The extraordinary energy metabolism of the bloodstream *Trypanosoma brucei* forms: a critical review and a hypothesis

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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) exhibits unique metabolic features including: *i)* reduced expression and activity of mitochondrial enzymes; *ii)* respiration mediated by the glycerol phosphate shuttle (GPSh) and the *Trypanosoma* alternative oxidase (TAO) that is intrinsically uncoupled from generation of mitochondrial membrane potential; *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 oxidants. Here, we critically review the main metabolic features of BSF and provide a hypothesis to explain the unusual metabolic network and its biological significance for this parasite form. We postulate that intrinsically uncoupled respiration provided by GPSh-TAO system would act as a preventive antioxidant defense by limiting mitochondrial superoxide production and complementing the NADPH-dependent scavenging antioxidant defenses to maintain parasite redox balance. Given the uncoupled nature of the GPSh-TAO system, BSF would avoid programmed cell death processes by maintaining mitochondrial membrane potential through the reversal of ATP synthase activity using the ATP generated by glycolysis. This unique "metabolic design" in BSF has no biological parallel outside of Trypanosomatids and highlights the enormous diversity of the parasite mitochondrial processes 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 affects 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 nutritional challenges as a result of the distinct host environments (Marchese et al 2018). 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 2022; Zíková et al 2017; Butter et al 2012; Matthews 2005).

The mechanisms involved in energy provision may change in 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 other eukaryotes. For example, in BSF the dominant mechanism of energy provision is mediated by substrate-level phosphorylation (SUBPHOS) through glycolysis that takes place in a unique peroxisome-derived organelle (named glycosomes) (Opperdoes et al 1977; Visser, Opperdoes 1980; Visser et al 1981; Creek et al., 2015). Glycosomes are essentially found in trypanosomatids along their distinct life-cycles. In contrast to *T. brucei* BSF, the key mechanism of PCF ATP production relies on oxidative phosphorylation (OXPHOS) which takes place within the parasite single mitochondrion (Dewar et al 2022).

Considering the unique energy metabolic pathways in BSF, we will critically revise the knowledge framework on glycosomal, mitochondrial and redox metabolism to provide readers a more complete picture of the enormous complexity of the BSF metabolic network. We will also propose a hypothesis to explain the metabolic signatures of BSF mitochondria as a preventive antioxidant mechanism to complement the classical scavenger redox defenses. Importantly, we discuss the potential consequences of this unusual "metabolic design" at cellular level in BSF that might be explored for future therapeutic interventions and key 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 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 demonstrated for *T. brucei* the compartmentalization of the enzymatic activity of the first seven glycolytic enzymes (from hexokinase, HK, to phosphoglycerate kinase, PGK) and the other two enzymes involved in glycerol metabolism (glycerol-3-phosphate dehydrogenase, G3PDH, and glycerol kinase, GK) (Opperdoes, Borst 1977). Interestingly, glycosomes were proposed to be peroxisome-like organelles, although the *T. brucei* glycosomes are devoid of catalase or oxidase activity involved in hydrogen peroxide (H_2O_2) metabolism, the hallmark of peroxisomes. When catalase activity was found in glycosomes in other kinetoplastids such as the bodonid flagellate *Trypanoplasma borelli*, the evolutionary origin of glycosomes from peroxisomes was sealed (Deschamps et al 2011; Opperdoes et al 1988). Subsequent studies demonstrated the presence of this organelle in other Kinetoplastida organisms and in Diplonema, but not in Euglena. This indicates that glycosome development was a feature that arose in one of the common ancestors of the two Euglenozoa subclades, but after the separation from Euglenida (Reviewed in Gualdrón-López et al 2012; Gabaldón et al 2016).

Although the *T. brucei* glycosomes are authentic peroxisomes, they have certain peculiarities (Gabaldón et al 2016; Gualdrón-López et al 2012). Glycosomes compartmentalize key metabolic pathways such as most reactions of glycolysis, the pentose phosphate pathway (PPP), purine salvage pathway and sugar-nucleotide biosynthesis, which contrasts with most eukaryotic species (reviewed in Michels et al 2021; Allmann, Bringaud 2017; Gualdrón-López et al 2012; Opperdoes 1987). Compartmentalization of the initial glycolytic reactions in glycosomes resulted in the loss of HK and phosphofructokinase (PFK) regulation by their reaction end-products (Bakker et al 2000). In eukaryotes where glycolysis occurs in cytosol, the lack of regulation of these two enzymes would imply an acceleration of glycolytic flux by feedback occurring when ATP produced as an output is used as a substrate in the same pathway. This would lead to an accumulation of phosphorylated intermediates of glycolysis, which affects cell physiology by two ways: i) by restricting the access to the energy invested in these high-energy bonds (HEB) for other cellular energy demands; ii) by causing osmotic disturbances generated by the accumulation of such intermediates. 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 into two distinct

