The Remarkable Mitochondrion of Trypanosomes and Related Flagellates

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Abstract While the single mitochondrion of trypanosomatid flagellates contains many of the hallmarks that are known from mitochondria in other conventional model organisms, it also possesses several unique features, making it a subject of intense research. Here, we summarize current knowledge of the (1) structure, maintenance and replication of the extensive kinetoplastid DNA network, (2) byzantine organellar RNA metabolism, including insertion/deletion RNA editing,

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W. de Souza (ed.), *Structures and Organelles in Pathogenic Protists*,
Microbiology Monographs 17, DOI 10.1007/978-3-642-12863-9_10,
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(3) translation of mitochondrial-encoded proteins, and, finally, (4) distinctive aspects of energy metabolism of the organelle. While we focus on the mitochondrion of *Trypanosoma brucei*, particularly in the context of its radical physiological and biochemical changes during the life cycle of the parasite, in order to get a more complete picture of the workings of this fascinating organelle, we also discuss significant findings obtained from other trypanosomatids.

The single mitochondrion of trypanosomes and their relatives is a remarkable organelle, containing almost all of its hallmarks as well as some unique features. Among the latter are exceedingly complex mitochondrial DNA and RNA editing. Furthermore, the organelle is very different in the two principal stages of the life cycle, as it is metabolically active in the procyclic stage transmitted by the insect vector (Fig. 1d), while its morphology and metabolism are highly reduced in the bloodstream stage (Fig. 1e). Thanks to initiatives such as description of the mitochondrial proteome of the procyclic stage, our knowledge of the organelle of *Trypanosoma brucei* and *Leishmania* species increased substantially within the last decade. In this chapter, we focus on mitochondrial DNA and its transcription and translation and conclude with a brief description of the function of mitochondrial-encoded proteins and energy metabolism. Within the last decade, these topics were subject to several authoritative reviews (Besteiro et al., Trends Parasitol 21:185–191, 2005; Bringaud et al., Mol Biochem Parasitol 149:1–9, 2006; Liu et al., Trends Parasitol 21:363–369, 2005; Lukeš et al., Eukaryot Cell 1:495–502, 2002, Curr Genet 48:277–299, 2005; Rubio and Alfonzo, Top Curr Genet 12:71–86, 2005; Shlomai, Curr. Mol. Med. 4:623–647, 2004; Schnaufer et al., Int J Parasitol 32:1071–1084, 2002; Schneider, Int J Parasitol 31:1403–1415, 2001; Simpson et al., RNA 10:159–170, 2004, Trends Parasitol 22, 168–174, 2006; Stuart et al., Trends Biochem Sci 30, 97–105, 2005). In this chapter, we briefly summarize our present knowledge with somewhat more detailed treatise of the findings obtained mostly within the last 5 years.

1 The Kinetoplast DNA

Protists of the class Kinetoplastea derive their name from the mitochondrial genome, termed kinetoplast (k) DNA, for a good reason. Thanks to its enormous size (and complexity, as we shall see later), it is likely the first organellar DNA observed (Ziemann 1898), which until present arguably belongs to the best studied organellar genomes. It also represents a unifying feature that these protists carry in their mitochondrion. Indeed the presence of an extranuclear DNA, easily stainable with the Giemsa solution, is a strong hint that the cell in question belongs to the kinetoplastid flagellates.

The kDNA of trypanosomes and related flagellates belongs arguably to the most complex DNA known. Since its description by light and electron microscopy, it is the advent of molecular biology methods that enables dissection of this

fascinating structure. Principal progress in our understanding of the kDNA structure and replication was recently achieved mainly via functional analyses using RNA interference in *Trypanosoma brucei*.

1.1 kDNA: Its In vivo Structure and Diversity

Initial studies by transmission electron microscopy in the 1960s revealed the presence of a compact electron-dense structure invariably located in a region of the single reticulated mitochondrion that is adjacent to the flagellar basal body (Fig. 1a, b). This structure represents the kDNA, which in all trypanosomatid flagellates exists in the form of a disk-like structure, with DNA strands aligned in parallel to the axis of the disk (Fig. 1f) (Shapiro and Englund 1995). Numerous transmembrane filaments attach the kDNA to the flagellar basal body (Zhao et al. 2008). During

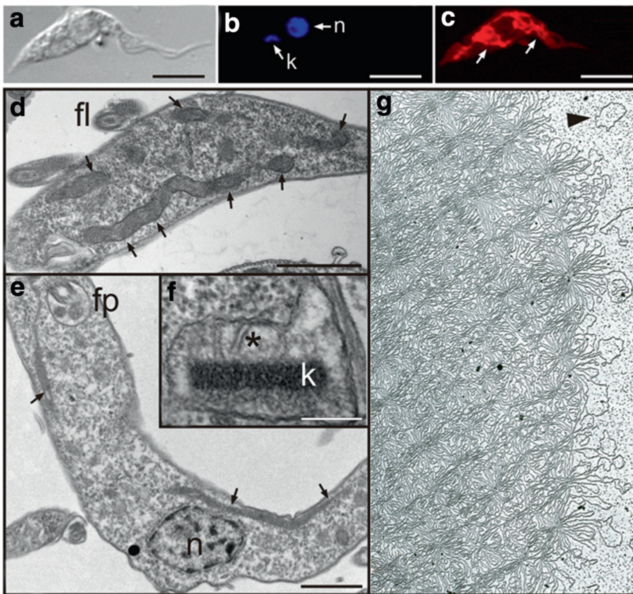

Fig. 1 *Light and electron microscopy of Trypanosoma brucei* (**a**) Procyclic cell of strain 29-13; (**b**) The same cell stained with 4′,6-diamidino-2-phenylindole (DAPI) revealing the large oval nucleus (n) and distinct kinetoplast (k); (**c**) Tubular reticulated mitochondrion stained in the same cell by mitotracker red (indicated by arrows); (**d**) Transmission electron microscopy of a procyclic cell, showing multiple cross-sections thru the peripherally located mitochondrial network (*arrows*). Cross-sectioned flagella at the periphery are indicated (fl); (**e**) Transmission electron microscopy of a bloodstream cell, revealing the reduced thin mitochondrion of this stage (*arrows*). Nucleus (n) and flagellar pocket (fp) are also indicated; (**f**) Longitudinal section thru the kinetoplast (k) in the extended periflagellar portion of the mitochondrion. Peripherally located tubular cristae are indicated with an *asterisk*. (**g**) Electron microscopy of the kinetoplast DNA network and free minicircles (*arrowhead*). *Bars*, 3 μm (**a–c**), 1 μm (**d**), 0.5 μm (**e**, **f**)

the cell cycle, kDNA division follows after the formation of a new flagellum, which is among the first and most conspicuous morphological signs of cell division (Woodward and Gull 1990). Duplication of the nuclear DNA in the form of closed mitosis, with the formation of an intranuclear spindle in the nucleus with an intact envelope terminates the cell cycle (Ogbadoyi et al. 2003).

