

# Iron Metabolism in Pathogenic Trypanosomes

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## 1. Introduction

### 1.1 Unique features, evolution and life cycle of trypanosomes

Trypanosomatids comprise a large group of flagellated unicellular protists with free-living and parasitic lifestyles. Several members of this family are widely known for being vertebrate pathogens of biomedical and veterinary importance. They belong to the order *Kinetoplastida*, which together with the groups *Diplonemida* (deep-sea organisms) and *Euglenida* (photosynthesizing organisms) form the phylum Euglenozoa (Simpson *et al.*, 2006; Roger & Simpson, 2009). Kinetoplastids represent one of the most ancient eukaryotic lineages that diverged after the acquisition of the mitochondrion and share unique biochemical and subcellular features including the nuclear and mitochondrial gene expression mechanism, the energy and thiol redox metabolism and the organization of the mitochondrial DNA, named kinetoplast, and the compartmentalization of the almost entire glycolytic pathway in a peroxisome-like organelle called the glycosome. Phylogenetic studies support the theory that hemoflagellate trypanosomes evolved from a free-living bodonid that subsequently became an insect parasite, which later gained capacity to adapt to mammalian hosts (Adl *et al.*, 2005, 2007; Hamilton *et al.*, 2004; Simpson *et al.*, 2002). An early split of the genus *Leishmania* from the trypanosome taxon together with the existence of two well-defined clades within the genus *Trypanosoma* (the “*brucei*” clade and the “*cruzi*” clade) are strong evidences that the different lineages of pathogenic trypanosomes have evolved independently. Although they share a common ancestor, trypanosomes from the “*brucei*” and “*cruzi*” clades evolved and are geographically confined to the African and New World (South-America and Oceania) continents, respectively. In contrast to members from the “*cruzi*” clade, several lines of evidence indicate that African trypanosomes co-evolved with their mammalian hosts and insect vectors (Hamilton *et al.*, 2007; Maslov *et al.*, 1996; Stevens *et al.*, 1998; Stevens *et al.*, 2001). Parasitic trypanosomes are naturally transmitted by arthropods, *e.g.* tsetse fly for *T. brucei*, kissing bug for *T. cruzi*, and sand fly for *Leishmania* spp. (Fig. 1). Occasionally, bats can also act as vector agents (Dávila & Silva, 2000) and, more recently, rats and ticks are discussed as potentially involved in transmission of *T. cruzi* and *Leishmania* parasites to mammals (Herrera & Urdaneta-Morales, 2001; Colombo *et al.*, 2011; Dantas-Torres, 2011). The natural infectious cycle of these parasites is initiated when non-dividing infective trypanosomes (metacyclic stage) are mechanically introduced in the mammal *via* insect bites or by involuntary deposition of infected feces from insects in the mucosa or injured dermis of mammals. Depending on the trypanosomatid species, parasites can reside in the host dermis (*L. major*), infect mucosa (*L. brasiliensis* and *L. amazoniensis*) or

internal tissues/organs (*T. cruzi*, *L. donovani* and *L. infantum*) or remain in the extracellular fluids (*i. e.*, *T. brucei* ssp.). Inside the mammal hosts, parasites shuttle between proliferative and non-proliferative forms. Indeed, the non-proliferative stages play an important role in the perpetuation of the life cycle since they are pre-adapted to survive in the poikilothermic arthropod (Comini *et al.*, 2011). They are taken up during insect blood meal of an infected host and further differentiate to dividing forms. The transformation to the quiescent infective stages occurs in the gut (*T. cruzi*) or salivary glands (*T. brucei* and *Leishmania* spp.) of the invertebrate. Trypanosomes undergo important metabolic and morphological changes to adapt to the growth conditions imposed by the different hosts and environments (extracellular medium and cell tissues) they inhabit. Fine tuning of energy metabolism, organelle reorganization and dedicated nutrient uptake are some of the mechanisms the parasite exploits to survive in the human host, as will be discussed below.