compartments: glycosomes and cytosol. Glycosomal membranes are impermeable to solutes with molecular masses above 400 Da (e.g. NAD^+/NADH and adenylates) (Hammond et al 1985, Quiñones et al 2020), thus no free ATP/ADP/AMP exchange occurs 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, the net ATP production is zero. The other two ATP molecules from glycolysis are produced in the cytosol by the pyruvate kinase (PK) reaction. As a result, equilibrium of ATP consumption and production is established in glycosomes and the glycolytic reaction rate is limited by the glycosomal ATP pool, preventing accumulation of HEB intermediates 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 including the ATP producing phosphoglycerate kinase (PGK) are found within this organelle (Misset et al 1986; Hart et al 1984). As a consequence of the glycosomal heterogeneity along parasite life-cycle, the main metabolic end-products are also different in distinct parasite forms. While BSF excretes pyruvate as the major end product and maintain proper redox (NAD^+/NADH) using glycerol-3-phosphate shuttle (GPSh) and ATP/ADP balance using PGK, PCF metabolizes glucose to succinate in glycosomes as well to acetate and alanine in mitochondria. Glycosomal succinate fermentation is a consequence of specific fumarate reductase activity found in glycosomes to recycle NAD^+ to sustain glycolysis (Besteiro et al 2002) and phosphoenolpyruvate carboxykinase (PEPCK) activity to replenish the ATP pool. Although fumarate reductase activity was postulated to exist only in PCF (Besteiro et al 2002), a small but consistent succinate production from glucose metabolism in BSF was reported (Mazet et al 2013). Although PEPCK activity in BSF is quite low compared to PCF (Durieux et al 1991; Hart et al 1984), it is surprisingly essential to this parasite form as silencing PEPCK caused BSF growth arrest (Creek et al 2015). Nevertheless, massive production and excretion of pyruvate as the metabolic product of glycolysis strengthens the critical role of the GPSh shuttle to regenerate glycosomal NAD^+ in BSF as will be later discussed.

1.2. Reduced mitochondrial function to support glycosomal redox balance

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 (Chandel 2015; Chandel, Martínez-Reyes 2020; Haigis, Spinelli 2018; Zhang et al 2010), as well as in the pathogenesis of numerous human diseases (Betarbet et al 2000; Narendra et al 2010). For example, there is no doubt that superoxide and other ROS play central roles in cell physiology as natural by-products of mitochondrial metabolism (Brand 2020; Sies, Jones 2020; Boveris, Chance 1973; Boveris et al 1972).

The body of knowledge generated over the years on *T. brucei* mitochondria is quite extensive and comprises 1151 original papers from 1964 to 2021 (Figure 1). This represents ~49 % of the papers published in all trypanosomatid species, indicating that *T. brucei* is considered a true model organism for mitochondrial research in

trypanosomatids. However, the share of papers dealing with BSF stages falls to only 299, representing only ~26 % of what is known about the mitochondria of *T. brucei*. This clearly indicates that we have a 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.

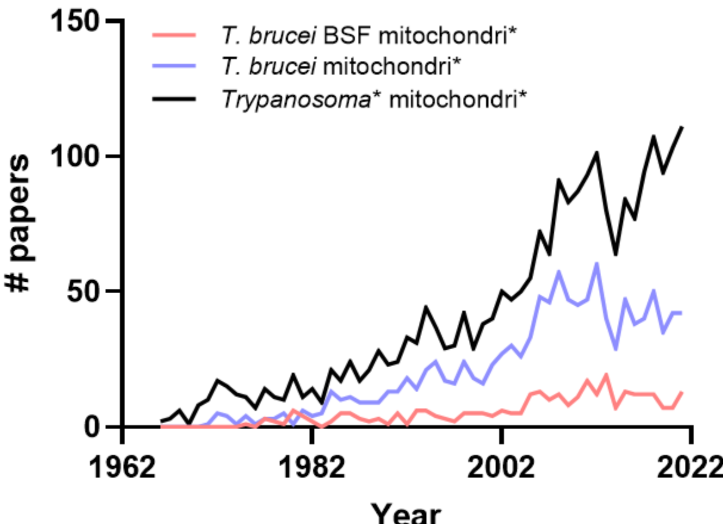

Figure 1. Evolution of knowledge on mitochondrial processes in trypanosomatids and *T. brucei*. Timeline of original publications of mitochondria between 1964 and 2021 in trypanosomatids (black line), *T. brucei* (blue line), and BSF (red line).

As the natural diversity of mitochondrial morphology, functions and interactions with other organelles 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 mitochondrion undergoing striking remodeling of its structure and activity along the parasite life cycle (Bílý et al 2021; 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 series of 25-30 circular DNA pieces of 25-50 kbp, and about 30,000 circular DNA pieces of about 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 mitochondrial gene expression (Benne et al 1986); *iv)* the absence of 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 (ETS) and ATP synthesis (Zíková 2022; Surve et al 2012; Clarkson et al 1989).