While kDNA of all the studied members of the family Trypanosomatidae exists in the form of a disk (Fig. 1f), the homologous structure assumes a variety of forms in the sister family Bodonidae. In *Bodo* species, which are considered to be free-living predecessors of obligatory parasitic trypanosomatids (Simpson et al. 2006), kDNA is also confined to the periflagellar position, yet it is formed by a bundle of DNA strands, comprising an arrangement termed pro-kDNA (Lukeš et al. 2002). In other bodonids, the kDNA is either evenly or unevenly distributed throughout the mitochondrial lumen. In the former case, first described from the fish parasite *Trypanoplasma borreli*, the so-called pan-kDNA seems to contain a comparable amount of nucleic acids as the nucleus, at least as judged by staining with the DNA-binding dyes. The free-living and commensalic bodonids of the genera *Dimastigella* and *Cruzella* also contain a huge amount of DNA in their mitochondrion. However, it is present in the form of multiple foci evenly distributed throughout organellar lumen, in an arrangement termed poly-kDNA (Lukeš et al. 2002). The morphologically most unusual structure is the globular kDNA found in the highly diverged kinetoplastid *Perkinsella*, a parasite of amoebae found on the gills of fish (Dyková et al. 2003). The interesting finding that bodonids are eukaryotes with the largest amount of DNA in their mitochondrion deserves thorough research, which is unfortunately not forthcoming, so we know close to nothing about its organization, gene content and function.

Initial characterization of the morphologically prominent kDNA commenced with the studies of the nonpathogenic insect parasite *Crithidia fasciculata*, which can be cultivated in a cheap medium and to high cell densities. Treatment of the purified kDNA with topoisomerase II showed that it is composed of circular DNA molecules that are mutually catenated into a single large network (Englund 1979). Following restrictions with different endonucleases demonstrated that there are two classes of DNA circles, termed maxicircles and minicircles (Fig. 2a) (Shapiro and Englund 1995). As it turned out, these circular molecules have strikingly different functions (see below). Subsequent studies have shown a very similar arrangement of kDNA in other trypanosomatid species, such as *Leishmania tarentolae*, the plant pathogen *Phytomonas serpens*, and *T. brucei*, *T. cruzi* and *Leishmania* spp., the causative agents of African sleeping sickness, Chagas disease and Leishmaniasis.

1.2 Maxicircles

Maxicircles are homologs of classical mitochondrial DNA of other eukaryotes, as they contain many typical protein-coding genes, a single subunit of mitochondrial

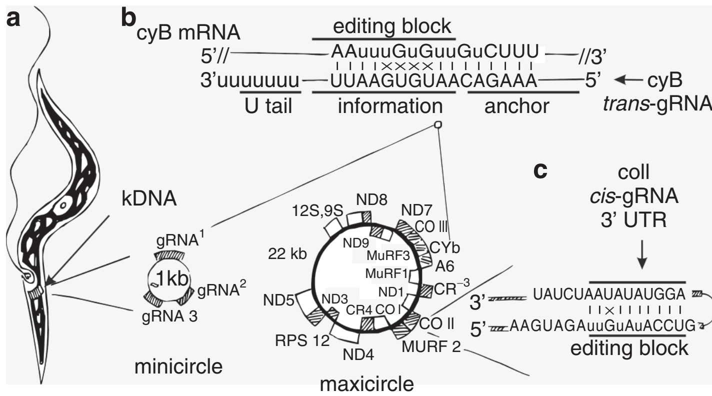

Fig. 2 *Integration of kinetoplast (k) DNA with RNA editing* (**a**) Genomic organization of kDNA maxi- and minicircles of *T. brucei*. The ~1 kb minicircle contains on average three guide (g) RNA genes. The ~22 kb maxicircle contains the complement of mitochondrial genes encoding subunits of the respiratory chain and mitoribosomal components. Genes undergoing RNA editing are shaded. Never-edited genes are left open. The genes are abbreviated as follows: mitoribosomal rRNAs 9 S (9S) and 12 S (12 S); ATPase subunit 6 (A6); cytochrome oxidase subunits 1 (COI) 2 (COII) and 3 (COIII); cytochrome reductase subunit b (cyB); maxicircle unknown reading frames 1 (MURF1), 2 (MURF2), and 3 (MURF3); NADH dehydrogenase subunits 1(ND1), 3 (ND3), 4 (ND4), 5 (ND5), 6 (ND6), 7 (ND7), 8 (ND8), and 9 (ND9); ribosomal protein S12 (RPS12); unknown open reading frames also labeled by CR3 and CR4. (**b**) CyB gRNA:mRNA duplex, with features of the gRNA molecule *highlighted*. Noncanonical U:G pairings are depicted as *crosses*, and inserted Us are shown in *lowercase*. (**c**) The *cis*-acting gRNA of COII, located in the 3′-UTR. Noncanonical U:G pairings are depicted as *crosses*, and inserted Us are shown in *lowercase*

ribosome, and the 9S and 12S mitoribosomal RNAs (Fig. 2a) (Estevez and Simpson 1999). They are composed of a gene-coding region or conservative region and a variable or divergent region, in which putative replication origin is located (Liu et al. 2005; Lukeš et al. 2005). Transcripts of some genes are readily translatable, however, most has to undergo (extensive) uridine insertion and deletion type of RNA editing (see section on RNA editing) to be rendered translatable (Stuart et al. 2005). It was proposed that approximately a dozen maxicircles, which along with minicircles constitute the kDNA disk, are mutually interlocked, forming a network within a network (Shapiro and Englund 1995). Maxicircles were considered to be homogeneous in sequence, but recent evidence from *Leishmania major* indicates that a cell may contain several maxicircle classes that differ in the variable region (Flegontov et al. 2009). The size of maxicircles has been established only in a handful of species, with a median size of 20 kb. In *T. brucei evansi* and *T. brucei equiperdum*, maxicircles are subject to deletions eventually leading to their complete loss. As a consequence, the mitochondrion loses the potential to produce key subunits of the respiratory complexes, turning it effectively into a petite mutant of *T. brucei* that cannot be transmitted via the tse-tse fly (Lai et al. 2008).

1.3 Minicircles

Initial restriction analyses and sequencing of minicircles did not bring any clue as to their "raison d'etre," yet revealed their extensive sequence heterogeneity, with the size being species-specific, ranging from 0.5 to 10 kb (Shlomai 2004). In fact the size of minicircles can also be determined by electron microscopy, since their DNA strands are packed in parallel to the axis of the disk (Lukeš and Votýpka 2000).

