Redesigned and reversed: architectural and functional oddities of the trypanosomal ATP synthase

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Abstract

Mitochondrial F-type adenosine triphosphate (ATP) synthases are commonly introduced as highly conserved membrane-embedded rotary machines generating the majority of cellular ATP. This simplified view neglects recently revealed striking compositional diversity of the enzyme and the fact that in specific life stages of some parasites, the physiological role of the enzyme is to maintain the mitochondrial membrane potential at the expense of ATP rather than to produce ATP. In addition, mitochondrial ATP synthases contribute indirectly to the organelle’s other functions because they belong to major determinants of submitochondrial morphology. Here, we review current knowledge about the trypanosomal ATP synthase composition and architecture in the context of recent advances in the structural characterization of counterpart enzymes from several eukaryotic supergroups. We also discuss the physiological function of mitochondrial ATP synthases in three trypanosomatid parasites, Trypanosoma cruzi, Trypanosoma brucei and Leishmania, with a focus on their disease-causing life cycle stages. We highlight the reversed proton-pumping role of the ATP synthase in the T. brucei bloodstream form, the enzyme’s potential link to the regulation of parasite’s glycolysis and its role in generating mitochondrial membrane potential in the absence of mitochondrial DNA.

Introduction

F-type adenosine triphosphate (ATP) synthases (also called F-ATPases of F₁F₀-ATPases) are bidirectional turbine-like enzymes coupling ATP synthesis or hydrolysis with proton translocation through biological membranes in bacteria and their endosymbiotic descendants, mitochondria and chloroplasts (for recent reviews see Junge and Nelson, 2015; Walker, 2017; Kuhlbrandt, 2019). When operating in the forward mode, ATP synthases utilize the proton motive force to produce ATP and thus represent fundamental constituents of the oxidative phosphorylation pathway. In the reverse mode, they act as ATP-consuming proton pumps contributing to the generation of electrochemical membrane potential. Bacteria employ both modes depending on the species and growth conditions (Cotter and Hill, 2003). By contrast, mitochondrial ATP synthases in most aerobic eukaryotes function as a major source of cellular ATP and the reversal of their activity is a manifestation of pathophysiological conditions (Campanella et al., 2009). Unlike the monomeric bacterial and chloroplastic counterparts, all mitochondrial ATP synthases characterized so far occur in dimers (Arnold et al., 1998; Dudkina et al., 2005). The wide phylogenetic distribution of the ATP synthase dimers suggests that the enzyme’s dimerization is a common feature of all aerobic eukaryotes with oxidative phosphorylation and was possibly present already in the last eukaryotic common ancestor. Dimerization of ATP synthases induces curvature of the inner mitochondrial membrane (Dudkina et al., 2006; Davies et al., 2012) and the intrinsic propensity of dimers to self-assemble into rows facilitates membrane shaping and governs cristae formation (Anselmi et al., 2018; Blum et al., 2019). Spatial separation of ATP synthases and electron transport chain (ETC) complexes on rims and flat regions of cristae, respectively, creates a local proton concentration gradient in cristae lumen, enhancing oxidative phosphorylation (Davies et al., 2011). Therefore, apart from the enzymatic function, ATP synthases affect mitochondrial physiology by aiding in the determination of submitochondrial ultrastructure.

In Trypanosoma brucei, a medically relevant digenetic parasite, the mitochondrial ATP synthase exhibits a 2-fold deviation from its counterparts from traditional model organisms. First, its architecture differs markedly from canonical ATP synthases. Second, its role switches during the parasite’s life cycle, from being an ATP producer in the insect form to an ATP consumer maintaining the vital mitochondrial membrane potential in the mammalian bloodstream form (BSF). This switch is associated with major rearrangement of mitochondrial morphology, possibly involving altered ATP synthase oligomerization. Unlike in T. brucei, in Trypanosoma cruzi and Leishmania, ATP synthase acts true to its name and participates in the oxidative phosphorylation providing ATP in all life forms of the parasite. In the first part of this review, we discuss the recent progress in our understanding of ATP synthase architecture based on structural characterization of enzymes from various eukaryotic supergroups.

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with a focus on trypanosomal ATP synthase complex. In the second part, we summarize our knowledge of the ATP synthase importance for the disease-causing forms of medically relevant trypanosomatid parasites.