From the functional side, the fundamental mitochondrial processes are drastically altered in trypanosomatid life forms, including the activities of tricarboxylic acid (TCA) cycle, the ETS which directly impacts 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, Alencar et al 2021). Mitochondrial ROS production in *T. cruzi* epimastigotes is low compared to bloodstream

trypomastigotes. Importantly, the mitochondrial ETS remodeling during the bloodstream trypomastigote differentiation favors electron leakage and production of mitochondrial H_2O_2 making these parasite forms more resistant to redox challenges induced by the environmental change (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. In any case, the mitoproteome between the two parasite forms showed that the BSF mitochondria are similarly complex to the PCF mitochondria, a remarkable observation considering the morpohological and metabolic reduction of the BSF mitochondria. (Zíková et al 2017).

Indeed, TCA cycle and ETS are strongly reduced in BSF, while these metabolic pathways are fully functional in PCF (Priest, Hajduk 1994; Njogu et al 1980, Clarkson et al 1989; Doleželová et al 2020; Smith et al 2017; Markos et al 1989). Noteworthy is the fact that the inability of BSF to perform OXPHOS is not due to the absence of ETS, but to a remarkable remodeling of its function. Indeed, ETS in BSF is essentially carried out by a reduced form of electron transfer which does not involve proton-pumping and cytochrome-containing complexes but two other 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).

A key aspect regarding BSF mitochondria is their apparent inability to generate oxidants compared to PCF. In this sense, early observations demonstrate that PCF mitochondria can produce H_2O_2 at fairly high rates which strikingly contrast with low (or even undetected) oxidant generation in BSF (Penketh and Klein, 1986; Fang, Beattie 2003; Turrens 2008). As will be described later in this review, TAO activity plays a key role in regulating cellular redox metabolism since superoxide production in both parasite forms was boosted when TAO was pharmacologically inhibited (Penketh and Klein, 1986).

As the energy from redox reactions of the ETS through the GPSh-TAO system is not conserved as *pmF* ($\Delta\Psi_{mt}$ and ΔpH), this represents the case of an intrinsically uncoupled respiration from the *pmF* generation. 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 use *pmF* to 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 TAO, cannot harness energy from electron transfer to proton translocation due to the inherent structural nature of its components.

Given the non-conservative nature of respiration in BSF, these parasites indeed maintain $\Delta\Psi_{mt}$, 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 $\Delta\Psi_{mt}$ generated by reversal of F_1F_o ATP synthase is close to 130-150 mV, (Vercesi et al 1992, Nolan, Voorheis 1992), which allows 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 each of these components in more detail below.

Although the discussion on programmed cell death (PCD) 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, these parasites 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). Despite the pointed differences between the process of PCD in pathogenic trypanosomatids and those in higher eukaryotes, similarities such as the involvement of typical markers of classical PCD can be pointed out. Indeed, trypanosomatids show DNA fragmentation, PS externalization, loss of $\Delta\Psi_{mt}$ 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 a key role in the PCD process in trypanosomatids.

Glycerol phosphate shuttle as a redox bridge linking glycosomes and mitochondria

The crucial 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 quite simple system and is composed of a cytosolic (NAD-dependent, cG3PDH) and a mitochondrial (FAD-dependent, mtG3PDH) 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 mtG3PDH transferring the electrons to FAD and 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 mtG3PDH which can be used as a precursor for gluconeogenesis. Also, G3P is a necessary component for phospholipids and triacylglycerol production (Mráček et al 2013).

Mammalian mtG3PDH has been known for decades (Green 1936), it is ~74 KDa and is one of the simplest components of the entire ETS (Mráček et al 2013). The activity of mtG3PDH is directly linked to respiration and mitochondrial ATP synthesis which mediates an electron transfer independent of TCA cycle and Complex I. GPSh is mostly regulated at mtG3PDH 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 ETS components 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 (Complex IV) activity by adenylate levels (Gaviraghi et al 2019).

In most mammalian cells, the content of mtG3PDH 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 mtG3PDH was found to have the highest activity than any other tissue (Houstěk et al 1975; Ohkawa et al 1969). Importantly, cG3PDH and mtG3PDH 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 mtG3PDH activity might give some hints about this process. In this regard, considering that mtG3PDH 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 NAD^+ dependent G3PDH (Systematic number Tb927.8.3530 at https://tritrypdb.org) and surprisingly two FAD^+ dependent mitochondrial G3PDH sequences (Tb927.11.7380 and Tb927.1.1130), a feature only shared with *Leishmania major* (Škodová et al 2013). Tb927.8.3530 codes for a protein of 37.8 KDa and 354 amino acids, which is quite similar to human cG3PDH. On the other hand, both mitochondrial sequences are slightly smaller than mammalian mtG3PDH: Tb927.11.7380 codes for a 67 KDa 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 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 mtG3PDH 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 mtG3PDH and promotes mitochondrial superoxide production by this enzyme (Wernette et al 1981;

Orr et al 2012). Conceivably, the absence of Ca^{2+} regulation renders *T. brucei* mtG3PDH insensitive to this cation as a possible mechanism to reduce mitochondrial superoxide production.