An estimated 5,000 minicircles per kinetoplast constitute a single large network (Fig. 1g), in which each and every minicircle is interlocked with three of its neighbors (Chen et al. 1995). Characteristic features of the minicircles include lack of supercoiling, conserved replication origins and unique sequence features such as the bent helix formed by evenly spaced polyadenine tracks (Liu et al. 2005). The seminal discovery of small genes encoding guide (g) RNAs on minicircles (Blum et al. 1990) uncovered their functional integration with maxicircles via the process of RNA editing (see Sect. 2). The coding capacity varies from a single to three gRNAs per minicircle (the assignment of some regions for gRNA is only tentative) (Fig. 2a), depending on the trypanosomatid species. Sequence heterogeneity of minicircles differs between species, but it is reasonable to assume that each trypanosomatid encodes hundreds of gRNAs in its minicircle kDNA.

In *T. brucei evansi* and *T. brucei equiperdum*, the (partial) deletion of maxicircles triggers loss of minicircle heterogeneity, although initially their abundance remains unaltered. However, further diminution of the kDNA is characterized by homogenization down to a single minicircle sequence class (dyskinetoplastic strains), which is eventually lost altogether, turning the cells into the akinetoplastic form (Lai et al. 2008; Jensen et al. 2008).

1.4 Replication and Maintenance of kDNA

It is apparent that the extremely complicated network composed of mini- and maxicircles (Fig. 1g) has to faithfully divide, in order to equip both daughter cells with a full kDNA complement. For that purpose, a sophisticated and likely also a highly exact mechanism evolved, which is expected to entail the participation of more than a hundred different proteins for this task (Liu et al. 2005).

In a nonreplicating network, all DNA circles are covalently closed and interlocked with their neighbors. By the action of topoisomerase II, individual minicircles are released from the network into a region between the mitochondrial membrane and the kDNA disk, or so-called kinetoflagellar zone, marking the initiation of replication (Drew and Englund 2001). It is in this region where DNA primase, two DNA polymerases (Klingbeil et al. 2002), and universal minicircle sequence-binding protein (UMSBP) (Abu-Elneel et al. 2001) perform replication of the free minicircles, in addition to the several other anticipated protein yet to be identified. Interaction of UMSBP with the minicircle replication origin is uniquely

affected by its redox state. While oxidation drives oligomerization of UMSBP and eliminates its binding to DNA, binding is activated under reduced conditions, which favors monomers (Sela and Shlomai 2009). Next, the newly replicated minicircles migrate into antipodal sites, which are two protein-rich regions flanking the kDNA disk 180° apart. Specific antibodies have been used to characterize the content of these well-defined protein centers, which contain topoisomerase II, structure-specific endonuclease 1 (SSE1), DNA polymerase β (Torri and Englund 1995), DNA ligase (Downey et al. 2005) and p38 (Liu et al. 2006). The antipodal sites were recently visualized also by electron microscopy (Gluenz et al. 2007). The reattachment of newly replicated minicircles occurs within the antipodal centers via the action of topoisomerase II, and all replicated minicircles are marked with the retention of at least one gap (Wang and Englund 2001). Proteins identified thus far leave no doubt about the extreme complexity of the system, as the mitochondrion of *T. brucei* contains at least six DNA polymerases (Klingbeil et al. 2002; Saxowsky et al. 2003), six DNA helicases (Lindsay et al. 2008) and two DNA ligases (Sinhaet al. 2006). Moreover, there are at least four histone-like proteins, called kinetoplastid-associated proteins (KAPs) 1–4, that have been implicated in stabilizing the kDNA disk in its compacted structure (Avliyakulov et al. 2004).

Downregulation, mostly by RNAi, of proteins associated with the kDNA leads to colorful phenotypes, many of which illuminate the intricate mechanisms behind its maintenance and replication. After topoisomerase II was ablated, the kinetoplast shrinks (Wang and Englund 2001) and accumulates the holes that remain after the release of minicircles from the dividing kDNA (Lindsay et al. 2008). In the absence of SSE1, the attachment of minicircles is altered (Liu and Englund 2007), while the elimination of HsIVU protease causes over-replication of minicircles, triggering growth of the kDNA disk to an enormous size (Li et al. 2008). Giant kinetoplasts were observed also in cells that interfered against some other proteins. Their presence in cells lacking p166 is explained as a consequence of the disrupted tripartite attachment complex (Zhao et al. 2008), whereas missegregation of minicircles in the absence of UMSBP triggers a similar outcome (Milman et al. 2007). The advent of methods of forward and reverse genetics, to which trypanosomes and related protists are well amenable, promises to eventually disentangle the functions of dozens, perhaps hundreds of proteins engaged in the faithful replication and maintenance of kDNA.

Comparative analysis of the distribution of newly replicated minicircles in the kDNA networks of *T. brucei* and *C. fasciculata* produced an unexpected conundrum, which might be solved by the recent data. While in *T. brucei* and some other flagellates, the newly replicated minicircles accumulate close to the two antipodal sites, labeling of the gapped minicircle progeny with [^{3}H]thymidine in *C. fasciculata* and *L. tarentolae* revealed their ring-like distribution around the network periphery. In order to explain this distribution, a mechanism has been suggested by which the kDNA of the latter species rotates between the antipodal sites (Liu et al. 2005). Therefore, it was postulated that two dramatically different mechanisms of kDNA replication evolved independently in trypanosomatids. Using fluorescent microscopy, Liu and Englund (2007) have shown that the kinetoplast either

rotates (*C. fasciculata*) or oscillates (*T. brucei*), resulting in the strikingly different patterns of distribution of replicated minicircles.

2 RNA Editing

2.1 Mechanism of RNA Editing

In 1986, Benne and colleagues made the seminal discovery that four uridine (U) residues not encoded in the gene are posttranscriptionally inserted into specific sites in the cytochrome oxidase subunit (co) 2 mRNA. This process was named RNA editing. A surge of reports followed indicating that mRNAs from 12 of the 20 genes residing on *T. brucei* maxicircles required this process for their maturation (Fig. 2a). Translatable open reading frames are created by insertions and/or deletions of hundreds of uridine (U) residues into/from maxicircle transcripts. Molecules undergoing this kind of maturation are conceptually grouped as pre-, partially- and fully edited RNAs, depending on their current stage in the process, while RNA molecules that bypass this route are referred to as never-edited (for review in RNA editing see Simpson et al. 2004; Stuart et al. 2005).

Another breakthrough in the field was the discovery of small RNA molecules (50–70 nts long) almost entirely encoded on the minicircles of the kDNA network. These primary transcripts were called guide (g) RNAs because they provide the genetic information defining the editing sites on a given pre- and/or partially-edited mRNA (Blum et al. 1990; Sturm and Simpson 1990). Examination of the three regions that make up the primary structure of a gRNA suggests how they act as blueprints for RNA editing events (Fig. 2b). The 5′-positioned anchor domain is a small stretch of $\sim$10 nts that hybridizes to a complementary sequence on the mRNA, just downstream of the editing sites. The information domain starts at the first base mismatch, providing a template for the appropriate U insertion/deletion. The transfer of information the gRNA to mRNA relies on both Watson–Crick and noncanonical G:U base pairing between the two molecules (Sturm and Simpson 1990). After completion of editing, the edited part of the mRNA, called the editing block, is complementary to the information domain. The third part of the gRNA molecule, the 3′-oligo(U) tail, which is added to the molecule posttranscriptionally and demonstrated to interact with the purine-rich sequences upstream of the editing block (McManus et al. 2000). Almost all gRNAs act in *trans* in the described fashion. A notable exception is the editing of cox2, which utilizes a *cis*-acting gRNA in its 3′-UTR (Golden and Hajduk 2005) (Fig. 2c).