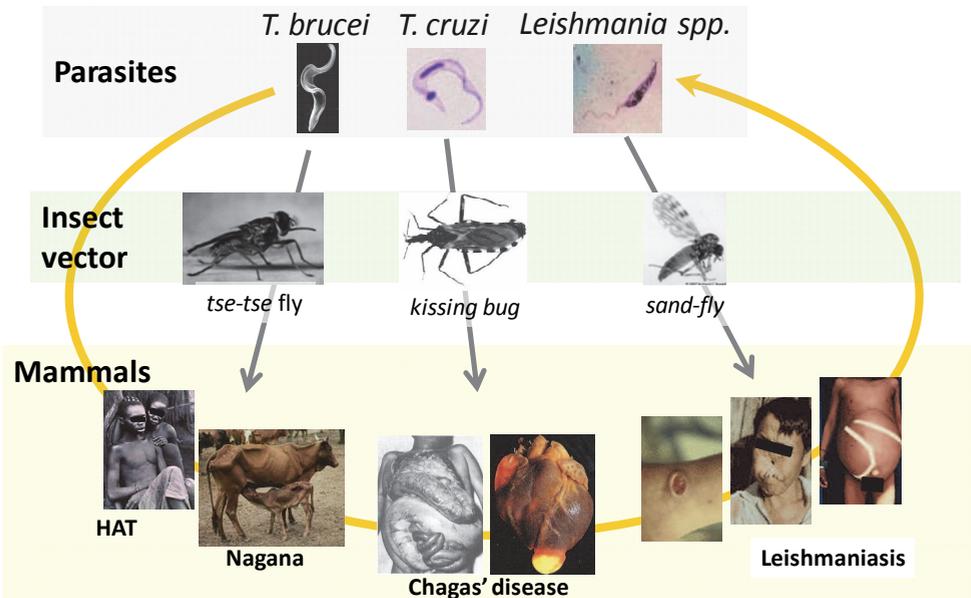


Fig. 1. Life cycle and diseases caused by trypanosomatids.

The genus *Trypanosoma* and *Leishmania* include species that are pathogenic for mammals. The natural cycle of these parasites involves their transmission by different but specific arthropods (insect vectors). Inside the host each species exhibit a peculiar tropism for different tissues, organs and systems resulting in different pathologies. For instance, *T. brucei* is an extracellular parasite that resides in the host's bloodstream and cerebrospinal fluid; different strains of *T. cruzi* invade cardiomyocytes or nervous tissue from the digestive system; depending on the *Leishmania* species, the parasites invade the dermis, mucosa or internal organs (*e. g.*, spleen). The diseases associated with infection are devastating and fatal if left untreated. Human pathogens from the "brucei" complex (*T. b. rhodesiense* and *T. b. gambiense*) cause sleeping sickness or human African trypanosomiasis (HAT; see section1); *T. b. brucei* produces nagana disease of cattle; *T. cruzi* is the etiologic agent of Chagas disease in Latin-America; *L. infantum* and *L. donovani* produces visceral leishmaniasis, *L. major* and *L. amazonensis* causes cutaneous leishmaniasis, and *L. brasiliensis* is responsible for

a mucocutaneous infection. During their life cycle, the parasites undergo important biochemical and ultrastructural remodeling to adapt to the different hosts. The cycle is closed when insect feeds on infected mammals.

## 1.2 Trypanosomiasis: Disease, burden and treatment

Different members of the Trypanosomatida family are etiologic agents of highly disabling and often fatal diseases of humans and livestock (see Fig. 1). With respect to the parasites that are the major focus of this chapter, members from the *T. brucei* complex are transmitted by tsetse flies of the genus *Glossina* spp., which is found exclusively in equatorial Africa. The human pathogens *T. b. gambiense* and *T. b. rhodesiense* differ in their geographical distribution with the first subspecies being endemic of west and central regions while the last is mainly present in east and southern Africa (Barrett *et al.*, 2003).