While subcellular localization of Tb927.1.1130 remains to be determined, proteomic analyses and ectopic expression of Tb927.11.7380 supported its mitochondrial localization (Guerra et al 2006). Glycosomal and mitochondrial G3PDH activities were first identified in *T. brucei* BSF (Opperdoes et al 1977), but a functional GPSh has also been demonstrated in *T. brucei* PCF establishing a functional redox link between glycosomal and mitochondrial metabolism (Guerra et al 2006). Although glucose is considered the main nutrient for BSF, glycerol can also be oxidized in a glycerol kinase and TAO-dependent manner and 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). Interestingly, respiration supported by glycerol oxidation was insensitive to uncoupling agents or inhibition of F_1F_o ATP synthase, suggesting that collapse of $\Delta\Psi_{mt}$ does not affect mitochondrial glycerol metabolism (Pineda et al 2018). Regarding genetic disruption of GPSh, silencing of mtG3PDH impaired BSF growth while causing no apparent effects on PCF (Škodová et al 2013). Surprisingly, 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 mtG3PDH silencing caused no apparent effects on NADH-induced respiration in PCF (Škodová et al 2013). Nevertheless, GPSh plays a key metabolic role to support BSF growth.

***Trypanosoma* alternative oxidase mediates respiration by shortcutting electron transfer**

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 Complex IV, 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 *pmF*, 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 with cellular redox balance since its expression and activity are regulated by and regulates cellular oxidant levels (Wagner 1995).

A remarkable metabolic feature of *T. brucei* life-stages is the change in respiratory mode. While in PCF molecular oxygen reduction to water is mediated by a cyanide-sensitive Complex IV activity, in BSF respiration is carried out by a cyanide-resistant and salicylhydroxamic acid-sensitive Trypanosome Alternative Oxidase (TAO)(Chaudihuri et al 2002). *T. brucei* TAO is coded by two nuclear-encoded genes (Tb927.10.9760 and Tb927.10.7090) that are mostly expressed in BSF (Butter et al 2012). The protein

products of Tb927.10.9760 and Tb927.10.7090 genes have predicted molecular masses of 39.8 and 37.6 KDa and have high probabilities to be exported to mitochondria (Mitoprot II = 0.97 and 0.93), which was confirmed by experimental studies (Hamilton et al 2014). The first molecular structure for AOX was determined for the *T. brucei* enzyme and revealed that TAO has two iron pockets that binds molecular oxygen, which distinguishes it from classical cytochrome *a* binding at Complex IV (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 Complex IV 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, are there benefits from heat production for a unicellular organism, or it's an unavoidable consequence of this particular "metabolic design"? A simple answer for these intriguing questions remains open and has not yet been directly addressed in *T. brucei*. Heat production in unicellular eukaryotes was considered 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 collected to date suggests that at least part of the chemical energy made available via GPSh-TAO can be dissipated as heat at the subcellular level. It is important to stress that this would not imply that BSF would contribute to mammalian host thermogenesis, but rather this would be a simple consequence of the first law of thermodynamics at the sub-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; Bokhari 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 measurements. Also, a recently developed temperature-sensitive fluorescent probe (MitoThermo Yellow, MTY) allows the assessment of mitochondrial heat production at the sub-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 Complex IV activity. However, when Complex IV-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 heat production as a result of AOX-mediated uncoupling.

Nevertheless, direct assessment of mitochondrial heat production 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 with increased sensitivity to glycerol (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). Even more, the 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 the Complex IV subunit IV and cytochrome *c*1 while up regulating the expression of the surface coat protein GPEET (Walker et al 2005).

F_1F_o ATP synthase runs “backward” to avoid programmed cell death

Another singular aspect of BSF mitochondria is the reversal of F_1F_o ATP synthase activity, which uses the energy released from ATP hydrolysis for *pmF* generation (Gahura et al 2021). In other eukaryotes, reversal of F_1F_o ATP synthase activity is observed in conditions where the flow of electrons through the ETS is somehow limited, either by hypoxia or activation of mitochondrial permeability transition (Rego et al 2001; Power et al 2014; Simbula et al 1997). However, in other eukaryotes, ATP hydrolysis to maintain reverse proton pumping by F_1F_o ATP synthase is an acute effect that, if prolonged, can deplete cellular ATP to critical levels (indeed ~40 % of cellular ATP) (Ichikawa et al 1990; Campanella et al 2008; Leyssens et al 1996). Thus, short term maintenance of *pmF* by means of ATP synthase reversal is limited by the availability of cellular ATP mostly supplied by glycolysis (Chalmers-Redman et al 1999). The uniqueness of reversal of F_1F_o ATP synthase activity in BSF is that it operates chronically along this parasite life-form, which, to the best of our knowledge, has no parallel in nature.