While gRNAs represent the informational component of RNA editing, a cascade of enzymatic activities is also required (reviewed in Simpson et al. 2004; Lukeš et al. 2005; Stuart et al. 2005) (Fig. 3). An endonucleolytic cleavage occurs at the editing site, dividing the mRNA into 5′ and 3′ fragments that are bridged by the bound gRNA. What occurs next depends on whether a U insertion or deletion event

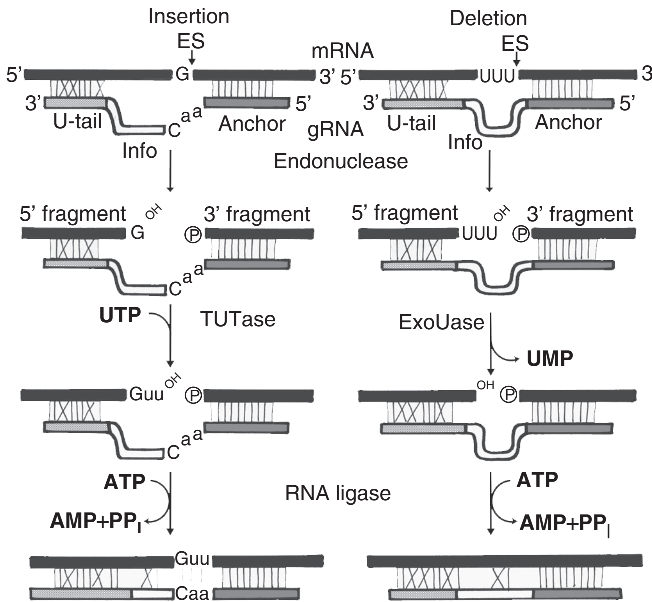

Fig. 3 *Mechanism of RNA editing*. The first base pair mismatch between mRNA (*top*) and anchor domain (*dark gray*) defines the editing site (ES), which is cleaved by an endonuclease. A 3′ terminal uridylyltransferase (TUTase) adds Us to the 5′ fragment while an exonuclease (exoUase) deletes extra Us. The two fragments are joined back together by an RNA ligase. The 3′ oligo(U) tail of the gRNA is indicated by light grey shading

is designated by the gRNA. In the case of the former, free UTP is added to the 3′ hydroxyl group of the 5′ fragment, the number of which is dictated by A and G residues in the information domain. A 3′→5′ exonuclease prunes away 3′-protruding U(s) from the 5′ fragment in the case of deletion. Once the processing step results in a fully complementary duplex, the two mRNA fragments are rejoined by an RNA ligase. After an editing block is completed, the current gRNA in unwound from the mRNA to allow the upstream hybridization of a subsequent gRNA for the next round. As a consequence, editing of pan-edited mRNAs proceeds with a 3′ to 5′ polarity (Maslov and Simpson 1992).

2.2 *The RNA Editing Core Complex*

The macromolecular RNA Editing Core Complex (RECC) confers the core editing activities required for mitochondrial biogenesis (Simpson et al. 2010). It has also been called in the literature the (20S) editosome, a name reflecting the settling rate in Svedberg units of the active complex in glycerol gradient ultracentrifugation experiments (Stuart et al. 2005), and the L-complex (Simpson et al. 2004).

The three-dimensional structure of the complex that has been resolved recently shares a similar structure with a larger version that sediments at 35–40S and contains extra elements such as substrate RNAs (Golas et al. 2009; Li et al. 2009). The $\sim$20 protein subunits comprising the complex often occur in sets or pairs sharing motifs, domains and/or functions. Some of these related subunits are further divided into the two subcomplexes of RECC, each of which confers *in vitro* insertion or deletion editing activities (Schnaufer et al. 2003). Three distinct types of RECCs exist that differ in their incorporation of one of the three endonucleases that catalyze the initial mRNA cleavage step (Carnes et al. 2008). The residing endonuclease determines whether the complex has the capacity to cleave RNA editing substrates with U deletion or insertion editing sites (Carnes et al 2005; Trotter et al. 2005), or processes cox2 editing mediated by its *cis*-gRNA (Carnes et al. 2008).

The finding that RNA editing ligase is essential in the bloodstream stage was surprising (Schnaufer et al. 2001), as the existence of dyskinetoplastic trypanosomes lacking the gRNA repertoire for processing mRNAs, ostensibly implied that RNA editing is not required during this stage of its life cycle (Schnaufer et al. 2002). However, further studies clearly demonstrated that editing is essential for survival of flagellates both in the tse-tse fly and mammalian host (Fisk et al. 2008; Hashimi et al. 2009). The solved crystal structure of RNA editing ligase (Deng et al. 2004) has primed a study for drug-like inhibitors of its function (Amaro et al. 2008), which may potentially be developed for drug treatment of the various diseases caused by trypanosomes.

2.3 Other Proteins Involved in RNA Editing and/or Processing

Several other proteins and complexes that have a role in RNA editing aside from imparting the core enzymatic activities in this process have been described. A DExD/H-box RNA helicase found unstably associated with RECC was proposed to have gRNA-unwinding role (Missel et al. 1997). Another 3′ terminal uridylyltransferase that acts independently of RECC is responsible for the posttranscriptional addition of the 3′-oligo(U) tail to gRNA molecules, and is essential for RNA editing (Aphasizhev et al. 2003a).

The participation of RNA binding proteins has always been an expected feature of RNA editing. RBP16 is an example of such a protein, which has a demonstrated gRNA/mRNA annealing activity (Ammerman et al. 2008). Its silencing by RNAi had a pleimorphic phenotype, affecting some never-edited transcripts as well as those undergoing this process (Pelletier and Read 2003). The mitochondrial RNA binding proteins MRP1 and 2 associate in a heterotetrameric complex that was shown to have in vitro RNA matchmaking activity (Schumacher et al. 2006; Zíková et al. 2008b). Although RNAi-knockdowns of these proteins also affected a subset of both never-edited and edited mRNAs (Vondrušková et al. 2005), the crystal structure of this complex, in which positively-charged amino acids on its surface

bind gRNAs, is consistent with a role in gRNA:mRNA duplex formation (Schumacher et al. 2006; Zíková et al. 2008a). TbRGG1 has affinity for poly(U) (Vanhamme et al. 1998), and its RNAi-silencing affects steady state levels of edited mRNAs but not gRNAs (Hashimi et al. 2008).