HAT present two main clinical phases with compromise of the hemolymphatic system (acute phase) and the central nervous system (CNS, chronic phase). The *T. b. rhodesiense* infection develops rapidly as an acute disease that is characterized by a high parasite load, severe anemia and thrombocytopenia accompanied by hypertrophy of the reticuloendothelial system. If untreated, the pathology evolves to a pancarditis with congestive heart failure, pulmonary edema and physiological collapse (Barrett *et al.*, 2003). Due to this fast and lethal development, along with the limited sanitary conditions of endemic regions, HAT caused by *T. b. rhodesiense* is usually under-diagnosed explaining - at least partly - why 90% of the reported cases of African sleeping sickness are ascribed to *T. b. gambiense* (Birkholtz *et al.*, 2011). In contrast, HAT produced by *T. b. gambiense* displays a more discrete development without characteristic symptoms in the early phase, unless the patient develops a generalized lymphadenopathy, which hinders an accurate diagnosis. The second stage of HAT starts when the parasites invade internal organs, including the CNS. For *T. b. rhodesiense* and *T. b. gambiense* this phenomena can take place within few weeks and up to years upon infection, respectively. Invasion of the CNS by the parasite is typically accompanied by intense headache, sleeping disorders and mental dysfunction, leading to a comatose state and sudden death (Barrett *et al.*, 2003). The differential pathogenicity and clinical manifestations of both diseases have been formerly explained on the basis of mutual host-parasite adaptations that shaped pathogen virulence and host resistance during evolution (Fèvre *et al.*, 2006). However, the hypothesis of a co-evolutionary virulence attenuation of *T. b. gambiense* has recently been challenged by studies on phylogenetic relationships within the *T. brucei* taxon (Balmer *et al.*, 2011).

The World Health Organization (WHO) estimates that sixty million people are at risk of infection as a consequence of at least 300 separate active foci in 36 African countries (Jacobs *et al.*, 2011), most of them in rural areas of extreme poverty. Around 300,000 people are currently infected with trypanosomes and 48,000 of them die per year (Cavalli & Bolognesi, 2009). The lack of local human and financial resources combined with the burden of conflicts in most of the endemic countries impedes to achieve full control of HAT (Cavalli & Bolognesi, 2009). However, after continued control programs spanning vector eradication, early diagnosis and treatment, and surveillance (Barrett *et al.*, 2003; Cavalli, 2009) the number of annual infections fell almost 5- to 7-fold in the last three decades<sup>1</sup>. Unfortunately, this progress is not

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<sup>1</sup> <http://www.who.int/mediacentre/factsheets/fs259/en/>

accompanied by the development of new chemotherapeutic options (see below) and is endangered by the increasing drug resistance of the naturally circulating strains of *T. brucei* ssp. (Cavalli & Bolognesi, 2009; Delespaux & de Koning, 2007; Jacobs *et al.*, 2011; Matovu *et al.*, 2001). In addition to HAT, animal trypanosomiasis represents a major problem for the agricultural and nutritional development of endemic regions. About ten million square kilometers of arable land are infested by tsetse flies (Matovu *et al.*, 2001) capable of transmitting *T. b. brucei*, *T. congolense* and *T. vivax* (Nagana-disease) or *T. evansi* (Surra-disease) between domestic and wild (reservoirs) animals. For Africa, the total economic losses due to animal trypanosomiasis are estimated to be US\$ 4.75 billion per year<sup>2</sup>. The recent detection of members from the “*brucei*” clade in countries from Asia, Central- and South-America should raise alarm considering the serious threat these pathogens entails for the well-developed agricultural economies of these regions (Luckins & Dwinger, 2004; Batista *et al.*, 2007, 2009; Da Silva *et al.*, 2011; Dávila & Silva, 2000; Mekata *et al.*, 2009).