Unexpected diversity of mitochondrial ATP synthases

Structural studies of mitochondrial ATP synthases have been key for the seminal research on the structure of the matrix-facing soluble F₁-ATPase subcomplex isolated from the bovine heart (Abrahams et al., 1994) initiated two decades of dominance of X-ray crystallography in the field. Numerous crystal structures of subcomplexes of bovine and yeast ATP synthases provided atomic models of the enzyme’s rotor and the extrinsic part of the peripheral stalk, and revealed the mechanism of the conversion of the rotational force into the cyclic catalysis of ATP formation (reviewed in Junge and Nelson, 2015; Walker, 2017). Due to the striking similarity of the blueprints for bacterial and opisthokontal mitochondrial F-type ATP synthases and their fundamental role in cellular energetics, it was assumed that the mitochondrial enzymes do not differ substantially between eukaryotic lineages. However, only the advance of cryo-electron microscopy (cryo-EM) techniques in the last few years allowed researchers to build atomic models of complete mitochondrial ATP synthases not only from conventional model organisms [mammals (Gu et al., 2019; Pinke et al., 2020; Spikes et al., 2020) and Saccharomyces cerevisiae (Guo et al., 2017)], but also from protists representing other eukaryotic phyla [Polytoma (Murphy et al., 2019), Euglena (Mühleip et al., 2019), Tetrahymena (Flygaard et al., 2020) and Toxoplasma (Mühleip et al., 2021)]. From the mechanistic point of view, structures revealed by cryo-EM elucidated how the proton motive force across biological membranes is converted into the torque of the enzyme’s rotor (Klusch et al., 2017; Hahn et al., 2018) and how the symmetry mismatch of the rotor and catalytic subcomplexes is compensated by the flexibility of the peripheral stalk (Sobti et al., 2019). From the evolutionary perspective, the structures demonstrated that although the functional core is universally conserved in all F-type ATP synthases, peripheral parts and the dimerization interface of mitochondrial ATP synthases have diverged substantially among eukaryotic lineages. Notably, cryo-EM structures not only allowed identification of novel lineage-specific components, but in some species also revealed conserved subunits, which had not been previously identified by traditional homology searches using known genomes and proteomes (Fig. 1).

All F-type ATP synthases are composed of two subcomplexes, the membrane-embedded F₀ and the soluble F₁, connected by the rotary central stalk and a stationary peripheral stalk (Fig. 2). The globular F₁ subcomplex, also referred to as F₁-ATPase, is a pseudo-symmetrical assembly of three heterodimers of α- and β-subunits organized around single γ-subunit, the main constituent of the central stalk. The functional core of F₀ consists of the α-subunit and a ring of several identical c-subunits (c-ring), providing together a path for protons across the membrane. Proton translocation drives the rotation of the c-ring and the tightly attached central stalk. The torque of the asymmetric γ-subunit induces cyclic conformational changes of three active sites on the interfaces of α- and β-subunits, resulting in the stepwise binding of ADP and phosphate, their condensation into ATP, and its release, emptying the nucleotide binding site for the next catalytic cycle (Abrahams et al., 1994). The (αβγδε)₆-hexamer is prevented from rotation by interaction with the oligomycin sensitivity-conferring protein (OSCP), the uppermost constituent of the extrinsic part of the peripheral stalk.

The entire F₁-ATPase consisting of subunits α₁, β₁, γ₁, δ₁ and ε₁ (stoichiometry of subunits indicated by the subscripts), OSCP and both proton translocating components (α-subunit and c-ring) are present in all reported mitochondrial ATP synthases. The rest of the enzyme is markedly less conserved (Fig. 2). The highest diversity is observed in the soluble part of the peripheral stalk and in the luminal and peripheral F₀ regions, often composed of lineage-specific subunits or lineage-specific extensions of conserved proteins. Subunits involved in the anchoring of the peripheral stalk to the membrane and in clamping the α-subunit to the c-ring (b, d, e, f, g, i/j, k and l), originally identified in opisthokonts, appear to be conserved in several other eukaryotic groups (Fig. 1). In many cases, their proposed homology is based largely on tertiary and quaternary structure resemblances revealed by cryo-EM. Low sequence similarity precludes identification of the respective homologs in lineages without structurally characterized ATP synthases. Although dimerization is a general hallmark of all reported mitochondrial ATP synthases, subunits constituting the dimer interface differ between eukaryotic lineages, likely due to divergent evolution. Consequently, the shape of dimers and topography of oligomeric assemblies vary in the characterized enzymes, which is reflected in diverse cristae morphology (Fig. 1; Dudkina et al., 2010; Davies et al., 2012; Mühleip et al., 2016; Mühleip et al., 2017; Mühleip et al., 2021).

