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A unifying hypothesis for the extraordinary energy metabolism of bloodstream *Trypanosoma brucei*

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Abstract

The parasite *Trypanosoma brucei* is the causative agent of sleeping sickness and involves an insect vector and a mammalian host through its complex life-cycle. *T. brucei* mammalian bloodstream forms (BSF) have unique metabolic features including: *i)* reduced expression and activity of mitochondrial enzymes; *ii)* intrinsically uncoupled respiration mediated by the glycerol phosphate shuttle (GPSh) and the *Trypanosome* alternative oxidase (TAO); *iii)* maintenance of mitochondrial membrane potential by ATP hydrolysis through the reversal of F_1F_o ATP synthase activity; *iv)* strong reliance on glycolysis to meet their energy demands; *v)* high susceptibility to a variety of oxidants. Here, we provide a unifying hypothesis for this unusual metabolic network and its biological significance for BSF. We postulate that strong reliance on glycolysis would minimize the use of glucose by the pentose phosphate pathway that generates NADPH to maintain reduced thiols and scavenging antioxidant defenses. To this end,

intrinsically uncoupled respiration provided by GPSh-TAO system would act as the main antioxidant defense by preventing mitochondrial superoxide production. This would reduce parasite investment in maintaining NADPH-dependent reduced thiols, sparing glucose to generate ATP by glycolysis. On the other hand, mitophagy and apoptosis-like processes would be limited by the maintenance of mitochondrial membrane potential through the reversal of ATP synthase activity. This unique "metabolic design" in BSF has no biological parallel and highlights the enormous diversity of mitochondrial processes present in trypanosomatids to adapt to distinct environments.

1. Sleeping sickness and the *Trypanosoma brucei* life-cycle

Trypanosoma brucei is the etiologic agent of sleeping sickness, also known as Human African Trypanosomiasis (HAT). The infection targets the central nervous system and causes severe neurological disorders, leading to coma and, if left untreated, death (Rodgers 2010; Kennedy, Rodgers 2019; Kennedy 2013). Throughout its complex life cycle, *T. brucei* transits between invertebrate hosts of the genus *Glossina* (tse-tse flies) and mammals (Shuster et al 2021; Marchese et al 2018). At each stage of its life-cycle, the parasite undergoes differentiation processes and faces several physical, chemical and metabolic challenges as a result of the distinct host environments (Marchese et al 2018). In order to adapt to these remarkable environmental variations, the parasite alters not only its morphology, gene expression and signaling pathways, but also its metabolism. Indeed, metabolic rewiring observed along the transition from the insect forms (procyclic, PCF) and the mammalian forms (bloodstream, BSF) is impressive and absolutely critical for parasite survival and proliferation (Zíková et al 2017; Butter et al 2012; Matthews 2005).

The mechanisms involved in energy provision may change from different cell types to meet their energy demands. In this sense, the energy metabolism network existent in trypanosomatids starkly contrasts with the predominant paradigm for eukaryotes. For example, in BSF substrate-level phosphorylation through glycolysis represents the dominant mechanism of energy provision that occurs in a unique peroxisome-derived organelle, the glycosome (Mazet et al 2013; Creek et al 2015). On the other hand, in PCF, oxidative phosphorylation within the single mitochondrion is the key mechanism of parasite ATP production.

Considering the unique energy metabolism pathways in BSF we propose a unifying hypothesis to understand how the glycosomal and mitochondrial metabolism works in a concerted way not only to regulate glycolysis in glycosomes but also to mediate respiration and regulate reactive oxygen species (ROS) production in mitochondria. Importantly, we discuss the potential consequences at cellular level of this unusual "metabolic design" in BSF that might be explored for future therapeutic interventions. To accomplish this, we will firstly present the knowledge framework on glycosomal, mitochondrial and redox metabolism to provide readers a broader picture of the

enormous complexity of the BSF metabolic network. Finally, we will present our hypothesis and discuss some future perspectives and open questions to be explored.

1.1. Glycosomes: peroxisomes turned in sugar-fueled metabolic powerhouses

Glycosomes are single-membrane-enclosed intracellular organelles found mostly in the Kinetoplastida group. Glycosomes are oval-shaped electron dense structures enclosed by a single membrane that constitute approximately 4% of the cell volume in trypanosomatids (Opperdoes, Borst 1977; reviewed in Allman, Bringaud 2017). When glycosomes were first evidenced in BSF, they were described as microbody-like organelles, bounded by a single lipid-bilayer membrane and heterogeneous in morphology. In 1977 Opperdoes and Borst evidenced in BSF the compartmentalization of the enzymatic activity of the first seven glycolytic enzymes (from hexokinase, HK, to phosphoglycerate kinase, PGK) and the other two involved in glycerol metabolism (glycerol-3-phosphate dehydrogenase, G3PDH, and glycerol kinase, GK) (Opperdoes, Borst 1977). However, it was later shown that glycosomes are not just organellar microbodies with glycolytic enzymes. Initially, glycosomes were seen as peroxisome-like organelles but this association was not obvious since the organelle found in *T. brucei* was devoid of catalase or oxidase activity involved in hydrogen peroxide (H_2O_2) metabolism. Despite that, after catalase activity was found in glycosomes in other Kinetoplastidea organisms such as in the bodonid flagellate *Trypanoplasma borelli*, this association was defined (Deschamps et al 2011; Opperdoes et al 1988).

Despite the glycosomes turned out to be authentic peroxisomes, they have certain peculiarities (Gabaldón et al 2016; Gualdrón-López et al 2012). Subsequent studies demonstrated the presence of the organelle in other kinetoplastid organisms and in Diplonema, but not in Euglenida. This indicates that glycosome development was a feature that arose in one of the common ancestors of the two Euglenozoa subclades (Reviewed in Gualdrón-López et al 2012; Gabaldón et al 2016). Glycosomes compartmentalize key metabolic pathways such as most of the reactions of glycolysis, pentose phosphate pathway (PPP), purine salvage pathway, sugar-nucleotide biosynthesis and ether-lipid biosynthesis, which contrasts with most eukaryotic species (reviewed in Michels et al 2021; Allmann, Bringaud 2017; Gualdrón-López et al 2012; Opperdoes 1987). Beyond its compartmentalization, other peculiarities of glycolysis in *T. brucei* include the lack of regulation of HK and phosphofructokinase (PFK) by their reaction end-products (Bakker et al 2000). In other eukaryotes, this lack of regulation could imply the acceleration of the glycolytic flux by the feedback occurring when the ATP produced as an output is used as a substrate by the same pathway. This would cause the accumulation of phosphorylated intermediates of glycolysis, restricting the access of the energy invested in these high-energy bonds (HEB) to other cellular energy demands. This type of buildup is known as the "turbo design" of glycolysis (Haanstra et al 2008). However, this risk is mitigated in trypanosomatids by splitting the glycolytic pathway in two distinct compartments: most taking place within the glycosomes and some in the cytosol. Glycosome membranes are permeable to solutes with molecular masses below 400 Da (Quiñones et al 2020). This means that there are no free ATP/ADP/AMP exchanges between the glycosome and the cytosol. Since in glycosomes two ATP molecules are invested per mol of glucose from HK to PFK reactions and only two ATPs are produced by the PGK reaction, the net ATP production is zero. The further two ATP molecules from glycolysis are produced in the cytosol by the pyruvate kinase (PK)

reaction. As a result, a balance of ATP consumption is established by limiting the reaction rate by the glycosomal ATP pool, preventing intermediates with HEBs from accumulating in excess due to the turbo glycolysis (Bakker et al 2000; Clayton, Michels 1996; Opperdoes 1987; Visser et al 1981).