Confronted with the lack of prospect for vaccine development against trypanosomiasis, chemotherapy remains as the only short- and mid-term therapeutic choice for these diseases. Nevertheless, the few drugs currently available against HAT (acute phase: pentamidine and suramine, chronic phase: melarsoprol and efluornithine<sup>3</sup>) are far from optimal: most of them were originally developed for veterinary use, lacking safety compliance, and present a limited efficacy against late-stage disease (Steverding, 2010). An additional drawback associated with the inappropriate use of these drugs lies on the emergence of resistance. Unfortunately, pharmaceutical companies are less prone to engage and invest in drug discovery and development against diseases that affect the world’s poorest people (Barrett *et al.*, 2003; Cavalli & Bolognesi, 2009; Matovu *et al.*, 2001). However, in the last years, scientists, policy-makers and non-profit institutions (WHO, TDR, Médecins Sans Frontières, FioCruz Institute, Drugs for Neglected Disease initiative), together with a few pharmaceutical companies (Glaxo SmithKline®, Bayer®), have joined efforts to improve this situation. As mentioned above, trypanosomes present several unique biochemical and biological features that can be exploited for the development of specific therapies. These include several organelles (glycosomes, acidocalcisomes, kinetoplast) that are absent in the mammalian host, and metabolic pathways and cellular functions that differ significantly from host counterparts, namely carbohydrate metabolism, protein and lipid modification, thiol-redox metabolism, cell cycle, programmed cell death, etc. (Naula & Burchmore, 2003). Despite the obvious indispensability of iron for pathogenic trypanosomatids, the mechanisms and components comprising the uptake, storage and usage of this metal have been poorly investigated. In the next sections will be reviewed the state-of-the-art regarding iron-homeostasis and metabolism in African trypanosomes.

## 2. Iron acquisition and homeostasis

Owing the extracellular and parasitic lifestyle of African trypanosomes, it deems important to comment first on the mechanisms and components controlling iron homeostasis in the human host. Dietary ferric iron (Fe<sup>3+</sup>) is reduced to its ferrous form (Fe<sup>2+</sup>) by a ferrireductase

<sup>2</sup> Amounts in terms of agricultural Gross Domestic Product, data taken from: <http://www.fao.org/ag/againfo/programmes/en/paat/disease.html>

<sup>3</sup> It must be noted that efluornithine is active only against *T. b. gambiense* but not *T. b. rhodesiense* (Cavalli & Bolognesi, 2009; Jacobs *et al.*, 2011; Matovu *et al.*, 2001).

present in the membrane of enterocytes, and then incorporated *via* a divalent metal transporter. Cytosolic iron is exported to the circulation from the basolateral membrane of enterocytes through ferroportin with the concomitant reoxidation to  $\text{Fe}^{3+}$  by the multicopper oxidase hephaestin (Kosman, 2010). Iron circulates in plasma bound to the glycoprotein transferrin (Tf), which is internalized by cells *via* a specific transferrin receptor (TfR1) in a clathrin-dependent mechanism. In the acidic environment of the endosome Tf, iron and TfR1 disassemble. Apo-Tf is released to circulation whereas TfR1 is recycled back to the membrane.  $\text{Fe}^{3+}$  is exported from the late endosomal vesicle through the concerted action of transporters and metalloreductases (Hentze *et al.*, 2010; Kurz *et al.*, 2011). How this “labile iron pool” (LIP, Hider & Kong, 2011; Kakhlon & Cabantchik, 2002) is trafficked within the cell remains poorly understood (Hentze *et al.*, 2010; Anderson & Vulpe, 2009; Atanasiu *et al.*, 2007; Hentze *et al.*, 2010; Subramanian *et al.*, 2011). Other major source of iron comprises the recycling of heme-iron from senescent erythrocytes, a task carried out by specialized macrophages of the reticuloendothelial system known as Kupffer cells (Anderson & Vulpe, 2009; Schultz *et al.*, 2010). The liver is both the major site for iron storage and also the central metabolic regulator. Induced by different stimuli –from iron availability to inflammatory stresses– (Zhang & Enns, 2009), hepatocytes produce and secrete a 25-aminoacid hormone, hepcidin (Atanasiu *et al.*, 2007), which regulates the levels of systemic iron by a negative-loop mechanism that involves ferroportin turnover. Intracellular iron homeostasis is regulated post-transcriptionally by the iron regulatory protein 1 (IRP1, see section 3.3.3.1). The vast majority of iron is dedicated to hemoglobin or myoglobin synthesis, but also the biogenesis of iron-sulfur (Fe/S) proteins demands this metal. Both processes occur mostly in the mitochondria, representing the main subcellular compartment for iron utilization (Levi & Rovida, 2009). Iron is stored bound to ferritin heteropolymers, which can hold up to 4500  $\text{Fe}^{3+}$  atoms (Andrews, 2010). Heme-iron is also essential in mammalian physiology (see section 4) but it poses an independent mechanism of absorption that remains mostly unsolved (Schultz *et al.*, 2010). As envisaged, iron homeostasis is tightly controlled at several levels –from absorption to mobilization and utilization– and in an interdependent manner in humans.