Unique structural features of ATP synthases in Trypanosomatida

The knowledge about the architecture of mitochondrial ATP synthases in Trypanosomatida, a group of protozoan parasites with monoxenous or dixenous life cycles, is based mostly on studies performed with cultured insect trypanosomatids of T. brucei. The composition of the mitochondrial ATP synthase in these cells was determined by mass spectrometry (MS) identification of proteins co-immunoprecipitated with F₁ and tandem affinity purified using TAP-tagged conserved or newly identified subunits as baits. These biochemical analyses complemented by a comprehensive genome search revealed 23 subunits (Zikova et al., 2009), including the α-subunit detected by MS later (Skodova-Sverakova et al., 2015). Characterization of the trypanosomal F₁-ATPase released by chloroform from mitochondrial membrane fragments and purified by two-step chromatography (Gahura and Zikova, 2019) showed that the catalytic subcomplex contains three copies of the euglenozoan-specific p18-subunit in addition to the universally conserved subunits α, β, γ, δ and ε (Gahura et al., 2018b). The F₁-ATPase structure determined by X-ray crystallography further revealed that each copy of p18-subunit, an α-helical protein with three pentatricopeptide repeats, associates peripherally with one of the three α chains (Fig. 3A), and does not contribute directly to the catalytic mechanism (Montgomery et al., 2018). Although the atomic structure did not explain the function of p18, the elaboration of the F₁ head in Euglenozoa by this additional subunit is extraordinary, because the αβγδε subcomplex is invariant in all other known F-type ATP synthases. In addition, the α-subunit was found to be proteolytically cleaved at two sites separated by eight amino acid residues, producing two fragments α₁₂³ and α₁₅–₅₀₀, both stably associated with the complex. The split of α-subunit into two parts was also demonstrated in other euglenozoan protists Crithidia fasciculata (Speijer et al., 1997), Leishmania tarentolae (Nelson et al., 2004) and Euglena gracilis (Sathish Yadav et al., 2017). The structure of the trypanosomal F₁-ATPase showed that the cleavage occurs at the region corresponding to a loop on the surface of F₁-ATPases from other organisms and splits the N-terminal β-barrel domain from the rest of the protein (Montgomery et al., 2018; Fig. 3A and B). Nevertheless, the architecture of the entire (αβγδε)-headpiece, including the nucleotide binding pockets, is highly similar to
the prototypical bovine F1-ATPase (Bowler et al., 2007) and the biological relevance of the α-subunit cleavage remains unclear. So far, there is no available structure of a complete ATP synthase from any trypanosomatid species. However, low-resolution structures obtained by subtomogram averaging documented general morphological similarity between ATP synthase dimers from T. brucei and E. gracilis, a representative of euglenids, a lineage closely related to kinetoplastids (Muhleip et al., 2017). An atomic model of dimeric ATP synthase from E. gracilis has recently been determined by cryo-EM (Muhleip et al., 2019). Apart from the universally conserved components of mitochondrial ATP synthases (α, β, γ, δ, ε, a, c and OSCP), the E. gracilis enzyme contains seven subunits with previously recognized homology (Sathish Yadav et al., 2017) to proteins found in the complex from T. brucei (Zikova et al., 2009). To get a deeper insight into the trypanosomal ATP synthase structure, we performed systematic pairwise sequence comparison of all remaining subunits in the complex from E. gracilis with all non-conserved trypanosomal ATP synthase components, and propose homology of four additional protein pairs, which exhibit >20% sequence identity (Fig. 3C). Overall, 19 of 23 subunits of the trypanosomal ATP synthase have homologs found in the structure from E. gracilis. Taken together, although the ATP synthases from E. gracilis and T. brucei are not compositionally identical, it is conceivable to assume that the structural features shaped by the conserved subunits are shared between the two species (Fig. 3C), and possibly among Euglenozoa in general.