In *T. brucei,* glycosomes have different enzymatic content and play distinct roles along parasite life-cycle (Hart et al 1984). For example, in PCF the first six reactions of glycolysis take place within the glycosomes, while in BSF the first seven enzymes are found within this organelle (Misset et al 1986; Hart et al 1984). Another feature of glycosomes is the selective permeability to solutes including NAD^+/NADH and adenylates, which do not cross glycosomal membranes (Hammond et al 1985). Therefore, specific mechanisms are required to maintain proper redox (NAD^+/NADH) and energy (ATP/ADP) balance within glycosomes. As a consequence of the glycosomal heterogeneity along parasite life-cycle, the main metabolic end-products are also different in distinct parasite forms: while in BSF pyruvate represents the major excretion product, PCF mostly produce acetate and succinate. Succinate fermentation is a consequence of a specific fumarate reductase activity found within glycosomes to recycle NAD^+ to sustain glycolysis (Besteiro et al 2002). Although fumarate reductase activity was postulated to exist only in PCF (Besteiro et al 2002), a small but consistent succinate, acetate, and alanine production from glucose metabolism in BSF was reported (Mazet et al 2013). Thus, massive production and excretion of pyruvate as the metabolic product of glycolysis strengthens the critical role of the glycerol phosphate shuttle (GPSh) to regenerate glycosomal NAD^+ in BSF, as will be later discussed.

1.2. Reduced mitochondrial functionality as a way to maximize glycolysis and minimize oxidant production

Over the years, the classical paradigm of mitochondria as the key organelles in providing cellular ATP has been extended to a myriad of other processes beyond energy metabolism (Kowaltowski, Oliveira 2019). Today, mitochondria must be seen as key organelles directly involved in a diverse of cellular events including differentiation (Chen et al 2003), growth (Son et al 2013), and signaling (Zhang et al 2010), as well as in the pathogenesis of numerous human diseases (Betarbet et al 2000; Narendra et al 2010). For example, we know that superoxide and other ROS are natural by-products of mitochondrial metabolism, playing central roles in cell physiology (Boveris, Chance 1973; Boveris et al 1972).

Although the discussion about programmed cell death in unicellular organisms remains open, there is no doubt about the role of mitochondria as one of the main actors in the cell death process. Trypanosomatids do not have the classic caspases described in the 1990s in mammals (Tsuji et al 1977, Thornberry et al 1992, Yuan et al 1993). However, it does have a group of proteins called metacaspases, described as ancestors of the caspases of multicellular organisms, preserving typical domains found in caspases, as well as similarity in tertiary structure (Minina et al 2017, Welburn et al 2006, Ameisen et al 2006, Meslin et al 2011, Kaczanowski et al 2011). The process of apoptosis-like cell death in pathogenic trypanosomatids involves the appearance of typical markers of classical apoptosis in higher eukaryotes, including DNA fragmentation, PS externalization, loss of $\Delta\Psi_m$ and cytochrome c release and formation of the mitochondrial transition pore (Ameisen et al 1995; Debrabant et al 2003; Das et al 2001; Duszenko et al 2006; Menna-Barreto 2019, Morciano et al 2021, Dewar et al 2018, Bustos et al 2017). Therefore, we

can safely assume that there is sufficient evidence to state that mitochondria play an important role in the cell death process in trypanosomatids.

The body of knowledge generated over the years on *T. brucei* mitochondria is quite relevant and comprises 1159 original papers from 1964 to 2022 (Figure 1). This represents ~49% of papers published in all trypanosomatid species, indicating that *T. brucei* is a true model organism for mitochondrial research in trypanosomatids. However, the share of papers dealing with BSF stages falls to just 302, representing only ~26% of the knowledge on *T. brucei* mitochondria. This clearly indicates that we have a very limited understanding of the mitochondrial processes in BSF stages. Despite this, the knowledge accumulated so far provides us with a very interesting scenario that challenges those interested in understanding energy metabolism in these exquisite parasite forms.

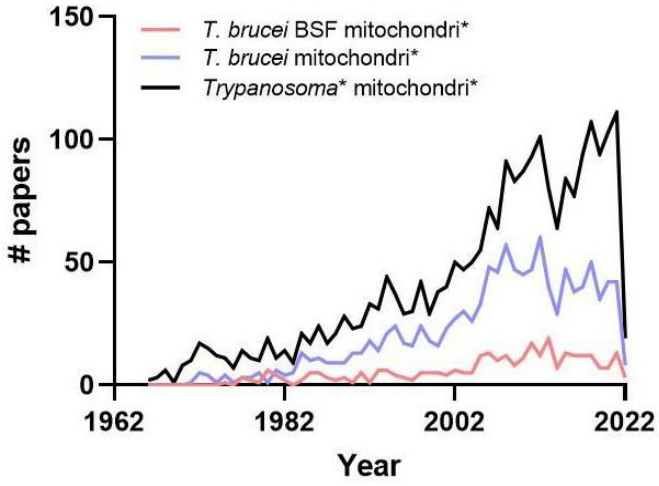

Figure 1. Evolution of knowledge on mitochondrial processes in trypanosomatids and *T. brucei*. Timeline of original publications of mitochondria between 1964 and 2022 in trypanosomatids (black line), *T. brucei* (blue line), and BSF (red line).

Although the natural diversity of mitochondrial morphologies, interactions with other organelles and functions are enormous, some aspects in trypanosomatid mitochondria are unique or are much more represented than in other organisms and includes: *i)* the presence of a single and branched mitochondrion along all parasite life-cycles (Hecker et al 1972); *ii)* the presence of kinetoplast, a structure that holds the mitochondrial genome comprised by a compacted network of the so-called kinetoplastid DNA (kDNA). The kDNA consists of a set of 25-30 pieces of circular DNA of 25-50 kbp, and some 30,000 pieces of circular DNA of approximately 1 kbp. The mitochondrial genome codes for some mitochondrial proteins as well as rRNA (Gluenz et al 2011; Ogbadoyi et al 2003). *iii)* the mechanism of mitochondrial mRNA editing, adding another layer of regulation of gene expression (Benne et al 1986); *iv)* the absence tRNA coding genes in mitochondrial genomes, requiring the mitochondrial import of tRNA (Hancock, Hajduk 1990); *v)* the remarkable heterogeneity of protein complexes involved in the electron transfer system and ATP synthesis (Surve et al 2012; Clarkson et al 1989).