TbRGG1 associates with a macromolecular complex in a RNA that mediates fashion (Hashimi et al. 2008). This putative complex was provisionally named the mitochondrial RNA binding complex 1 (MRB1) since many of its constituents contain motifs and domains involved in RNA binding and metabolism. The composition of MRB1 shares a significant degree of overlap with other isolated complexes, including the recently discovered mitochondrial poly(A) polymerase complex (Etheridge et al. 2008; Panigrahi et al. 2008; Weng et al. 2008). Several of the interactions within MRB1 appear to be also mediated by RNA interactions (Fisk et al. 2008; Weng et al. 2008) and there is a diverse array of RNA phenotypes, lending credence to the idea that MRB1 represents a collection of smaller complexes and/or monomers that assemble around RNA (Weng et al. 2008; Hashimi et al. 2009). The so-called gRNA binding complex (GBRC) contains two orthologs, known as the gRNA associated proteins (GAPs) 1 and 2 that appear to have a role in gRNA stability and/or processing (Hashimi et al. 2008; Weng et al. 2008).

2.4 The **raison d'etre** *of RNA Editing*

Shortly after its discovery, it was proposed that kinetoplastid RNA editing may be a relic of an ancient "RNA world," when only these molecules existed. The lack of catalytic activity of the substrate RNAs and the participation of a sophisticated protein complex has negated this idea. In addition, a possible link of seemingly cumbersome process to parasitism has been invalidated by its existence in free-living bodonids. Several hypotheses have suggested the evolutionary advantages bestowed by RNA editing, including (1) extra level of regulation of mitochondrial gene expression, (2) fixing mutations that have accumulated in a nonfunctional mitochondrion, (3) accelerated evolution by creating more genetic variation, (4) multiple proteins coded by one gene, and several other hypotheses (for review see Speijer 2008).

The persistent editing of some transcripts in the bloodstream stage, despite the fact that proteins encoded by these mRNAs are obviously not required at this stage (see below), has spurred the exploration of the idea that RNA editing contributes to protein diversity. An interesting study has recently provided evidence for a protein product of an alternatively edited cox3 mRNA that has a role in kDNA maintenance (Ochsenreiter et al. 2008a). Although more alternatively edited RNAs of other mt gene transcripts have been described (Ochsenreiter et al. 2008b), hard evidence of their translation is needed to confirm this exciting theory.

3 The Mitochondrial RNA Metabolism

RNA editing is integrated into what is emerging as a byzantine RNA metabolism (Fig. 4). While only a single mitochondrial RNA polymerase appears to be required for mini- and maxicircle RNA synthesis (Fig. 4) (Grams et al. 2002; Hashimi et al. 2009), their transcripts undergo different maturation pathways before the gRNAs are duplexed with their cognate preedited mRNAs. Minicircles are thought to be transcribed polycistronically and cleaved by a 19S protein complex into one or more gRNAs (Grams et al. 2000), before being polyuridylylated by the terminal uridylyl transferase 1 (RET1) (Fig. 4) (Aphasizhev et al. 2003a). These molecules are believed to assume a secondary structure with two hairpin loops, perhaps as a way of being recognized by the protein machinery of editing (Schumacher et al. 2006). The two mitoribosomal rRNAs (9S and 12S) also undergo posttranscriptional modification, forming their short 3′ oligo(U) tails (Fig. 4) (Adler et al. 1991).

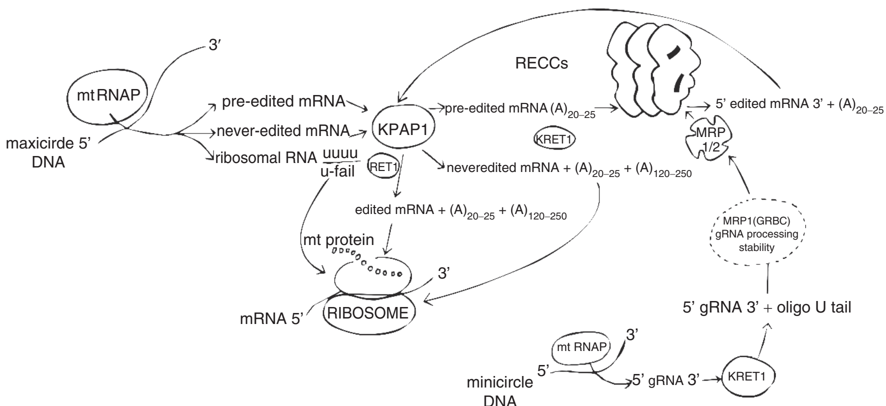

Fig. 4 *Mitochondrial RNA metabolism in procyclic T. brucei* Maxicircle kDNA is transcribed by the mitochondrial RNA polymerase (mtRNAP) into three types of transcripts: preedited mRNAs, never edited mRNAs and mitoribosomal RNAs. Preedited mRNA is equipped with a short 20–25 (A) tail in the kinetoplast poly (A) polymerase 1 complex (KPAP1), and subsequently undergoes editing in one of the RECCs involved in insertion, deletion or *cis*-gRNA mediated editing. 120–250 (A/U)-tail is appended to the fully edited transcript, which is then transcriptionally competent, perhaps with the involvement of the kinetoplastid RNA editing 3′- terminal uridylyl transferase 1 (RET1). Never-edited mRNA is equipped with both short and long tails in the KPAP1 complex and is transported to ribosome to be translated. Minicircle kDNA is transcribed by mtRNAP into gRNA to which oligo-U tail is added by KRET1. gRNAs are then probably stabilized and/or processed by mitochondrial RNA binding complex 1 (MRB1) (also termed GRBC). The heterotetrameric complex comprised of mitochondrial RNA binding proteins 1 and 2 (MRP1/2) stabilize the gRNA molecule in unfolded conformation suitable for pre-mRNA–gRNA hybridization in the initial stage of RNA editing in the 20S editosome. This model depicts a possible sequence of steps a mitochondrial RNA species requires for their maturation, although the precise order of these events remains to be elucidated. The depictions of the 20S editosome and the MRP1/2 complex are based on their resolved three-dimensional structures

The dense gene structure of the T. brucei maxicircle (Fig. 2) indicates that it is transcribed polycistronically. Editing mediated by *trans*-gRNAs occurs independently of cleavage of these precursors into monocistronic transcripts, sometimes even preceding this event (Koslowsky and Yahampath 1997). The resulting mRNAs are polyadenylated by kinetoplast poly(A) polymerase (Fig. 4) (Etheridge et al. 2008). Interestingly, this enzyme appears to be in association with some of the subunits of the MRB1 complex (Fig. 4) and mitochondrially-targeted pentatricopeptide proteins (Pusnik et al. 2007), although the nature of these interactions remains uncertain.