The peripheral stalk in euglenozoan ATP synthases differs markedly from other lineages. The universally conserved OSCP features a long and partially flexible C-terminal extension, which bridges the core of the protein on the top of F1 with ATPTB2, a highly divergent and extended homolog of the d-subunit from opisthokontal ATP synthases anchoring the peripheral stalk to the membrane. The interaction of ATPTB2 with the extension of OSCP is clamped between two laterally positioned lineage-specific globular proteins ATPTB3 and ATPTB4 on one side and one copy of p18 on the other side. The contacts of the peripheral stalk with p18 contribute to the immobilization

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**Fig. 1.** Structural diversity of mitochondrial ATP synthases mapped on the phylogenetic tree of eukaryotes. The figure summarizes structural and proteomics studies of mitochondrial ATP synthases. The phylogenetic tree is based on Burki et al. (2020). Organisms with ATP synthases with atomic models obtained by single particle cryo-EM, visualized by cryo-ET (see first two columns) or characterized by MS, and several relative species were included. Major groups without experimental data are shown in small font. Numbers of subunits showed in grey are based on proteomic characterization of purified complexes without available single particle cryo-EM analysis and might be revised in future. ‘Canonical’ subunits are proteins originally identified in opisthokonts, which have divergent homologs in other lineages. Categorization of ATP synthases in types I to IV is based on Kuhlbrandt (2019). Cristae morphology is adopted from Panek et al. (2020). The numbered references are following: 1 (Muhleip et al., 2017), 2 (Zikova et al., 2009), 3 (Muhleip et al., 2019), 4 (Sathish Yadav et al., 2017), 5 (Davies et al., 2012), 6 (Guo et al., 2017), 7 (Davies et al., 2011), 8 (Spikes et al., 2020), 9 (Muhleip et al., 2021), 10 (Salunke et al., 2018), 11 (Huet et al., 2018), 12 (Flygaard et al., 2020), 13 (Muhleip et al., 2016), 14 (Blum et al., 2019), 15 (Murphy et al., 2019), 16 (Vazquez-Acevedo et al., 2006), 17 (Klodmann et al., 2011), 18 (Bultema et al., 2009), 19 (Senkler et al., 2017).
of the (αβ)₆-hexamer (Fig. 3D). However, the reinforcement of the interaction between the F₁ head and the peripheral stalk is unlikely the only role of p18, because the protein is essential for the integrity of the F₁-ATPase (Gahura et al., 2018b), in contrast to the peripheral stalk components OSCP (Hierro-Yap et al., 2021) and ATPTB₂/d-subunit (Subrtova et al., 2015). In E. gracilis, the extensions of OSCP and d-subunit compensate for the reduction of the b-subunit, the major constituent of the peripheral stalk in bacteria and mitochondria of mammals and yeast. Sequence comparison did not reveal a homolog of the b-subunit in the ATP synthase of T. brucei, suggesting that it might be further reduced or completely absent in Trypanosomatida.

In all reported F-type ATP synthases, the proton channel-forming a-subunit contains two horizontal membrane-embedded helices H5 and H6, which are directly involved in proton translocation (Guo et al., 2017; Murphy et al., 2019). In mammals (Spikes et al., 2020) and yeast (Guo et al., 2017) the two helices are lined by a series of six parallel transmembrane helices, contributed by one helix of each of subunits b, f, 8, i/j and k, and by a transmembrane helix of the a-subunit itself. The same configuration is observed in E. gracilis (Fig. 3E), suggesting the conservation of the contributing subunits. Although the ATP synthase in T. brucei contains homologs of E. gracilis subunits k and i/j, the homologs of subunits b, f and 8 were not identified. The dimer interface of the ATP synthase in E. gracilis is formed largely by subunits ATPEG₁ and ATPEG₂ without discernible homology outside Euglenozoa, but with apparent homologs in Trypanosomatida (called ATPTB₉ and ATPTB₈ in T. brucei; Perez et al., 2014; Fig. 3C and F). The presence of these two proteins in T. brucei together with the fact that the dimers from both species arrange into short left-handed helices, as showed by cryo-electron tomography (cryo-ET; Muhleip et al., 2017), strongly suggests that the dimerization is mediated by the same elements. The dimer in E. gracilis is further stabilized by a homotypic interaction of extensions of d-subunit adopting a ferredoxin-like fold on the matrix side of the membrane. This extension is specific to Euglenids and the interaction is therefore completely missing in T. brucei, possibly explaining the poor stability of its ATP synthase dimers (our observation). Notably, the dimer interface in Euglena contains several ordered phospholipid molecules, predominantly cardiolipins (Muhleip et al., 2019; Fig. 3F).