From the functional side, several mitochondrial processes are drastically altered along trypanosomatid life forms, including the activities of tricarboxylic acid (TCA) cycle, the electron transfer system (ETS) which directly impacts mitochondrial membrane

potential ($\Delta\Psi_m$) and protonmotive force (*pmF*), Ca^{2+} metabolism, and ATP synthesis (Docampo, Vercesi 2021; Gonçalves et al 2011; Zíková et al 2017; Priest, Hajduk 1994; Bringaud et al 2021). For example, *T. cruzi* epimastigotes have fully functional TCA cycle, ETS and OXPHOS, which maintains *pmF* to allow mitochondrial ATP production (Gonçalves et al 2011, Barisón et al 2017). Mitochondrial ROS production in *T. cruzi* epimastigotes is low compared to bloodstream trypomastigotes. Importantly, mitochondrial ETS remodeling would favor electron leakage and mitochondrial hydrogen peroxide (H_2O_2) production in bloodstream trypomastigotes making these parasite forms resistant to redox challenges (Gonçalves et al 2011).

Regarding the *T. brucei*, it is long known that BSF exhibits remarkable alterations of mitochondrial functionality and morphology compared to PCF (Vickerman 1965; Bílý et al 2021). In this regard, high-resolution 3D reconstruction of *T. brucei* revealed that mitochondria in PCF are reticulated structures with numerous disk-like cristae which occupy a higher volume than in tubular shaped organelles in BSF (Bílý et al 2021). In addition, BSF mitochondria have multiple small cristae which occupy ~10 times less volume than cristae from PCF mitochondria (Bílý et al 2021). Considering that cristae represent the fundamental bioenergetic unit of mitochondria (Wolf et al 2019), cristae ultrastructure indirectly reflects mitochondrial energy metabolism.

Indeed, in *T. brucei* PCF TCA cycle, ETS and OXPHOS are fully functional, which contrasts with strong reductions in these metabolic pathways in BSF (Priest, Hajduk 1994; Njogu et al 1980, Clarkson et al 1989; Doleželová et al 2020; Smith et al 2017). Noteworthy is the fact that such limited OXPHOS is not directly related to the absence of a functional ETS, but rather to a dramatic reduction and change of its components. Indeed, BSF ETS is essentially carried out by a reduced form of electron transfer which does not involve cytochromes but two critical components: *i)* the glycerol phosphate shuttle (GPSh) composed of glycosomal and mitochondrial glycerol 3 phosphate dehydrogenases (G3PDH) (Škodová et al 2013; Opperdoes et al 1977); *ii)* the *Trypanosoma* alternative oxidase (TAO), which bypasses the electron flux through Complex III-cytochrome *c*-Complex IV path (Clarkson et al 1989; Chaudhuri et al 1995). The GPSh-TAO system thus allows complete oxygen reduction to water through the redox cycling of ubiquinone without contributing to proton translocation across mitochondrial inner membrane (Clarkson et al 1989; Opperdoes et al 1977). Therefore, as the energy from redox reactions of the ETS through the GPSh-TAO system is not conserved as *pmF* ($\Delta\Psi_m$ and ΔpH), we think this represents the case of an intrinsically uncoupled respiration. Intrinsically uncoupled respiration distinguishes from inducibly uncoupled respiration as seen in brown adipocytes under thermogenic stimuli. Inducibly uncoupled respiration in brown fat is a reversible phenomenon that is mediated by uncoupling protein 1 (UCP-1) which under certain signals divert the energy of *pmF* from the ATP synthase causing massive proton leak, reduction in OXPHOS efficiency and promoting heat production (Hittelman et al 1969; Heaton et al 1978). However, brown adipocytes do not uncouple their mitochondria all the time and under basal (non-thermogenic) conditions, mitochondria can synthesize ATP at fairly high rates (Benador et al 2018). Thus, the energy from *pmF* can be used either to generate ATP (coupled) or heat (uncoupled) depending on the stimuli and UCP-1 activation. In this regard, intrinsically uncoupled respiration, as is the case of AOX, cannot harness energy from electron transfer to proton translocation due to the inherent structural nature of its components.

As a result of the non-conservative nature of respiration in BSF, one might expect that protons would not be accumulated at the intermembrane space. However, a completely unexpected finding revealed that BSF indeed maintains $\Delta\Psi_m$, not by the activity of the ETS complexes, but rather by the reversal of F_1F_o ATP synthase activity (Nolan, Voorheis 1992). To accomplish this, F_1F_o ATP synthase hydrolyses ATP to allow proton translocation across the mitochondrial inner membrane towards the intermembrane space. The *pmF* generated by reversal of F_1F_o ATP synthase is close to 130-150 mV, (Vercesi et al 1992, Nolan, Voorheis 1992), which seems to allow the transport of ions and metabolites as well as nuclear-encoded proteins across the mitochondrial inner membrane (Bertrand, Hajduk 2000). Therefore, given the unique features of ETS and F_1F_o ATP synthase in BSF, we will describe in further detail below each one of these components.

1.2.1. Glycerol phosphate shuttle as a redox bridge linking glycosomes and mitochondria

The central importance of GPSh to BSF deserves a closer look on the general roles of this metabolic pathway in other organisms. In most eukaryotic cells, GPSh directly regulates cellular redox balance by two interconnected ways: first, by providing a mechanism for cytosolic NADH oxidation to NAD^+ to maintain glycolysis and serine biosynthesis; second, by transferring cytosolic reduced NADH potential to mitochondria. GPSh is a very simple system and is composed of a cytosolic (NAD-dependent, cG3PDH) and a mitochondrial (FAD-dependent, mG3PDH) glycerol 3 phosphate dehydrogenase (Mráček et al 2013). The activity of GPSh involves the cytosolic reduction of dihydroxyacetone phosphate (DHAP) to glycerol 3 phosphate (G3P) by cG3PDH using NADH as the electron donor and generating NAD^+. Then, G3P is oxidized back to DHAP by mG3PDH transferring the electrons to FAD and ultimately to ubiquinone at the mitochondrial inner membrane. In this regard, GPSh represents a critical metabolic hub that interconnects glucose and lipid metabolism, as well as respiration and mitochondrial ATP production. Indeed, glycolysis is one of the key metabolic sources of DHAP for GPSh and G3P can be converted to DHAP by mG3PDH which can be used as a precursor for gluconeogenesis. On the other hand, G3P is a necessary component for phospholipids and triacylglycerol production (Mráček et al 2013).