In BSF, reversal of F_1F_o ATP synthase activity generates a *pmF* higher than 190 mV and is classically regulated in many organisms by the inhibitory factor 1 (IF1) which impairs specifically the ATPase but not ATP synthase activity (Schnaufer et al 2005; Pullman, Monroy 1963; Nolan and Voorheis, 1990; Nolan and Voorheis, 1992). *T. brucei* expresses IF1 (TbIF1) only in PCF exhibiting specific inhibitory effects of the ATPase activity (Panicucci et al 2017). As expected, overexpression of TbIF1 in BSF collapses $\Delta\Psi_{mt}$ and promote cell death underscoring the critical role of reversal of F_1F_o ATP synthase activity for BSF survival (Panicucci et al 2017).

The energy provision to sustain this $\Delta\Psi_{mt}$ is a critical aspect to be considered as many cellular processes compete for the ATP. In this sense, the likely ATP source to sustain the $\Delta\Psi_{mt}$ by F_1F_o ATP synthase reversal is the cytosolic ATP pool generated by PK (reviewed in Michels et al 2021; Nolan, Vooheis 1992). Possible mechanisms for ATP transport into the mitochondrial matrix would be the adenine nucleotide translocator (ANT) or the mitochondrial Ca^{2+}-dependent ATP-Mg^{2+}/Pi exchanger (SLC25A25). Indeed, ANT seems to be the major route for cytosolic ATP to reach the F_1F_o ATP synthase (Hierro-Yap et al 2021). An alternative source of ATP can be provided by the coupled activity between acetate:succinate CoA transferase (ASCT) with succinyl-CoA synthetase (SCS) generating acetate and ATP in BSF via succinyl-CoA production within the mitochondrial matrix (Mochizuki et al 2020).

Regardless the ATP source, the $\Delta\Psi_{mt}$ is required for import of nuclear encoded mitochondrial proteins (Neupert, 1997) and allows the transport of ions and metabolites including Ca^{2+} (Huang et al 2013; Docampo, Lukeš 2012; Lukeš, Basu 2015). For example, maintenance of mitochondrial respiration in BSF is essential for mtDNA stability and for the establishment of the parasite's life cycle. From the regulatory point of view, reversed F_1F_o ATP synthase function in BSF is responsible for maintaining proper level of intramitochondrial ATP by controlling adenylate levels (especially ATP) and modulating both G3PDH and TAO activities (Gahura et al 2021; Hierro-Yap et al 2021; Luévano-Martínez et al 2020).

1.3. Do PCF and BSF exhibit different redox susceptibilities?

Despite few studies have addressed redox susceptibility of PCF and BSF, the evidence available suggests that BSF seems to be more sensitive to various oxidants than PCF (Rossi and Dean 1988; Meshnick et al 1977). For example, the trypanolytic effect of extracellular H_2O_2 exposure was more prominent in BSF than for PCF (Rossi and Dean 1988). Indeed, exposure of PCF to 100 µM H_2O_2 for 3 h caused 35 % of cell lysis, while the same effect was produced at the same time by exposing BSF to only 1 µM (Rossi and Dean 1988). Assessment of the differential redox susceptibilities of PCF and BSF were also conducted by testing the effect of heme, the prosthetic group of hemeproteins which is a pro-oxidant and in the protein-free state mediates the oxidation of lipids, proteins, and nucleic acids (Ferreira et al 2018). Similarly to extracellular H_2O_2 exposure, the cytotoxic effects of free heme in *T. brucei* revealed that PCF were remarkably more resistant to heme exposure than BSF (Meshnick et al 1977). Although the mechanistic basis for the BSF redox susceptibility is not fully understood, the lack of glutathione reductases, thioredoxin reductases, and catalase and reduced activity of iron superoxide dismutase seem to partly explains this trait (Kabiri, Steverding 2001; Tomás, Castro 2013; Fang, Beattie 2003).

A key missing aspect in *T. brucei* redox biology is a clear definition of the main cellular sources and specific sites of mitochondrial ROS production during the parasite life-cycle. 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 has long been known that mitochondria represent the dominant source of cellular oxidants, which are generated at different sites (Boveris and Chance 1973; Wong et al 2017). The evidence available indicates that mitochondrial superoxide is produced at low levels in BSF especially when TAO inhibited (Fang, Beattie 2003). Although direct comparisons of endogenous ROS production between BSF and PCF were not yet carried out, it seems that BSF produces less mitochondrial superoxide than PCF as determined by electron paramagnetic resonance studies (Fang, Beattie 2003). Regarding the topology of mitochondrial oxidant production, Complex I and NDH2 (Fang, Beattie 2002), as well as mitochondrial fumarate reductase (Turrens 1987), were suggested as potential sites to support superoxide generation in these parasites.