Role of the ATP synthase in trypanosomatid parasites

The function of the ATP synthase in trypanosomatid parasites depends on the species and their life cycle stage. Digeneric trypanosomatid parasites (e.g. T. brucei, T. cruzi and Leishmania) possess a complex life cycle as they alternate between the insect vector and a mammalian host. In humans, they cause dreadful diseases (African trypanosomiasis, Chagas disease and leishmaniasis). The scarcity of effective treatments and weak vaccine prospects drive an ongoing urgent need to identify new therapeutic targets. Considering the ATP synthase’s central position in energy metabolism and its structural and compositional divergence from the mammalian counterpart, one may consider this complex as a promising drug target. The feasibility of targeting the ATP synthase is underscored by the recent approval of bedaquiline, a specific F-ATP synthase inhibitor that blocks the c-ring rotation, to treat multi-drug resistant tuberculosis (Preiss et al., 2015).

Whereas the function of the ATP synthase in T. brucei parasites is well understood, the exact role of the ATP synthase and its requirement for virulence and survival of Leishmania and T. cruzi parasites are not well defined yet.

Role of the ATP synthase in intracellular amastigotes of Leishmania and T. cruzi parasites

Leishmania parasites infect a mammalian host when an infected sand fly takes a blood meal. The promastigote form is released to the skin and is rapidly internalized by macrophages. Inside the macrophages, promastigotes differentiate to amastigate and non-motile amastigotes, which multiply in the acidic environment of the phagolysosome-like parasitophorous vacuole, whose exact metabolic content has not been determined yet. The environment most likely offers a wide range of carbon sources (sugars, amino acids and fatty acids) for the parasite to salvage due to macrophage polarization to the M2 state, which exhibits increased oxidative metabolism and promotes Leishmania parasites growth (Saunders and McConville, 2020). Despite this seemingly plentiful environment, intracellular amastigotes do not proliferate rapidly (Klohn et al., 2015), but rather activate a stringent metabolic response, a protective mechanism to confer resistance to multiple cellular stresses (oxidative, nutritional and pH). This programmed response is associated with reduced rates of glucose uptake and with low metabolic activity to possibly minimize reactive oxygen species (ROS)-sensitive processes and to lessen the levels of endogenously generated ROS (McConville et al., 2015). The glucose-sparing metabolism of the intracellular amastigotes questions the importance of oxidative phosphorylation, and hence ATP production by the ATP synthase, for the parasite. Nevertheless, amastigotes show increased reliance on mitochondrial metabolism, including tricarboxylic acid (TCA) cycle and fatty acid β-oxidation (Saunders et al., 2014). These pathways generate reduced electron carriers NADH and FADH₂ that are reoxidized by the ETC coupled to ATP synthase (Dey et al., 2010; Saunders et al., 2014). The ablation of aconitase, a TCA cycle enzyme, cytochrome c oxidase, or ATP synthase is protected to be lethal by an in silico-generated model of the metabolic network (Subramanian et al., 2015). Indeed, the inhibition of ATP synthase by the cell-permeable antimicrobial peptide histatin 5 arrests of Leishmania amastigotes’ growth (Luque-Ortega et al., 2008). Nevertheless, to prove that oxidative phosphorylation is critical for the Leishmania amastigote forms, further studies are necessary (Fig. 4).

Trypanosoma cruzi parasites are transmitted to humans by a blood-sucking triatomene bug through contact with its infected feces. In the skin wound, metacyclic trypanostages invade various types of cells, escape the lysosome-derived parasitophorous vacuole and establish infection in the host cell cytoplasm as
intracellular amastigotes (de Souza et al., 2010). During T. cruzi trypomastigotes’ transition to amastigotes, there is a dramatic shift from sugar-based metabolism to catabolism of amino and fatty acids manifested by repression of glucose transporters and increased levels of TCA cycle enzymes and proteins involved in fatty acid oxidation and oxidative phosphorylation. The oxidative metabolism of amino and fatty acids suggests a crucial role for oxidative phosphorylation to generate ATP (Atwood et al., 2005; Silber et al., 2009; Li et al., 2016; Shah-Simpson et al., 2016). This is further supported by the identification of highly selective compounds (i.e. GNF7686 and ELQ271) that target complex III and inhibit the growth of intracellular amastigotes in a dose-dependent manner (Khare et al., 2015).