Mammalian mG3PDH has been known for decades (Green 1936) it has ~74 KDa and is one of the simplest components of the entire ETS (Mráček et al 2013). The activity of mG3PDH is directly linked to respiration and mitochondrial ATP synthesis in a way that electron transfer occurs through a TCA cycle and complex I independent route. GPSh is mostly regulated at mG3PDH either through its content or by allosteric regulation by specific signals including free fatty acids and Ca^{2+} (Wernette et al 1981; Bukowiecki, Lindberg 1974). However, mitochondrial G3P oxidation is linked to other components of the electron transfer system and can indirectly be controlled by their respective regulators. For example, in *Aedes aegypti* mosquitoes mitochondrial G3P oxidation is indirectly controlled by ATP through allosteric regulation of cytochrome *c* oxidase (CIV) activity by adenylate levels (Gaviraghi et al 2019).

In most mammalian cells, the content of mG3PDH is quite low compared to cG3PDH which limits the GPSh activity. However, the role of GPSh in mammalian brown adipose tissue (BAT) is particularly relevant to note as mG3PDH was found to have the highest activity than any other tissue (Houstěk et al 1975; Ohkawa et al 1969). Importantly,

cG3PDH and mG3PDH in BAT were shown to have equivalent activities, a key requisite for a functional GPSh (Ohkawa et al 1969). Although the potential contribution of GPSh to BAT thermogenesis remains to be determined, the very nature of mG3PDH activity might give some hints about this process. In this regard, considering that mGPDH activity is not coupled to proton translocation across the mitochondrial inner membrane, energy conservation through GPSh is expected to be low (Masson et al 2017; Syromyatnikov et al 2013; Miwa et al 2003; Gaviraghi et al 2019; Soares et al 2015). Indeed, a rat model resistant to diet induced obesity is strongly associated with increased liver mGPDH expression (Taleux et al 2009). Also, in bumblebee flight muscles mitochondrial G3P oxidation is intrinsically and poorly coupled to ATP production with a strong thermogenic role especially under cold exposure (Syromyatnikov et al 2013; Masson et al 2017). The broader picture across different organisms reveals a clear association between increased GPSh function and reduced mitochondrial energy efficiency triggered by nutritional and environmental stresses.

T. brucei codes for a single glycosomal G3PDH (Systematic number Tb427.08.3530 at https://tritrypdb.org) and surprisingly two mitochondrial G3PDH sequences (Tb927.11.7380 and Tb927.1.1130), a feature only shared with *Leishmania major* (Škodová et al 2013). Tb427.08.3530 codes for a protein with 37.8KDa and 354 amino acids, which is quite similar to human cG3PDH. On the other hand, both *T. brucei* sequences are slightly smaller than mammalian mG3PDH: Tb927.11.7380 codes for a 67KDa protein with 603 amino acids with a high probability to be exported to mitochondria (Mitoprot II = 0.81); Tb927.1.1130 codes for a 66.2 KDa protein with 617 amino acids with a very high probability to be exported to mitochondria (Mitoprot II = 0.94). Importantly, despite both sequences presenting FAD dependent oxidoreductase domains, a remarkable distinction between these and mammalian mG3PDH is the apparent absence of the canonical EF-hand calcium binding motif at the C-terminal. In this regard, evidence indicates that Ca^{2+} is a potent activator of mG3PDH and promotes mitochondrial superoxide production (Wernette et al 1981; Orr et al 2012). Conceivably, the absence of Ca^{2+} regulation renders *T. brucei* mG3PDH insensitive to this cation as a mechanism to reduce mitochondrial superoxide production.

Despite subcellular localization of Tb927.1.1130 remains to be determined, proteomic analyses and ectopic expression of Tb927.11.7380 confirmed its mitochondrial localization (Guerra et al 2006). Indeed, glycosomal and mitochondrial G3PDH activities were formally identified in *T. brucei* BSF (Opperdoes et al 1977). In addition, a functional GPSh involving both gG3PDH and mG3PDH activities was demonstrated in *T. brucei* PCF and establishes a redox link between glycosomal and mitochondrial metabolism (Guerra et al 2006). Although glucose is considered the main nutrient for BSF, glycerol can not only be oxidized in a glycerol kinase and TAO-dependent manner, but also can replace glucose to support cell growth (Pineda et al 2018). In addition, gluconeogenesis and PPP are sustained by glycerol metabolism in BSF especially under glucose shortage (Kovářová et al 2018). Also, respiration supported by glycerol oxidation was insensitive to uncoupling agents or inhibition of F_1F_o ATP synthase, suggesting that collapse of $\Delta\Psi_m$ does not affect mitochondrial glycerol metabolism (Pineda et al 2018). Regarding genetic disruption of GPSh, silencing mG3PDH impaired BSF growth while causing no apparent effects on PCF (Škodová et al 2013). Interestingly, when the alternative rotenone-insensitive proxy of complex I (NADH:ubiquinone oxidoreductase, NDH2) was depleted in PCF, a compensatory increase in mitochondrial G3P oxidation was observed (Verner et

al 2013). However, the opposite is not true as mG3PDH silencing caused no apparent effects on NADH-induced respiration in PCF (Škodová et al 2013).

1.2.2. *Trypanosoma* alternative oxidase short circuits electron transfer as a preventive antioxidant defense

Alternative oxidases (AOX) are small enzymes that mediate respiration in many organisms from algae, bacteria, nematodes and even ascidians and higher plants (May et al 2017). AOX belongs to the non-heme di-iron carboxylate protein family, which is shared by many proteins including ribonucleotide reductase and others. In eukaryotes, AOX is a mitochondrial inner membrane protein facing towards the matrix side and found as a homodimer. Likewise CIV, AOX catalyzes the complete reduction of molecular oxygen to water but through unique mechanisms: *i)* AOX uses ubiquinol as an electron source rendering mitochondria cyanide-insensitive as a result of the alternative electron path that short-circuits the ETS (Chance, Hackett 1959; Clarkson et al 1989; Chaudhuri et al 1995); *ii)* the energy from electron flow is not conserved as proton motive force, and can be dissipated as heat as shown in thermogenic plants (Elthon, McIntosh 1987). The thermogenic role of AOX in higher plants has a key biological significance as the increase in temperature of the flowers induces the evaporation of compounds that attract pollinators (Wagner et al 2008); *iii)* AOX strongly associates to cellular redox balance since its expression and activity are regulated by and regulates cellular oxidant levels (Wagner 1995).

T. brucei TAO is coded by a single nuclear-encoded gene (Tb427.10.7090) that is mostly expressed in BSF. The protein product of Tb427.10.7090 has a predicted molecular mass ~37.6KDa which is within the range of AOX from other organisms. Also, *T. brucei* TAO has a very high probability to be exported to mitochondria (Mitoprot II = 0.93), which was confirmed by experimental studies (Hamilton et al 2014). The first molecular structure of AOX was determined from *T. brucei* and revealed that TAO has two iron pockets that binds molecular oxygen, which distinguishes these enzymes from classical cytochrome *a* binding at CIV (Shiba et al 2013).