In mitochondria, polyadenylation either stabilizes mRNAs, as in humans, or marks them for degradation, as is the case in plants. However, it turns out that the role of polyadenylation is more complex in *T. brucei*, in which the length of the poly(A) tail appears to be a key determinant of the fate of the molecule. Preedited mRNAs have short poly$(A)_{20–25}$ tails, while never- and fully-edited transcripts have either short poly$(A)_{20–25}$ or long poly$(A/U)_{120–200}$ extensions, in which oligo(U) tracts are interspersed among the poly(A) (Fig. 4) (Militello and Read 2000; Ryan and Read 2005; Etheridge et al. 2008). While the short tail destabilizes preedited molecules, it has the opposite effect in edited mRNAs (Kao and Read 2005). Moreover, another mitochondrial mRNA degradation pathway seems to exist that is independent of the poly(A) tail or UTP (Militello and Read 2000). The exact mechanism of all processing events has to be further explored since some reports claim A/U-tails are destabilizing elements, in which the RET1 enzyme marks the RNA for decay by polyuridylylation (Militello and Read 2000; Ryan and Read 2005).

The 12S rRNA and NADH dehydrogenase subunit 3 genes represent the 5′ ends of the major and minor strands of the maxicircle, respectively, as they are adjacent to the variable sequence domain (Fig. 2a). TbDSS-1, which is a homolog to the eponymous yeast mitochondrial degradosome exonuclease (Penschow et al. 2004), targets aberrant byproducts of these loci, which still contain their unprocessed 5′-ends (Mattiacio and Read 2008). Thus, this enzyme has a role in surveillance of the mitochondrial transcriptome for improperly processed RNAs.

4 Mitochondrial Transfer RNAs

Transfer (t) RNA genes are lacking in the kDNA and thus a complete set of mitochondrial tRNAs has to be imported from the cytosol (Rubio and Alfonzo 2005). The tRNA import system remains, despite intense study, poorly understood. While the requirement for elongation factor 1α and aminoacylation of tRNA for import were rigorously proven (Bouzaidi-Tiali et al. 2007), it was also proposed that thiolation of tRNAs acts as a negative determinant for organellar import in *L. tarentolae* (Kaneko et al. 2003). However, recent data indicate that this is not the case in *T. brucei* (Paris et al. 2009). In a similar vein, the postulated essentiality of the putative tRNA import complex in *L. tropica* (Mukherjee et al. 2007) was not

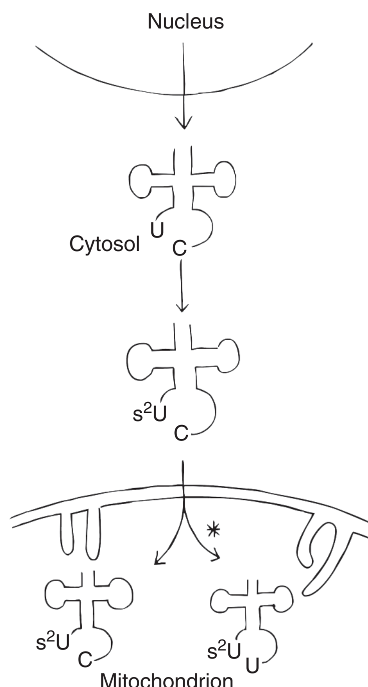

Fig. 5 *Import of tRNAs from the nucleus in T. brucei*. All mitochondrial tRNAs are coded in the nuclear genome and imported into the single mitochondrion from the cytosol. During mitochondrial import, tRNAs are thiolated (s^2U) (at position 33) and subsequently a fraction of the molecules (indicated with an *asterisk*) undergo C to U editing (at position 34)

confirmed in related *T. brucei*, where tRNA import is also independent on membrane potential (Paris et al. 2009). A unique feature of the trypanosomatid tRNA system is C to U editing in the anticodon of a single tryptophanyl tRNA, which allows it to decode the predominantly mitochondrial tryptophan codons (Alfonzo et al. 1999). We have shown recently that thiolation and editing of the neighboring bases in the tRNA molecule are intertwinned processes (Fig. 5) (Wohlgamuth-Benedum et al. 2009).

5 Mitochondrial-Encoded Proteins

Having started with kDNA we browsed through transcription and editing to end the story with translation. Since kDNA codes for components of mitoribosome and subunits of respiratory chain complexes, we are going to limit our protein description mainly to these two areas. For genes encoded on a maxicircle see Fig. 2a.

5.1 Mitochondrial Translation in T. brucei

Mitochondrial ribosomes are of the prokaryotic-type and their rRNA component has been minimized during evolution. In *T. brucei*, mitochondrial ribosomes

contain extremely reduced rRNAs (Zíková et al. 2008a). The protein composition of these mitoribosomes was determined by tandem affinity purification followed by mass spectrometry. The large ribosomal subunit sediments at 50S, while the small subunit sediments at 30S. The analysis identified 133 proteins, of which 77 were associated with the large subunit and 56 with the small subunit (Zíková et al. 2008a, b). Quite a similar protein composition was described in the related trypanosomatid *L. tarentolae* (Maslov et al. 2006). Comparisons of this set of proteins with the bacterial and mammalian mitoribosomal proteins identified a number of homologues of both large and small subunits, although the degree of conservation varied widely. Sequence characteristics of some of the component proteins indicated apparent functions in rRNA modification and processing, protein assembly and mitochondrial metabolism, implying possible additional roles for these proteins.

5.2 Composition of Mitochondrial Respiratory Complexes

The eukaryotic respiratory chain is canonically composed of five multisubunit complexes, commonly termed I thru V (Fig. 6a). As in most eukaryotes, the *T. brucei* complexes contain at least one mitochondrial-encoded subunit, with the exception of complex II (FAD-dependent succinate:ubiquinone oxidoreductase). However, in trypanosomes there are several important departures from this general arrangement, which will be discussed below.

Most of the genes encoded by the kDNA maxicircles belong to complex I (NADH:ubiquinone oxidoreductase) (Fig. 2a). Thus, the core complex, as defined by subunits also present in the bacterial homolog of complex I, is composed of nine mitochondrial-encoded subunits, all of which bind Fe–S clusters as cofactors, and six nuclear-encoded subunits. During the evolution of eukaryotes, complex I gradually acquired more and more proteins, reaching in humans a huge complex of over 40 subunits (Gabaldón et al. 2005). In the nuclear and mitochondrial genomes of *T. brucei*, different authors identified a total of 19 subunits (Opperdoes and Michels 2008; Pagliarini et al. 2008). In frame of an extensive mitochondrial proteome study, 17 obvious homologs of complex I subunits and 12 additional hypothetical conserved proteins associated with this complex were identified (Panigrahi et al. 2009).

No subunits of complex II are encoded in the mitochondrial genome, yet we still mention it here in order to sequentially describe the respiratory chain. This complex has a core composed of four subunits that are shared with prokaryotes. So far, a proteomic study revealed two subunits in *T. brucei* (Panigrahi et al. 2009). This number is certainly not final, since the same complex from *T. cruzi* is composed of at least 12 subunits (Morales et al. 2009).

Following the flow of electrons, the next complex to carry a mitochondrial-encoded subunit is complex III (cytochrome c reductase or complex bc1) (Fig. 6a).