After several rounds of intracellular replication, amastigotes differentiate to trypomastigotes, which are released from the cell by its rupture. Extracellular trypomastigotes disseminate and infect other cells or they are ingested by the triatomine bug to conclude the life cycle. Interestingly, the extracellular bloodstream trypomastigotes switch back to glycolysis to meet their energy demands but still employ ETC complexes to maintain the mitochondrial membrane potential. Moreover, disproportional changes in expression of ETC complexes II, III and IV create a bottleneck forcing electrons to pre- maturely reduce molecules of oxygen and generate significant levels of ROS (Goncalves et al., 2011). This induced production of ROS probably provides a redox-mediated pre-conditioning to evoke protection against host-induced oxidative challenge once the parasite enters the cell again. In this particular case, the ETC linked to ATP synthase also serves as an adaptive process allowing parasites to survive redox challenges imposed by the host. Presumably, inhibition of the ATP synthase would cause a decrease in ATP levels, which by itself can be lethal, but it would also further increase the already elevated ROS levels. Although there is evidence that the oxidative environment stimulates the parasite’s growth and ROS are critical for successful infection of T. cruzi in various cell types (Paiva and Bozza, 2014), very high levels of ROS reverse this favourable effect and are harmful for the parasite (Goes et al., 2016). Therefore, an additive effect of endogenous and exogenous ROS bursts may induce deleterious changes resulting in parasites’ elimination by oxidative damage (Fig. 4).

Despite some evidence that mitochondrial ETC and ATP synthase might be essential entities for the clinically relevant infectious stages of Leishmania and T. cruzi parasites, the knowledge of the mitochondrial energy metabolism in these parasites remains largely incomplete.

The F-ATPase in the bloodstream form of T. brucei

The disease-causing form of T. brucei is an extracellular parasite inhabiting various niches of its mammalian host, including the bloodstream, adipose tissue, skin or central nervous system.
Since these environments offer different nutrients, the BSF mitochondrion maintains its complexity and plasticity despite its largely reduced size compared to the insect stage organelle. Uniquely, the parasite’s mitochondrion employs ATP synthase as an ATP-consuming proton pump (Fig. 4).

The discovery of the oligomycin-sensitive F-ATP synthase in BSF by Opperdoes et al. (1977), elicited the curiosity of scientists, who back then considered the BSF mitochondrion a vestigial organelle with no obvious role in cellular metabolism. Seeking if this ‘promitochondrion’ possesses mitochondrial membrane potential, Nolan and Voorheis reported the existence of electrochemical gradients in BSF trypanosomes, documented by the accumulation of the radiolabelled lipophilic cation methyltriphenylphosphonium. One year after, the same authors reported that oligomycin caused a collapse of the mitochondrial membrane potential identical in magnitude to that achieved by the proton uncoupler FCCP, concluding that in BSF cells, the nature of the mitochondrial electrical gradient was exclusively attributable to the proton-pumping activity, or reverse mode, of the ATP synthase (Nolan and Voorheis, 1992). In more than 10 years, RNA interference silencing of two F1-ATPase subunits further corroborated the previous findings and confirmed the unusual and essential function of this enzyme for the survival of BSF trypanosomes (Schnaufer et al., 2005; Brown et al., 2006).

The role of the F-ATPase in BSF T. brucei strikingly contrasts with that of other eukaryotes, where the reverse operation of the ATP synthase is a rare and short-term phenomenon associated with hypoxia or anoxia. For example, under ischaemic conditions in mammalian tissues, respiration is halted and the ATP synthase reverses in response to decreased mitochondrial membrane potential, partially compensating for its loss by utilizing ATP produced by glycolysis. To limit the detrimental depletion of cellular ATP caused by an ATP-consuming F-ATP synthase (Rouslin et al., 1986; Jennings et al., 1991; St-Pierre et al., 2000), the activity of this enzyme is regulated by a short naturally occurring protein, the inhibitory factor 1 (IF1) (Pullman and Monroy, 1963; Walker, 1994). IF1 specifically inhibits the ATPase activity with no effect on ATP synthesis (Pullman and Monroy, 1963; Asami et al., 1970), although some authors propose an inhibitory effect of IF1 on both ATP synthase modes (Harris et al., 1979; Schwerzmann and Pedersen, 1981).