Phylogenetic studies indicate that fungal AOX sequences do not cluster with plant AOX, suggesting the existence of peculiar features between these groups. Indeed, *T. brucei* TAO and fungi AOX sequences are phylogenetically related as they cluster in taxonomic distribution analyses (Luévano-Martínez et al 2020). Importantly, adenylates were shown to regulate the activity of AOX from different unicellular eukaryotes including *T. brucei* (Woyda-Ploszczyca et al 2009; Sakajo et al 1997; Luévano-Martínez et al 2020). Although the mechanism by which adenylates regulate TAO activity remains elusive, it is possible that it shares some similarities with the allosteric regulation of CIV by ATP/ADP (Sakajo et al 1997). Conceivably, *T. brucei* TAO might have specific allosteric binding sites for adenylates that would reduce (ATP) or increase (ADP, AMP) its activity depending on the energy availability and the F_1F_o ATP synthase activity (Hierro-Yap et al 2021). In this regard, the reversal of F_1F_o ATP synthase activity seems to regulate *T. brucei* TAO activity by preventing matrix ATP accumulation (Luévano-Martínez et al 2020; Hierro-Yap et al 2021).

The fact that energy flow through the GPSh-TAO respiratory system in BSF is non conservative in nature, implies a central thermodynamic question: does BSF dissipate energy as heat? If so, what are the advantages brought by thermogenesis for a unicellular

organism? A simple answer for these intriguing questions remains open and was not yet directly addressed by calorimetry studies in *T. brucei*. Thermogenesis in unicellular eukaryotes was thought to be unlikely because of their microscopic size and the fast heat diffusion between cells and the environment (Jarmuszkiewicz et al 2010). However, we think that evidence accumulated so far is strong enough to support that at least part of the chemical energy made available via GPSh-TAO can be dissipated as heat at the cellular level. Indeed, heat production was already quantified by calorimetry not only in intact brown adipocytes, but even in isolated BAT mitochondria, with sizes even smaller than *T. brucei* (Ricquier et al 1979; Amri et al 2021; De Meis et al 2012). These studies could even determine the contribution of UCP-1-mediated thermogenesis during classical stressors, underscoring the specificity of these measures. Also, a recently developed temperature-sensitive fluorescent probe (MitoThermo Yellow, MTY) allows the assessment of mitochondrial heat production at the cellular level (Arai et al 2015). Although MTY was originally designed to sense intracellular temperature changes by the extracellular challenges, MTY fluorescence can be used to quantify heat generated by mitochondrial metabolism (Chrétien et al 2018). This was elegantly demonstrated by ectopically expressing AOX in human embryonic kidney 293 cells which caused no apparent effects on respiration and heat production when cells respire through the CIV activity. However, when CIV-dependent respiration was blocked, both processes were preserved strongly indicating that the energy made available by the electron short-circuit provided by AOX is engaged in heat production (Chrétien et al 2018). Therefore, we think that unicellular eukaryotes can engage in thermogenesis as a result of either UCP-mediated uncoupling or AOX-mediated uncoupling. Nevertheless, direct assessment of mitochondrial thermogenesis by calorimetry should be carried out in *T. brucei* to unambiguously confirm this possibility.

Several lines of evidence converge to a key role of AOX in regulating mitochondrial superoxide production (Popov et al 1997; Maxwell et al 1999; Cvetkovska, Vanlerberghe 2012; Fang, Beattie 2003; El-Khoury et al 2013) and by conferring tolerance to redox insults in different organisms (Giraud et al 2008). Despite TAO has a minor role in PCF mitochondrial metabolism, when these parasite forms were stressed by redox challenges TAO expression and activity increased (Fang, Beattie 2003). Importantly, pharmacological TAO inhibition in BSF strongly induces superoxide production and protein oxidation, indicating that TAO activity has a preventive antioxidant role (Fang, Beattie 2003). Structural studies revealed that binding of H_2O_2 to TAO is stronger than with molecular oxygen, and this interaction reversibly inhibits the enzyme activity at micromolar concentrations (Yamasaki et al 2021). This indicates that redox imbalance conditions may directly affect mitochondrial and glycosomal metabolism by inhibiting TAO activity.

A final aspect is to consider *T. brucei* TAO as a potential target for HAT chemotherapy given that it has no ortholog in mammals. Indeed, several TAO inhibitors were identified over the years and with different degrees of potency and specificity (Ebiloma et al 2019). From these studies, ascofuranone was revealed to be the most potent TAO inhibitor known capable of affecting BSF respiration, ATP production and viability (Yoshisada et al 1997; Yabu et al 2003). Modulation of *T. brucei* TAO expression by genetic approaches also revealed interesting phenotypes. For example, TAO silencing strongly reduced BSF growth and respiration while rendering parasites sensitive to glycerol toxicity (Helfert et al 2001). The mechanism of glycerol toxicity lies in its inhibitory effect on ATP production by glycerol kinase which partially sustains parasite energy demand especially under ETS

blockage or glucose deprivation (Kovářová et al 2018; Pineda et al 2018). On the other hand, overexpression of TAO in PCF caused no effects on parasite growth and as expected, increased the share of cyanide-resistant respiration by two-fold (Walker et al 2005). Curiously, TAO overexpression strongly reduced the expression of CIV subunit IV and cytochrome c1 while up regulating the expression of the surface coat protein GPEET (Walker et al 2005).

1.2.3 F_1F_o ATP synthase walks backward to avoid mitochondrial degradation and cell death

Another singular aspect of BSF mitochondria is the reversal of F_1F_o ATP synthase activity, which is the mechanism responsible for proton motive force generation. This has been well documented in prokaryotes (Futai et al 1983). Curiously, BSF is a unique case in nature, as in other eukaryotes, reversal of ATP synthase is observed in conditions where the flow of electrons through the ETS is somehow limited, either by the effect of specific inhibitors or by oxygen deprivation in hypoxic/anoxic environments (Rego et al 2001; Power et al 2014).

The energy provision to sustain $\Delta\Psi_m$ by the reversal of F_1F_o ATP synthase is a critical aspect to be considered as many cellular processes would compete by the ATP. In this regard, the most likely ATP source to sustain the $\Delta\Psi_m$ by F_1F_o ATP synthase reversal is the cytosolic ATP pool generated by PK. Indeed, it has been postulated that F_1F_o ATP synthase reversal generates a protonmotive force around 190 mV (Nolan and Voorheis, 1990; Nolan and Voorheis, 1992). Beyond PK, an alternative source of ATP for this purpose was also postulated. The coupled activity between acetate:succinate CoA transferase (ASCT) with succinyl-CoA synthetase (SCS) generates acetate and ATP in BSF via succinyl-CoA production within the mitochondrial matrix (Mochizuki et al 2020). This process, called the ASCT/SCS cycle, plays a role in BSF growth as silencing ASCT reduced proliferation. However, if the mechanism by which ASCT/SCS cycle directly contributes to sustain BSF $\Delta\Psi_m$ due to generation of matrix ATP remains to be determined.