Iron is an essential element also for *T. brucei*, therefore the parasite has developed exceptional mechanisms to guarantee metal supply from a host that (un)intentionally limits metal availability in response to infection (see section 5). For example, iron-deficiency induced by ferritin upregulation and reduction of iron-transferrin saturation is a classical immune-based response mounted during the acute phase of the infection to limit parasite proliferation (Chisi *et al.*, 2004). Also the chronic stage is accompanied by a notorious iron deprivation (*e.g.* anemia, see section 5) but, in this case, its origin is a chronic inflammatory disorder involving macrophage hyperactivation along with iron accumulation within the reticuloendothelial system (Stijlemans *et al.*, 2008). In the bloodstream, trypanosomes acquire the metal<sup>4</sup> *via* a high affinity receptor-mediated endocytosis of iron-loaded Tf (see Fig. 2; Grab *et al.*, 1992, 1993; Steverding *et al.*, 1995). The trypanosomal Tf receptor (TfR) was identified as a heterodimeric complex of proteins encoded by the expression-site-associated-gene (ESAG) 6 and ESAG 7 (Salmon *et al.*, 1994; Steverding *et al.*, 1995). ESAG 6 and 7 are truncated forms of variable surface glycoproteins (VSG) that lack the C-terminal domain, and, in the case of ESAG 7, the glycosphosphatidylinositol (GPI) anchor. Under normal growth

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<sup>4</sup> The incorporation of heme-bound iron is discussed in section 4.