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Formentini et al., 2012; Garcia-Bermudez and Cuevza, 2016). IF1 has also been implicated in cristae biogenesis, dimerization of the ATP synthase and metabolic reprogramming of cancer cells (Campanella et al., 2009). This regulatory protein has been identified in *T. brucei* (TbIF1) and related trypanosomatid parasites. In *T. brucei* the expression of TbIF1 is tightly regulated throughout the life cycle as it is expressed only in the insect stages, but not in BSF (Panicucci et al., 2017). In *T. cruzi* and *Leishmania* parasites, regulation of IF1 expression is reflected in its slight upregulation in the mammalian intracellular stages compared to the respective insect stages in agreement with increased mitochondrial activity of *T. cruzi* and *Leishmania* amastigotes (Li et al., 2016; Inbar et al., 2017). As in other organisms, TbIF1 inhibits the hydrolytic but not the synthetic activity of the mitochondrial ATP synthase in vitro (Panicucci et al., 2017; Gahura et al., 2018a). The artificial expression of TbIF1 is lethal for BSF trypanosomes, due to the abolishment of the mitochondrial membrane potential (Panicucci et al., 2017).

In addition to its role in the maintenance of mitochondrial membrane potential, a functional F-ATPase is also required by BSF parasites to prevent the intramitochondrial accumulation of ATP. Elevated ATP levels lead to inhibition of the alternative oxidase, the only terminal oxidase in BSF parasites, and consequently to a reduced respiration rate (Luevano-Martinez et al., 2020). This represents a novel and unique role of the F-ATPase connecting this enzyme to the regulation of the glycolytic pathway, which is intimately linked to respiration via the alternative oxidase (Bakker et al., 1999). Despite these fundamental roles, BSF trypanosomes can withstand a loss of approximately 90% of the membrane-bound F-ATPase complexes with no obvious deleterious effect on their viability in culture nor on the mitochondrial membrane potential in live cells (Hierro-Yap et al., 2021). We speculate that the long-term tolerance of reduced levels of F-ATPase complexes might be favourable for the emergence of trypanosomes lacking mitochondrial genome, as it might provide the time frame for the parasites to gain key nuclear mutation(s) in the F-ATPase that enable(s) generation of mitochondrial membrane potential in the absence of mitochondrial-encoded a-subunit.

**The vestigial F-ATPase in trypanosomes without mitochondrial genome**

The mitochondrial DNA of trypanosomatids, which is arranged in a distinct submitochondrial structure termed kinetoplast (hence kinetoplast DNA, kDNA), encodes mostly for the components of the oxidative phosphorylation pathway, key for the survival of *T. brucei* insect stages (Stuart, 1983; Schnauffer et al., 2002). In BSF trypanosomes, which do not carry out oxidative phosphorylation, kDNA is indispensable due to the requirement of the F-ATP synthase a-subunit involved in proton translocation across the inner mitochondrial membrane. To synthesize the a-subunit, BSF mitochondria express additional two kDNA-encoded proteins, uS3m and uS12m, core components of mitochondrial ribosomes (Ramrath et al., 2018). However, there are BSF trypanosomes that partially or completely lack their kDNA and they are referred to as dyskinetoplastic (DK) parasites (Agbe and Yielding, 1995). The DK trypanosomes thriving in nature, *T. brucei evansi* (Hoare, 1937) and *T. brucei equiperdum* (Tobie, 1951), cause surra and dourine, respectively, devastating diseases that affect a broad range of domestic and wild animals in Africa, Asia and South America (Brun et al., 1998). Stable kDNA-deficient strains can also be generated artificially using compounds targeting the kDNA network, such as ethidium bromide or acriflavine (Stuart, 1971; Riou et al., 1980; Riou and Benard, 1980). Although DK trypanosomes were thought to be locked in the long slender BSF stage, the absence of kDNA does not impair their differentiation into the stumpy form, a transmission pre-adapted stage with partially activated mitochondrial functions. However, the absence of kDNA does reduce the lifespan of the stumpy form (Dewar et al., 2018), hampering their differentiation into the procyclic form (Timms et al., 2002). Therefore, DK trypanosomes are transmitted between hosts during sexual intercourse or mechanically via blood-sucking insects or vampire bats (Brun et al., 1998), which facilitated their spread outside the area of the tsetse fly belt (Lun and Desser, 1995).

