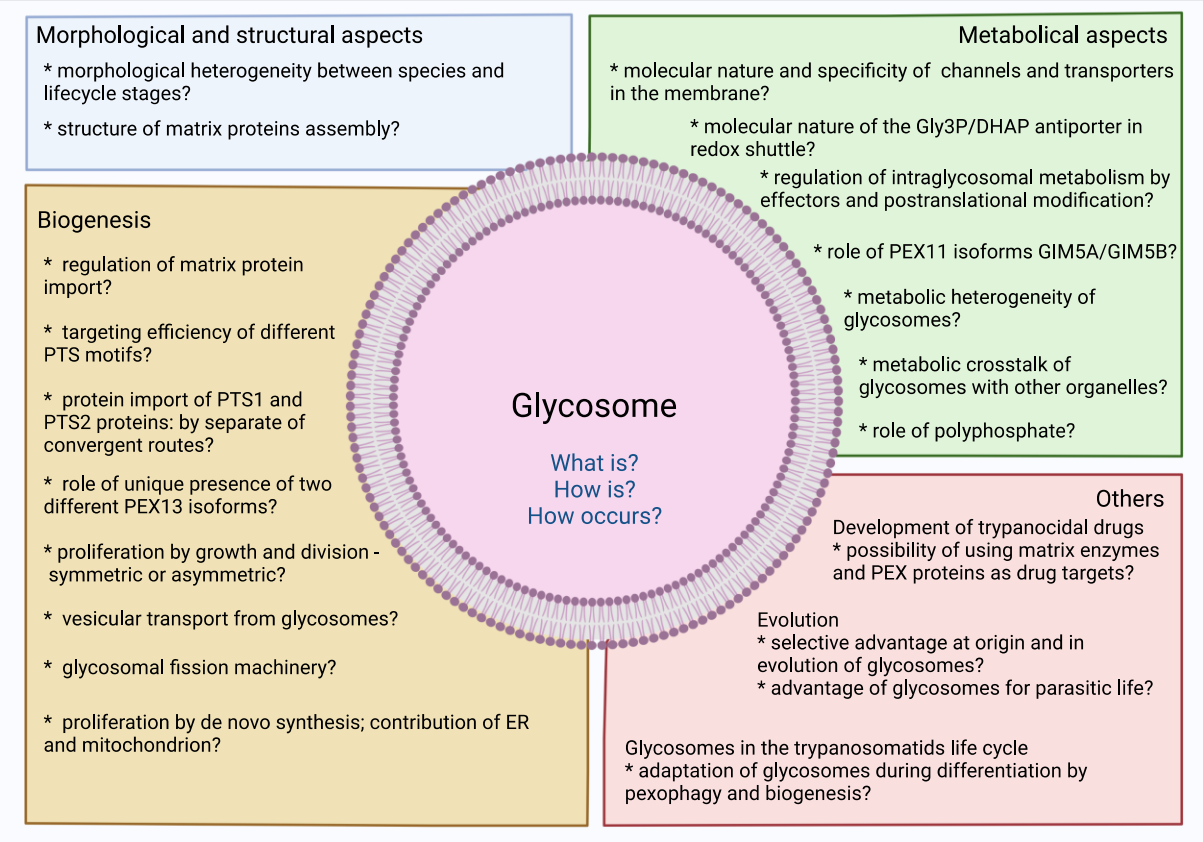

ER
I
II
pre-peroxisomal vesicle
PMP
PMP
Matrix proteins
PTS1
PTS2
B

Figure 5


Morphological and structural aspects
* morphological heterogeneity between species and lifecycle stages?
* structure of matrix proteins assembly?
Metabolical aspects
* molecular nature and specificity of channels and transporters in the membrane?
* molecular nature of the Gly3P/DHAP antiporter in redox shuttle?
* regulation of intraglycosomal metabolism by effectors and postranslational modification?
* role of PEX11 isoforms GIM5A/GIM5B?
* metabolic heterogeneity of glycosomes?
* metabolic crosstalk of glycosomes with other organelles?
* role of polyphosphate?
Biogenesis
* regulation of matrix protein import?
* targeting efficiency of different PTS motifs?
* protein import of PTS1 and PTS2 proteins: by separate of convergent routes?
* role of unique presence of two different PEX13 isoforms?
* proliferation by growth and division - symmetric or asymmetric?
* vesicular transport from glycosomes?
* glycosomal fission machinery?
* proliferation by de novo synthesis; contribution of ER and mitochondrion?
Glycosome
What is?
How is?
How occurs?
Others
Development of trypanocidal drugs
* possibility of using matrix enzymes and PEX proteins as drug targets?
Evolution
* selective advantage at origin and in evolution of glycosomes?
* advantage of glycosomes for parasitic life?
Glycosomes in the trypanosomatids life cycle
* adaptation of glycosomes during differentiation by pexophagy and biogenesis?