Regardless the ATP source, the $\Delta\Psi_m$ is required for several processes in BSF including: *i)* import of nuclear-encoded mitochondrial proteins (Neupert, 1997), *ii)* prevent mitochondrial permeability transition and cytochrome *c* release (leading to apoptosis-like processes and mitophagy) (Rego et al 2001), *iii)* allows the transport of ions and metabolites including Ca^{2+} (Huang et al 2013, Docampo, Lukeš 2012, Lukeš, Basu 2015) and *iv)* RNA editing (Read et al 2016, Schnaufer et al 2001). Finally, the maintenance of the basic functions of mitochondria in BSF are essential for the maintenance of the mtDNA and consequently, for the establishment of the parasite's life cycle progression. This is a critical aspect as partial (dyskinetoplastidy) or total (akinetoplastidy) loss of kDNA locks *T. brucei* in the BSF form and prevents the ability to establish proliferative infections in tse-tse flies (Schnaufer et al 2002; Lai et al 2008). Another intriguing aspect is the potential regulatory role of F_1F_o ATP synthase on TAO activity. In this sense, Luevano et al. showed that the ATPase function in BSF is responsible for maintaining safe levels of intramitochondrial ATP, since adenylates (specifically ATP in BSF) inhibit TAO activity by an as yet unknown mechanism (Gahura et al 2021; Luévano-Martínez et al 2020). This strongly indicates that new and unpredicted mitochondrial regulatory mechanisms exist in BSF and require our research effort for the future years.

1.3. Do bloodstream forms shift towards prevention instead of scavenging reactive oxygen species as a key antioxidant strategy?

Although trypanosomatids lack canonical scavenging antioxidant enzymes, they developed unique mechanisms to cope with redox insults, especially those involving polyamine and thiol metabolism (Krauth-Siegel et al 2007). In this regard, the trypanothione system is a complex redox network which plays a key protective role against oxidative insults in trypanosomatids. This system involves non-enzymatic antioxidants such as trypanothione (T(SH)2), tryparedoxin (Tpx) and ascorbate, but also antioxidant enzymes including the trypanothione reductase (TR), non-selenium glutathione peroxidase-type enzymes (Pxs) and 2-Cys-peroxiredoxins (Prxs) (Krauth-Siegel et al 2007; Tomás, Castro 2013; Diechtierow, Krauth-Siegel 2011; (Wilkinson et al 2003; Bogacz et al 2020). Under hydroperoxide exposure, Pxs and Prxs catalyze the decomposition of hydroperoxides to less reactive reduced alcohools. The oxidized Pxs and Prxs are then regenerated by the TR/T(SH)2/Tpx system in a NADPH-dependent way. Px and Prxs have distinct selectivities for ROS detoxification, since lipid hydroperoxides involve mostly Pxs (Hillebrand et al 2003; Schlecker et al 2005; Wilkinson et al 2003), while H_2O_2 and peroxynitrite (ONOO-) are preferentially detoxified by Prxs (Budde et al 2003; Tetaud et al 2001; Wilkinson et al 2003).

A key missing aspect in *T. brucei* redox biology is a clear definition of the main cellular sources and the specific sites of mitochondrial ROS production along the parasite life-cycle. Indeed, a critical limitation to address this issue is the absence of a systematic assessment of substrate preferences to sustain physiological mitochondrial superoxide production in different *T. brucei* life-forms. This is an important aspect as it is long known that mitochondria represent the dominant source of cellular oxidants (Boveris and Chance 1973). The scarce available evidence indicates that mitochondrial superoxide is produced in BSF especially when TAO inhibited (Fang, Beattie 2003; Fang, Beattie 2003). In addition, mitochondrial superoxide production in BSF seems to be lower than in PCF as determined by electron paramagnetic resonance (Fang, Beattie 2003). Regarding the specific sites of superoxide production, endogenous generation in *T. brucei* mitochondria was reported for complex I and NDH2 (Fang, Beattie 2002), as well as mitochondrial fumarate reductase (Turrens 1987). In any case, it seems that BSF are more susceptible to several oxidants including H_2O_2, hydroperoxides, heme and others than PCF (Rossi, Dean 1988; Meshnick et al 1977).

Although the mechanistic basis for the BSF redox susceptibility is not fully understood, reduced levels of endogenous antioxidant defenses seem to partly explain this trait. In this regard, like all trypanosomatids, BSF lacks glutathione reductases, thioredoxin reductases, and catalase while has very low iron superoxide dismutase activity (Kabiri, Steverding 2001; Tomás, Castro 2013; Fang, Beattie 2003). BSF exposure to H_2O_2 generated by xanthine oxidase is toxic and causes loss of parasite motility, indicating that these parasite forms have limited capacity to deal with this oxidant (Muranjan et al 1997). Curiously, depletion of mitochondrial PxIII is not lethal to BSF despite causing cardiolipin peroxidation and delayed cell growth (Diechtierow, Krauth-Siegel 2011). Also, silencing the BSF mitochondrial Prx caused no changes on redox sensitivity to H_2O_2 or on cell growth (Wilkinson et al 2003). This led to the suggestion that either mPxII or mPrx might be necessary for parasite growth and survival considering the low respiratory rates in BSF. Interestingly, silencing of mPrx in PxIII-depleted BSF caused no alterations in cell growth, strongly indicating that mitochondrial peroxidases are not

necessary for this parasite form (Bogacz et al 2020). Regarding Tpx, this protein is found as cytosolic (cTpx) and mitochondrial (mTpx) isoforms and cTpx content in BSF is 3-7 times higher than in PCF (Comini et al 2007). Importantly, cTpx silencing strongly reduced parasite growth despite the increased levels of reduced glutathione (GSH) and T(SH)2 suggesting a compensatory redox response against cTpx depletion (Comini et al 2007). Despite that, the sensitivity of cTpx-depleted BSF to extracellular H_2O_2 was significantly increased indicating that compensatory upregulation of GSH and T(SH)2 cannot provide redox protection against oxidant as efficiently as cTpx. A cautionary note is that effects were only observed in parasites with cTpx expression reduced to ~5% its original levels and for long periods of time. A similar scenario was observed in TR-silenced BSF, where arrest of cell growth and infectivity was only achieved in cells with >90% of reduction of TR expression (Krieger et al 2000).