Because the ATP synthase a-subunit is absent in DK trypanosomes, the molecular mechanism for the generation of mitochondrial membrane potential is analogous to that of mammalian ρ0 cells and yeast petite mutants, both of which lack mitochondrial genome. The mechanism involves ATP hydrolysis by the vestigial ATP synthase, which provides the substrate for the electrogenic exchange of mitochondrial ADP3− for cytosolic ATP4+ by the ATP/ADP carrier (AAC). Accordingly, oligomycin, an inhibitor that blocks the c-ring rotation, has virtually no effect on the growth of *T. b. evansi* (Schnauffer et al., 2005). Moreover, DK strains contain one of several point mutations in the nuclear-encoded subunits of the F1-ATPase, most often the L262P or A273P substitutions in the γ-subunit (Lai et al., 2008; Deen et al., 2013). These substitutions are sufficient to allow the loss of kDNA and enable the generation of mitochondrial membrane potential in an F0-independent manner, perhaps by enhancing the hydrolytic activity of the F1-ATPase (Dean et al., 2013).

The functional independence from the F0 section led to the long-standing belief that the F1 moiety exists as a soluble entity detached from the inner mitochondrial membrane in DK trypanosomes (Schnauffer et al., 2005; Jensen et al., 2008). However, the finding that the silenced expression of the peripheral stalk subunit ATPTB2/d-subunit slowed the growth of DK trypanosomes in vitro (Subrtova et al., 2015) indicates that the complex might be attached to the membrane. Considering the presence of a membrane-bound F-ATP synthase in cells devoid of mitochondrial genome in other organisms (Wittig et al., 2010; He et al., 2017), it is likely that the vestigial F-ATPase in DK trypanosomes also remains associated with the membrane to increase the spatial proximity between the AAC and the F1-ATPase, and ensure an efficient exchange of substrates between the two entities.

**Perspectives**

Although many architectural features of ATP synthase from *Euglena* can be extrapolated to Trypanosomatida, a high-resolution cryo-EM structure of a trypanosomatid ATP synthase will be necessary for an unbiased and complete description of the enzyme in this parasitic lineage. Such research will reveal structural diversification, predominantly in the membrane-embedded part of the enzyme, as suggested by the absence of apparent homologs of some conserved ATP synthase subunits in *T. brucei* (Fig. 1). If several rotational states of the enzyme are characterized, the study would explain how the apparently flexible peripheral stalk immobilizes the catalytic subcomplex and what is the contribution of its unprecedented contacts with the F1-ATPase (Fig. 3D). An atomic model of trypanosomal ATP synthase could also reveal details with potential relevance to structure-based drug development. For example, the IF1-F1-ATPase interaction interface can be exploited by peptidomimetics to develop inhibitors selective for ATP hydrolytic activity of the enzyme. In the insect stage of *T. brucei*, the dimers of ATP synthase contribute to the shaping of the inner mitochondrial membrane by assembling into short helices on the rims of discoidal cristae (Muhleip et al., 2017). However, it is not clear how the dimers...
arrange in the BSF cells, which exhibit reduced complexity of mitochondrial ultrastructure. The understanding of the molecular mechanisms of membrane reshaping during the T. brucei life cycle, which can now be mimicked in vitro (Kolev et al., 2012; Dolezelova et al., 2020), will further illuminate the principles underlying mitochondrial dynamics in eukaryotes in general. Furthermore, recent data suggested interaction of the ATP synthase complex with mitochondrial calcium unipporter complex, AAC and phosphate carrier (Huang and Doccampo, 2020). Further studies scrutinizing this putative megacomplex may reveal ATP synthase regulation by Ca\textsuperscript{2+} and strengthen the importance of the functional interplay between ATP synthase and both carriers in BSF and Dk cells. Considering our detailed knowledge of ATP synthase’s role in T. brucei parasites, it is surprising that very little is known about its function in medically important parasites, Leishmania and T. cruzi. Although this gap is caused most likely by the fact that these parasites are less amenable to genetic manipulation, this can now be overcome by using subgenomic CRISPR/Cas9 libraries to dissect the role of oxidative phosphorylation mechanisms of membrane reshaping during the BSF cells, which exhibit reduced complexity of mitochondrial dynamics in eukaryotes in general.

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