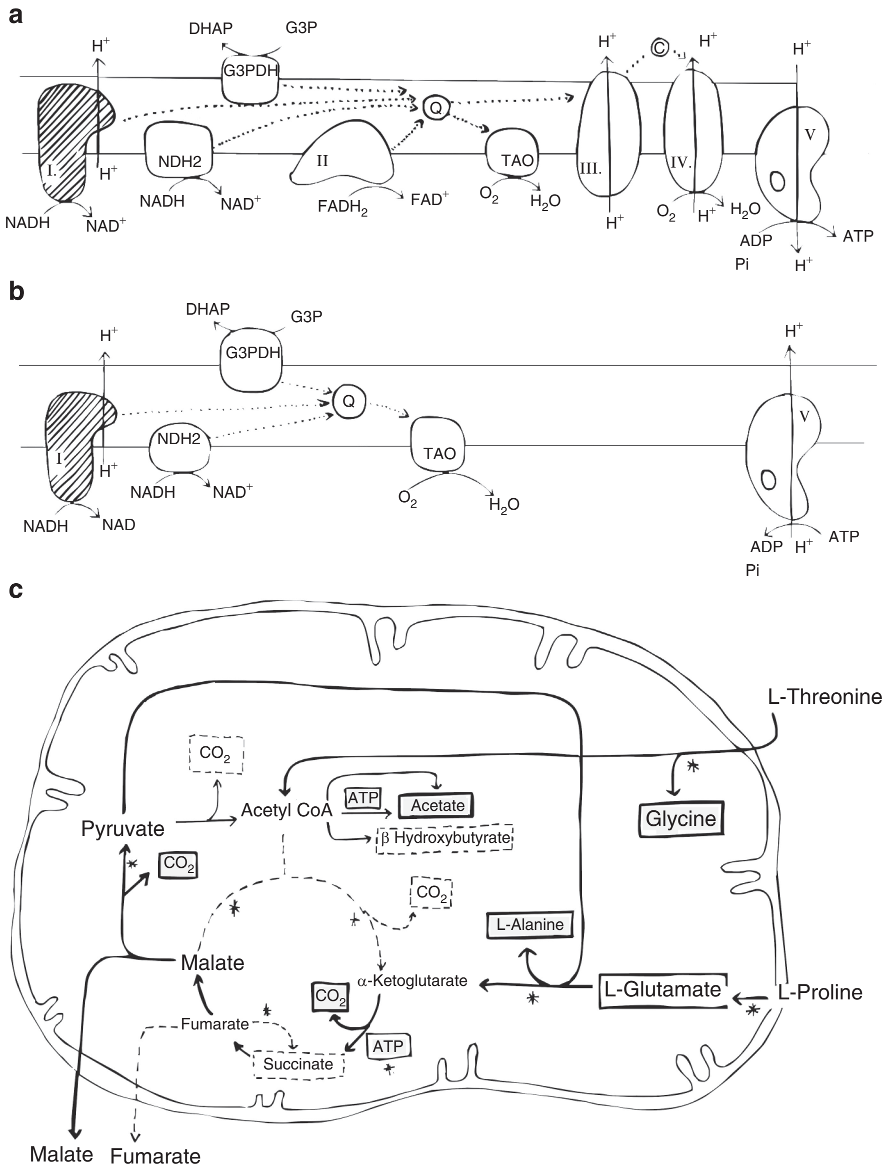

Fig. 6 *Respiratory chain and carbohydrate metabolism in the mitochondrion of T. brucei.* (**a**) *Procyclic stage from the tse-tse fly.* All canonical respiratory complexes (I–V) are physically present. Striped complex I emphasizes uncertainty of its biochemical function(s). Electrons are taken from complexes I and II and two single-peptide enzymes (alternative NADH dehydrogenase [NDH2] and glycerol-3-phosphate dehydrogenase [G3PDH]) and passed to ubiquinone [Q]. From here, about 25% flows to trypanosome alternative oxidase (TAO), the rest goes through cyanide sensitive pathway (complex III to cytochrome c) to complex IV. If not complex I, at least complexes III and IV generate proton-motive force that drives ATP synthesis by complex V. (**b**) *Bloodstream stage from mammalian blood.* Cytochromes (complex III, cytochrome c and complex

The prokaryotic homologue comprises three catalytic subunits, one of them having a Fe–S cluster and the others bearing heme. Eukaryotes added an additional six to eight more proteins to build their mitochondrial complex III (Attardi and Chomyn 1995). The *T. brucei* genome contains three subunits: Rieske Fe–S protein, cytochrome c1, and a putative cytochrome *c* hinge protein homologue. The two former proteins were also identified by mass spectrometry (Panigrahi et al. 2009). Since both of them, together with eight more proteins, were also isolated in the related parasite *L. tarentolae* (Horváth et al. 2000), their homologues are very likely to also be isolated in *T. brucei*. Moreover, experimental evidence is available for the Rieske Fe–S protein being a genuine component of complex III in *T. brucei* (Horváth et al. 2005).

Three subunits of complex IV (cytochrome *c* oxidase) are coded by maxicircle kDNA (Fig. 2a) and constitute the core shared between prokaryotes and eukaryotes. These proteins bear metal prosthetic groups (heme A and copper) that constitute three redox centers. Additional proteins were recruited during the evolution of eukaryotes, forming a complex of up to 13 subunits (Fontanesi et al. 2008). Seven nuclear-encoded subunits were identified in a mitoproteomic survey (Panigrahi et al. 2009).

Finally, complex V (F_1F_0-ATP synthase) requires only a single subunit coded by the organellar genome (Fig. 2a) (Hashimi et al. 2010). ATP synthase from *Escherichia coli* contains eight subunits with various degree of multimerization, while complex V from the yeast and bovine mitochondria contains additional ten subunits. The ATPase of *T. brucei* is more reminiscent of the bacterial homologue, as it is composed of just ten subunits (Zíková et al. 2009). However, since the apparent lack of hydrophobic nuclear-encoded subunits from these proteomic datasets may be due to technical reasons, other approaches are needed to verify the currently known composition of respiratory complexes in trypanosomatids.

Fig. 6 (continued) IV) are missing in this stage. Complex I is shown as we cannot unambiguously rule out its presence. Single-peptide alternative enzymes are present as in the procyclic stage. Notice that TAO is now the only electron sink. Complex V reverses its usual activity and burns ATP to produce proton gradient. (**c**) *"Krebs" cycle of the procyclic stage*. Proline is converted into glutamate that reacts with pyruvate to produce α-ketoglutarate. This follows standard Krebs cycle reactions to produce malate, which is either excreted or converted into pyruvate. Some of the pyruvate are used for production of acetyl coenzyme A. Most of pyruvate reacts with glutamate and closes the cycle. Acetyl coenzyme A is also produced from threonine. Through substrate phosphorylation, acetyl Coenzyme A gives rise to ATP and acetate, a major end-product. Note that there are two reactions leading to acetate; nevertheless, only one of them produces ATP. Part of acetyl coenzyme A is used for β-hydroxybutyrate, a minor end-product. Full lines and boxes depict main metabolic fluxes and end-products under glucose-low conditions; dashed lines and boxes represents minor or background reactions and end-products; asterisks indicate origins of reduced co-factors

6 Energy Metabolism of the *T. brucei* Mitochondrion

T. brucei faces two dramatically distinct environments during its life cycle. The mammalian bloodstream is an environment rich with glucose, where glycolytic ATP production is more than sufficient to support parasite growth. In contrast, the gut of the tse-tse fly is glucose-poor. Despite usage of amino acids extracted from this environment as a primary energy source, glucose is consumed at the same rate as in the bloodstream form (Cazzulo 1992). Amino acids enter the Krebs cycle through different intermediates. Reduced cofactors generated in this process are oxidized by the respiratory chain (Besteiro et al. 2005; van Weelden et al. 2005; Coustou et al. 2008).