In our view, it seems quite plausible to assume that low respiratory rates in BSF mitochondria through the GPSh-TAO system have a limited capacity to generate superoxide which, in turn, would require less investment in mitochondrial antioxidant defenses under normal conditions. However, this assumption should be balanced considering the source of oxidants to BSF (extra, cytosolic, or mitochondrial), as revealed by studies of cytosolic antioxidant defenses deletion which rendered BSF more susceptible to extracellular oxidants (Diechtierow, Krauth-Siegel 2011; Comini et al 2007; Wilkinson et al 2003). Thus, the GPSh-TAO system would represent a preventive antioxidant defense by limiting mitochondrial superoxide production in BSF.

2. A unifying hypothesis for the extraordinary BSF energy metabolism

Given the existence of unique, and apparently paradoxical, biochemical pathways in *T. brucei*, we propose a unifying hypothesis to explain the complex mechanisms involved in energy and redox metabolism for BSF growth and survival. Our proposal aims to reconcile previous observations and re-interpret novel ones in the light of specific phenomena that were not yet addressed in detail so far for BSF.

Figure 2 schematically depicts how GPSh-TAO and the F_1F_o ATP synthase work in a concerted way not only to regulate glycolysis in glycosomes, respiration and ROS production in mitochondria, but also the cellular significance for the BSF. In this regard, the preferential use of the GPSh-TAO system would have the following outcomes for BSF: *i)* regenerate glycosomal NAD^+ required for glycolytic ATP production which is the dominant mechanism to sustain the cellular energy demand. This is particularly critical as BSF mitochondria cannot generate ATP by OXPHOS and the glycosomal NAD^+ redox balance is essentially maintained by the GPSh activity, and not by fumarate reductase; *ii)* prevent mitochondrial superoxide production to compensate for the low investment in scavenging antioxidant defenses. As glycolysis represents the main pathway of ATP production, BSF cannot be limited by glucose availability. Therefore, low levels of scavenging antioxidant defenses, especially the NADPH-dependent thiols, would be a way to preserve glucose for glycolysis by limiting its use by the PPP to generate NADPH. *iii)* thermogenesis, as the respiration through the GPSh-TAO system is intrinsically uncoupled to proton translocation across the mitochondrial inner membrane. Conceivably, the dissipation of heat by the GPSh-TAO system would play a role in preventing the toxic effects of excess nutrients by increasing their consumption by intrinsically uncoupled mitochondria.

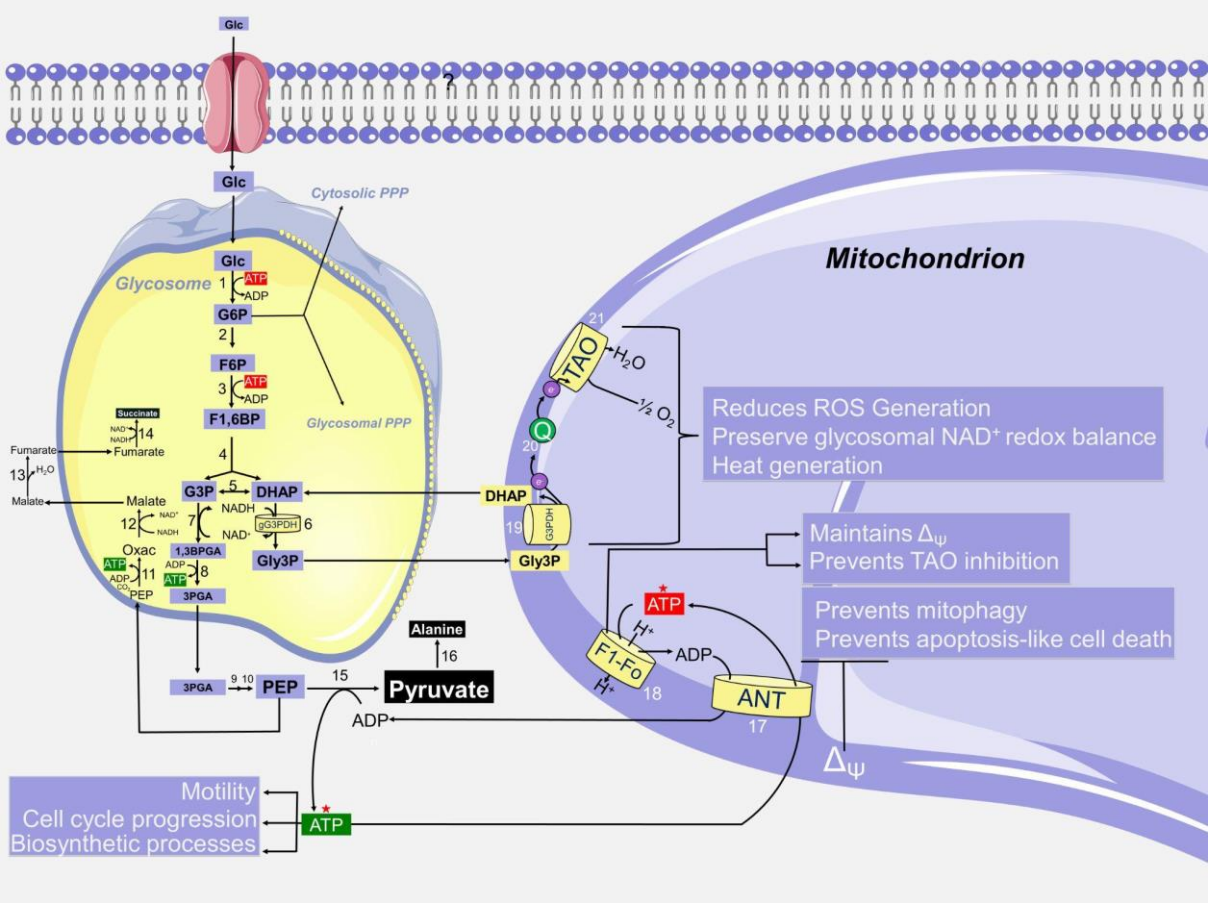

Figure 2. The complex energy metabolism of bloodstream *Trypanosoma brucei*. Glycosome and mitochondrial metabolism are linked by GPSh-TAO system and F_1F_o ATP synthase as a way to stimulate glycolysis and energy demand, limit superoxide production, prevent mitophagy and apoptosis-like cell death. For a descriptive explanation of this hypothesis please refer to item 2 above ("A unifying hypothesis for the extraordinary BSF energy metabolism "). Biochemical and cellular outputs provided by the GPSh-TAO-F_1F_o ATP synthase system are depicted in purple boxes within the mitochondrion. The cytosolic purple box indicates ATP generation by pyruvate is linked to motility, cell cycle progression and biosynthetic processes. $\Delta\Psi_m$ - Mitochondrial membrane potential; 1 - Hexokinase; 2 - Phosphoglucose isomerase; 3 - Phospho-fructokinase; 4 - Aldolase; 5 - Triosephosphate isomerase; 6 - Glycosomal glycerol 3 phosphate dehydrogenase; 7 - Glyceraldehyde 3 phosphate dehydrogenase; 8 - Phosphoglycerate kinase; 9 - Phosphoglycerate mutase; 10 - Enolase; 11 - Phosphoenol-pyruvate carboxykinase; 12 - Glycosomal malate dehydrogenase; 13 - Fumarase; 14 - Fumarate reductase; 15 - Pyruvate kinase; 16 - Alanine aminotransferase; 17 - Adenine nucleotide translocator; 18 - F_1F_o ATP synthase; 19 - Mitochondrial glycerol 3 phosphate dehydrogenase; 20 - Ubiquinone; 21 - *Trypanosoma* alternative oxidase. ATP production sites are depicted as green boxes while the ATP consuming sites are red boxes. The black boxes represent the main metabolic products excreted by BSF. The red star over the green ATP molecules in the cytosol represent those that sustain $\Delta\Psi_m$ by their hydrolysis through F_1F_o ATP synthase.