Despite trypanosomatids lack canonical scavenging antioxidant enzymes (e.g. catalase, glutathione reductase, thioredoxin reductase), they have evolved unique mechanisms to cope with redox insults (Krauth-Siegel et al 2007). In this regard, the Trypanosomatid-specific trypanothione (T(SH)2)-based system is a complex redox network which plays a key protective role against oxidative stress in these parasites. This system involves non-enzymatic antioxidants such as 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 alcohols. 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 are preferentially detoxified by Pxs (Hillebrand et al 2003; Schlecker et al, 2005; Wilkinson et al 2003), while H_2O_2 and peroxynitrite ($ONOO^-$) are scavenged by Prxs (Tetaud et al 2001; Wilkinson et al 2003).

Compartmentation of antioxidant enzymes also play a role in cell protection against redox insults. In this regard, cytosolic Tpx (cTpx) content in BSF is 3-7 times higher than in PCF and its silencing strongly reduces parasite growth and increased sensitivity to extracellular H_2O_2 (Comini et al 2007). This apparently contrasts with the higher redox susceptibility of BSF to oxidants. However, we have to consider two key aspects: i) the physiological effects caused by cTpx silencing were only observed when its expression was reduced to ~5 % its original levels and for long periods of time; ii) 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). Conversely, evidence demonstrates that mitochondrial peroxidases seem to play a critical role for BSF growth but not viability (Wilkinson et al 2003; Diechtierow, Krauth-Siegel 2011; Bogacz et al 2020). Conceivably, lower mitochondrial ROS production in BSF relative to PCF might explain the apparent dispensable role of mitochondrial peroxidases for BSF viability. However, experimental evidence is still needed to fully address this aspect.

In our view, it seems quite plausible to assume that ETS in BSF mitochondria through the GPSh-TAO system have a limited capacity to generate mitochondrial ROS. However, the redox susceptibility of BSF might be strongly dependent on the source of oxidant challenge (extracellular, cytosolic, or mitochondrial). In this regard, evidence indicated that depletion of cytosolic antioxidant defenses 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 to limit mitochondrial superoxide production in BSF (Figure 2).

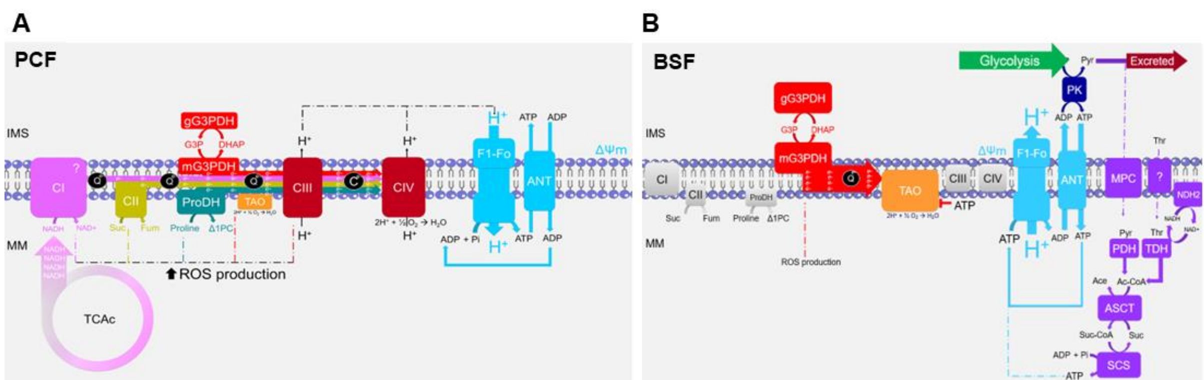

Figure 2. Schematic representation of the mitochondrial electron transport systems in PCF (A) and BSF (B). In PCF, mitochondrial metabolism is fully developed and comprises the reactions of tricarboxylic acid cycle (TCAc), electron transport system (ETS) and oxidative phosphorylation (OXPHOS). This contrasts with reduced mitochondrial metabolism in BSF. ETS reactions in PCF involves multiple dehydrogenases that channels electrons through the NADH dehydrogenase (CI, pink), succinate dehydrogenase (CII, light green), proline dehydrogenase (ProDH, dark green) and mitochondrial glycerol-3-phosphate dehydrogenase (mtG3PDH, red) as well as the electron-carriers ubiquinone (Q, black) and cytochrome *c* (C, black). Electron transfer mediated by mtG3PDH is directly linked to the glycosomal G3PDH through the glycerol phosphate shuttle (GPSh). On the other hand, the ETS in BSF is mainly fueled by a single dehydrogenase (mtG3PDH) and the GPSh. The fate of electrons flowing through the ETS is also quite distinct between *T. brucei* forms, since in PCF most of the molecular oxygen is reduced by cytochrome *c* oxidase (CIV) while in BSF it is essentially reduced only by a *Trypanosoma* alternative oxidase (TAO, orange). The energy from electron transfer in PCF is coupled to ATP synthesis by OXPHOS, which contrasts with BSF where electron energy transferred by the GPSh-TAO system is not coupled to proton translocation. Thus, the energy from *pmF* in PCF is utilized by the "forward" reaction of the F_1F_o ATP synthase to generate ATP and sustain cellular energy demands, in BSF *pmF* is maintained by the "reverse" F_1F_o ATP synthase activity that hydrolyses ATP to mediate proton transport across the inner mitochondrial membrane. There are possibly two cellular sources of ATP to maintain "reversed" F_1F_o ATP synthase activity: *i)* the coupled activity of acetate:succinate CoA transferase (ASCT) with succinyl-CoA synthetase (SCS) in the mitochondrial matrix and *ii)* the pyruvate kinase (PK, dark blue), which represents the main source of ATP. The use of cytosolic ATP to maintain BSF *pmF* also require the reversal of adenine nucleotide translocator (ANT) activity. Generation of mitochondrial superoxide and other reactive oxygen species (ROS) is a natural consequence of the ETS activity and seems to be higher in PCF compared to BSF. Components of the OXPHOS