6.1 Bloodstream Stage

In the stage that dwells in the bloodstream of vertebrate hosts, the mitochondrion is extremely suppressed. Pyruvate, the substrate of pyruvate dehydrogenase complex, is excreted as a main end-product of metabolism. The Krebs cycle thus has no substrate to use and its enzymes are absent, as are the cytochrome-containing respiratory complexes (Fig. 6b) (Hannaert et al. 2003). The biochemical presence of a genuine mitochondrial complex I remains the subject of an open debate (see Insect stage for details). Nevertheless, the presence of type II alternative NADH dehydrogenase, (Fang and Beattie 2003b) makes complex I expendable, at least for NAD regeneration. This alternative enzyme is represented by a single peptide, which is able to regenerate NAD, but cannot pump protons through the inner mitochondrial membrane (Fig. 6b).

The other electron source of respiratory chain of bloodstreams is mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase. This enzyme connects the mitochondrion with the glycosome via a glycerol-3-phosphate:dihydroxy-acetone phosphate shuttle, which helps to maintain a favorable redox state in the glycosomes. All gathered electrons are sent onto ubiquinone, from where they are subsequently transferred onto molecular oxygen at an enzyme called trypanosomal alternative oxidase (TAO) (Fig. 6b). This alternative oxidase catalyzes the same reaction as respiratory complex IV, but does not use cytochrome *c* as an electron mediator, and no protons are shuffled across the inner membrane (Hannaert et al. 2003).

Taken together, despite an electron flow, any enzymes or complexes that could use this flow to generate proton gradient seem to be absent in the respiratory chain of the bloodstream stage. Still, the proton gradient is indispensable for protein import (Schnaufer et al. 2005). Lacking any classical respiratory complexes to generate the proton gradient, a long lasting question was how bloodstreams cope with this challenge. This conundrum was solved when it was discovered that in the infectious bloodstream stage, complex V is able to reverse its action and maintain

the proton gradient at the expense of ATP consumption (Schnaufer et al. 2005; Brown et al. 2006). Hence, in this pathogenic stage of *T. brucei*, mitochondrion is not the powerhouse of the cell. Quite the contrary, the organelle is as ATP consumer (Fig. 6b).

6.2 Procyclic Stage

In contrast, the procyclic stage from the tse-tse fly contains a mitochondrion that is physiologically and metabolically similar to that found in conventional eukaryotic model systems. All classical complexes are present and most of them appear to be active from a biochemical point of view (Fig. 6a). Although nowadays we have a clear evidence for the physical presence of complex I, or at least a part of it (Panigrahi et al. 2008), its biochemical status remains problematic. Alternative NADH dehydrogenase catalyzes the same reaction and rotenone, a specific inhibitor of complex I in other eukaryotes, was shown to be rather nonspecific in the trypanosomal respiratory chain, failing to distinguish between either dehydrogenase (Hernandez and Turrens 1998). On the other hand, acyl-carrier protein, which is a subunit of complex I, was shown to be actively involved in fatty acids synthesis (Guler et al. 2008). Also, we cannot exclude the possibility that the activity of complex I is strain-dependent. The UC strain of *L. tarentolae* and the 1S LdBob strain of *L. donovani* lost some of their kDNA minicircles, thus losing the capacity to edit some subunits of the respiratory chain, and ultimately resulting in biochemically inactive complexes (Thiemann et al. 1994; Neboháčová et al. 2009).

The activities of the discussed enzymes are highly environment-dependent. Under standard in vitro condition, the procyclic cells are cultivated in a glucose-rich SDM-79 medium. This situation results in conditions under which the mitochondrion seems to be "semiactive," in that all enzymes involved in mitochondrial energy metabolism are present but ATP is mainly derived from glycolysis. The supposed in vivo conditions within the insect midgut can be mimicked by removing glucose from the medium, in which the cells resort to catabolism of amino acids. It is worth noting that such conditions lead to the remarkably higher activities of the respiratory complexes (Coustou et al. 2008).

The glycerol-3-phosphate:dihydroxy-acetone phosphate reaction shuttle, alternative dehydrogenase and alternative oxidase are also present in the procyclic stage (Fig. 6a) (Fang and Beattie 2003b; Guerra et al. 2006). The presence of the first of these three enzymes is considered unambiguous as glycolysis works the same way as in bloodstreams with only a couple of enzymes relocated into the cytosol. However, the presence of alternative dehydrogenase further questions any vital biochemical role of complex I. Similarly, we still have to learn a lot about the role of alternative oxidase. So far the most feasible theory connects its presence with the potential flexibility to cope with a different availability of nutrients (Chaudhuri et al. 2006). It has been also proposed to be retained during

the procyclic stage in order to reduce the level of reactive oxygen species (Fang and Beattie 2003a).

Mitochondrial energy generation is highly dependent on available carbon source (s). In vivo, with amino acids as a primary source, energy is usually obtained through the respiratory chain. Reduced cofactors are derived from reactions leading from amino acids to the intermediates of the Krebs cycle or acetyl-coenzyme A, and from reactions of this cycle itself (Fig. 6c). Proline is converted into glutamate, which enters the Krebs cycle through α-ketoglutarate and is metabolized in the canonical way until malate, which either leaves the mitochondrion and participates in gluconeogenesis, or is converted into pyruvate (Fig. 6c) (Coustou et al. 2008). The pyruvate is partly converted into acetyl-coenzyme A, while most of it is used for reaction with L-glutamate, thus completing the cycle (Fig. 6c) (Coustou et al. 2008).

When glucose is available, the *T. brucei* procyclics derive pyruvate mainly from glycolytic reactions. The ratio between the acetyl-coenzyme A and glutamate reactions is shifted in favor of the former pathway. Moreover, instead of being exported, malate is imported. Again, part of it is used for the production of pyruvate, while the rest goes in a reverse direction, producing succinate instead. Nevertheless, under both conditions a certain amount of ATP is produced by substrate phosphorylation. Acetyl-coenzyme A reacts with succinate producing succinyl-coenzyme A, which is converted back to succinate with concomitant production of ATP (van Weelden et al. 2005; Coustou et al. 2008) (Fig. 6c).

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