On the F_1F_o ATP synthase side, $\Delta\Psi_m$ is maintained by reversing its activity by hydrolyzing cytosolic/mitochondrial ATP and allows proton translocation across the mitochondrial inner membrane. Eukaryotic cells in general undergo mitochondrial cytochrome *c*-dependent apoptosis as well as mitochondrial autophagy (mitophagy) upon the collapse of $\Delta\Psi_m$ (Narendra et al 2010; Liu et al 2996). We postulate that reversal of F_1F_o ATP synthase activity in BSF represents a pro-survival mechanism not only to avoid apoptosis-like cell death, but also to prevent the loss of mitochondria through mitophagy. In this regard, fragmentation of dysfunctional mitochondria is a necessary step for mitophagy, while selective fusion of functional organelles is essential for mitochondrial quality control (Twig et al 2008). Although *T. brucei* encodes orthologs of mitochondrial fission proteins (Drp1 and Fis1) which regulate organelle division and morphology (Morgan et al 2004; Chanez et al 2006), experimental evidence supporting mitochondrial fusion remains scarce. Conversely, evidence for mitochondrial fusion is only indirect, either upon recovery of artificial fragmentation induced by Bax overexpression (Esseiva et al 2004) or by silencing the mitofusin orthologue TbMNFL (Vanwalleghem et al 2015). In any case, given that cell division and mitochondrial quality control to eliminate dysfunctional sites of the single organelle requires mitochondrial dynamics, this implies the existence and function of such mechanisms (Voleman et al 2019). Finally, reduction in mitochondrial matrix ATP levels provided by the reversal of F_1F_o ATP synthase would have a beneficial side-effect by preventing TAO inhibition through ATP (Luévano-Martínez et al 2020).

3. Future perspectives and relevant open questions

We foresee that understanding how the GPSh-TAO system prevents mitochondrial ROS production, as well as its contribution to energy dissipation and thermogenesis are valuable avenues for future research. We also envisage that studies devoted to understanding how reversal of ATP synthase activity controls mitophagy and apoptosis-like cell death will shed new light on these elusive processes. In addition, we think the following relevant questions would provide valuable insights to better understand how the knowledge of BSF energy metabolism can be exploited both in terms of basic science but also as innovative therapeutic interventions against HAT:

a) *What would be the metabolic consequences if BSF regenerated glycosomal NAD^+ through fumarate reductase instead of GPSh?*

   We anticipate that overexpression of fumarate reductase in BSF would rewire metabolic pathways in a way that PEP would be diverted from cytosolic PK towards glycosomal succinate production. As a consequence, ATP levels will drop, directly affecting the cellular energy demand and maintenance of $\Delta\Psi_m$ by F_1F_o ATP synthase. Ultimately, BSF will engage apoptosis-like cell death.

b) What would be the metabolic consequences if PCF regenerated glycosomal NAD^+ through GPSh instead of fumarate reductase?

   Previous evidence demonstrated that PEPCK deletion in PCF shifts energy metabolism towards proline oxidation to meet parasite energy demand (Ebikeme et al 2010). However, mitochondrial G3P oxidation by mG3PDH is also significantly affected. Importantly, doubling time of PEPCK-deleted

parasites caused no effect on parasite growth. This strongly suggests that blockage of succinate fermentation to PCF indirectly affects GPSh ultimately impacting glycosomal NAD^+ balance. Therefore, in PEPCK-deleted PCF energy metabolism shifts to proline oxidation that is independent of glycosomal NAD^+ balance. We anticipate that respiration will take place essentially through CIV instead of energetically intrinsically uncoupled TAO activity. This will increase superoxide production but maintains $\Delta\Psi_m$ and ATP levels to meet cellular energy demand.

c) *What would be the metabolic consequences if the PCF mitochondrial electron transfer involved mostly TAO instead of complex IV?*

Previous evidence demonstrated that TAO overexpression in PCF caused no effects on parasite growth and increased the share of TAO-mediated respiration (Walker et al 2005). Curiously, CIV expression and activity were also reduced suggesting that PCF shifts electron transfer to either one of the terminal oxidases. Despite G3P metabolism was not addressed in that study, we anticipate that TAO overexpression would shift energy metabolism towards GPSh, inducing a compensatory effect on ATP production via PK which would decrease the contribution of fumarate reductase as the key mechanism of glycosomal NAD^+ regeneration. We also expect reduced proline oxidation and glycerol excretion by TAO overexpression. Finally, considering that respiration mediated by TAO is energetically non-conservative, *pmF* should be maintained by F_1F_o ATP synthase reversal, while superoxide production will drop.

Abbreviations

ACH	acetyl-CoA thioesterase	HK	hexokinase
AOX	alternative oxidase	kDNA	kinetoplast
ASCT	acetate:succinate CoA-transferase	OXPHOS	oxidative phosphorylation
BAT	brown adipose tissue	PCF	procyclic form
BSF	Bloodstream forms	PFK	phosphofructokinase
CIV	Complex IV, cytochrome *c* oxidase	PGK	phosphoglycerate kinase
cPrx	peroxirredoxin	PK	pyruvate kinase
DHAP	dihydroxyacetone phosphate	*pmF*	protonmotive force
ETS	electron transfer system	PPP	pentose phosphate pathway
G3P	glycerol 3 phosphate	Px	peroxidase-type
G3PDH	glycerol-3 phosphate dehydrogenase	ROS	reactive oxygen species
GK	glycerol kinase	T(SH)2	trypanothione
GPSh	glycerol phosphate shuttle	TAO	Trypanosome alternative oxidase
HAT	Human African Trypanosomiasis	TCA	tricarboxylic acid
$\Delta\Psi_m$	mitochondrial membrane potential	Tpx	tryparedoxin
HEB	high-energy bonds	TR	trypanothione reductase
		UCP-1	uncoupling protein-1

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