"phosphorylation module" (F_1F_o ATP synthase and ANT) are represented in blue, while those involved in the "oxidation module" (TCAc, Complex I, II, III, IV, ProDH, mtG3PDH) are represented in other colors (pink, dark red, light green, dark green, red, orange). The mechanisms involved in ATP production to support "reversed" F_1F_o ATP synthase activity and the NAD^+/NADH mitochondrial balance are depicted in purple. Grey-shaded boxes in (B) represent components of the ETS which are detected by quantitative proteomic analyses but have no activity in BSF.

2. Hypothesis: GPSh-TAO system acts as a preventive and complementary antioxidant defense in BSF

Given the existence of unique, and apparently paradoxical, biochemical pathways in *T. brucei*, we propose a 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 have not yet been addressed in detail for BSF.

Figure 3 schematically depicts how GPSh-TAO and the F_1F_o ATP synthase work in a concerted way not only to regulate glycolysis in glycosomes, but also respiration and ROS production in mitochondria. In this regard, the use of the GPSh-TAO system has the following outcomes for BSF: *i)* regenerates glycosomal NAD^+ required for glycolytic ATP production which is the dominant mechanism for maintaining the cellular energy demand, *ii)* provides a safe mechanism for electron sink by preventing mitochondrial superoxide production, *iii)* complements the NADPH-dependent scavenging antioxidant defenses to cope with cellular oxidants, *iv)* contribute to heat production at sub-cellular level since GPSh-TAO system is intrinsically uncoupled to proton translocation across the mitochondrial inner membrane. Conceivably, heat generation by the GPSh-TAO system would play a role to boost mitochondrial enzyme activities to optimal levels just like observed in mammalian cells (Chrétien et al 2018; El-Khoury et al 2022).

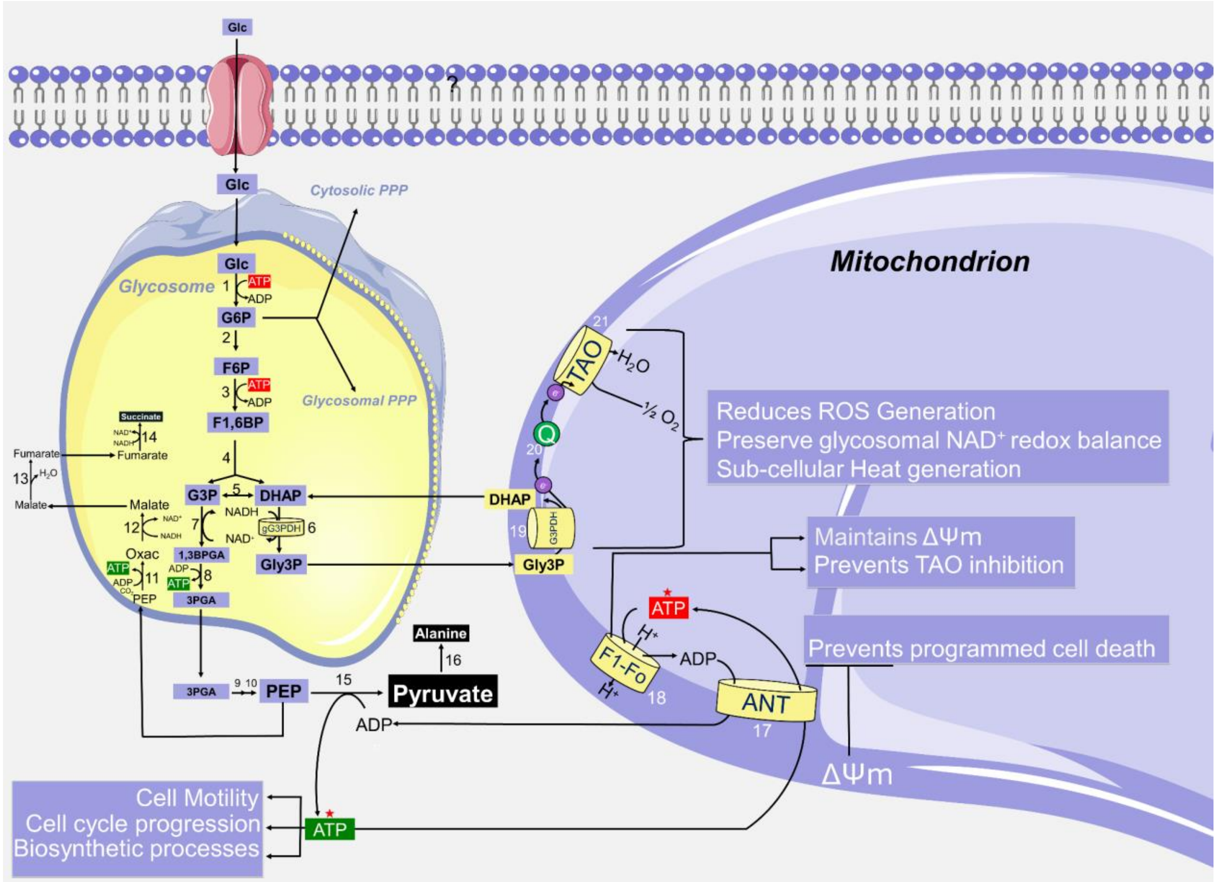

Figure 3. The complex energy metabolism of *T. brucei* BSF. Glycosome and mitochondrial metabolism are linked by GPSh-TAO system and F_1F_o ATP synthase as a way to stimulate glycolysis and energy demand, limit superoxide production and prevent PCD. For a descriptive explanation of this hypothesis please refer to item 2 above ("GPSh-TAO system acts as a preventive and complementary antioxidant defense in BSF"). 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. $\Delta\Psi_{mt}$ - 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 $\Delta\Psi_{mt}$ by their hydrolysis through F_1F_o ATP synthase.

On the F_1F_o-ATP synthase side, $\Delta\Psi_{mt}$ would be maintained by reversing its activity by hydrolyzing ATP and allowing proton translocation across the mitochondrial inner membrane. Given that eukaryotic cells in general undergo mitochondrial-dependent PCD

upon the collapse of $\Delta\Psi_{mt}$ (Narendra et al 2010; Liu et al 2006), we postulate that reversal of F_1F_o ATP synthase activity in BSF would represent a pro-survival mechanism avoiding PCD and preventing TAO inhibition by avoiding accumulation of mitochondrial ATP content (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 are valuable avenues for future research. In addition, we think the following relevant questions would provide valuable insights to better understand how knowledge of BSF energy metabolism can be exploited in both basic research and innovative therapeutic interventions against HAT:

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

We hypothesize that forced regeneration of glycosomal NAD^+ using the glycosomal succinic fermentation pathway in the BSF would lead to significant ATP perturbations as glycosomal ATP content would increase, while cytosolic ATP content would decrease. Potentially, this would directly affect cellular energy requirements and the maintenance of $\Delta\Psi_{mt}$ by F_1F_o ATP synthase, ultimately leading to BSF death.

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

We postulate that if the GPSh system (gG3PDH and mtG3PDH) was overexpressed in PCF to an extent comparable to that of BSF, the excess of electrons would fuel ETS, possibly leading to increased expression of TAO to avoid superoxide production. We also anticipate that glycosomal ATP balance would be maintained by pyruvate phosphate dikinase (PPDK) activity as a compensatory response in the presence of glucose as the main nutrient source.

c) What would be the metabolic/energetic consequences if the PCF mitochondrial electron transfer system involved mostly TAO instead of Complex IV?

Previous studies have shown that overexpression of TAO in PCF has no effect on parasite growth but leads to increased total cellular respiration as well as increased levels of TAO-mediated respiration (Walker et al 2005). Curiously, expression of the COIV subunit of Complex IV was reduced, suggesting that PCF shifts electron transfer to TAO. Regardless of nutrient availability, increased expression of TAO could lead to a reduction in mitochondrial superoxide because the TAO but not the Complex III/IV pathway is the dominant mechanism of oxygen reduction. In addition, overexpression of TAO could lead to a decrease in $\Delta\Psi_{mt}$ which promotes the reversal of F_1F_o ATP synthase and ultimately leading to cell death because of the lack of ATP to fulfill the energy demands of the cell.

Abbreviations

ACH	acetyl-CoA thioesterase	HK	hexokinase
AOX	alternative oxidase	kDNA	kinetoplastid DNA
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	peroxiredoxin	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
$\Delta\Psi_{mt}$	mitochondrial membrane potential	Tpx	tryparedoxin
HEB	high-energy bonds	TR	trypanothione reductase
PCD	programmed cell death	UCP-1	uncoupling protein-1

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