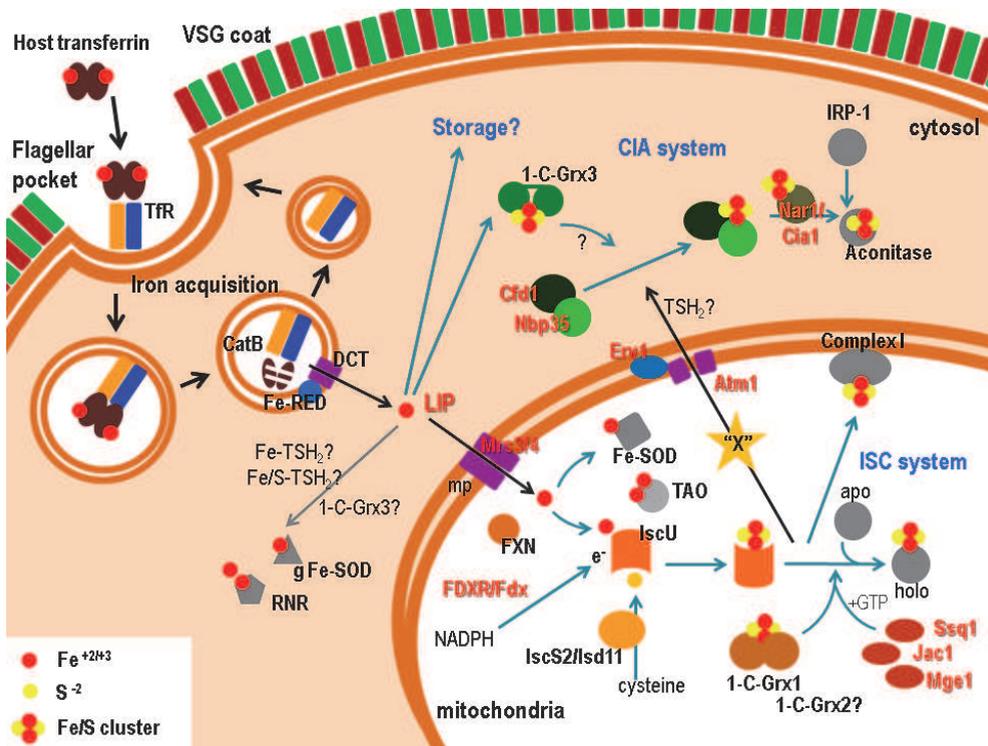


Fig. 2. Working model for iron acquisition and utilization in *Trypanosoma brucei*. Iron is incorporated from the host bloodstream through receptor (TfR) mediated internalization of loaded transferrin (Tf, upper left) at the flagellar pocket. In the late endosomes, Tf is degraded by a cathepsin B-like protease (CatB) and iron is released to the cytosol through the concerted action of a ferric reductase (Fe-RED) and a divalent cation transporter (DCT). Once in the cytosol iron is complexed to low molecular mass ligands forming the labile iron pool (LIP, see section 2) that probably fuels the synthesis of iron-dependent enzymes like the glycosomal superoxide dismutase (gFe-SOD) or ribonucleotide reductase (RNR), and the biogenesis of cytosolic Fe/S clusters (CIA system, section 3.3.1). Mitochondrial Fe/S cluster biogenesis depends on iron imported in a membrane-potential dependent (mp) fashion at expenses of the inner membrane carriers Mrs3/4. Mitochondrial iron is either incorporated to iron-centered enzymes (e.g. Fe-SOD and trypanosomal alternative oxidase, TAO) or participates in Fe/S cluster biogenesis (ISC system, see section 3.3.1.2). Black letters refer to proteins that have been reported in *T. brucei* and red letters denote components of the iron- and Fe/S cluster metabolism not yet identified in trypanosomes but with essential functions in yeast or mammals. CIA proteins are depicted in green tones, ISC proteins in orange-brown tones, membrane transporters in purple and Fe or Fe/S cluster receiving proteins in grey. Fe and sulfur atoms are shown as red and yellow spheres, respectively. Scheme based in Lill & Mühlenhoff, 2008; Hentze *et al.*, 2010 and Taylor & Kelly, 2010.

conditions the TfR localizes at the flagellar pocket wherefrom bound-transferrin is internalized and proteolytically degraded in the endolysosome by a cathepsin-B like protease (O'Brien *et al.*, 2008; Mussmann *et al.*, 2003), which finally releases iron. The relevance of the endocytic pathway as the sole source of iron for the parasite was demonstrated using antibodies anti-TfR that inhibited growth of *T. brucei in vitro* (Grab *et al.*, 1992). Expression of TfR in the parasite is regulated by iron availability and post-transcriptional control that does not involve the IRE/IRP1 system typical for mammals (Fast *et al.*, 1999). Iron starvation induced by iron chelators or species-specific transferrins lead to a 3- to 10-fold upregulation in the expression of TfR with a concomitant redistribution of the receptor from the flagellar pocket to the entire parasite surface (Fast *et al.*, 1999; Mussmann *et al.*, 2004). This, together with the rapid recycling of TfR (Kabiri & Steverding, 2000) and gene-specific (in)activation events (van Luenen *et al.*, 2004) allows trypanosomes to efficiently compete for limiting substrate and withstand iron-deprivation until a new set of higher affinity TfR is expressed. For instance, sequence polymorphisms in ESAG 6 and 7 were proposed to determine the affinities of TfR for transferrins from different mammalian species (Bitter *et al.*, 1998) permitting for a rapid adaptation of the parasite to distinct hosts. It was also suggested that the TfR repertoire may allow the parasite to overcome anti-TfR antibody response by the host (Gerrits *et al.*, 2002). However, estimations and studies by Steverding (1998, 2003, 2006) disproved this hypothesis and supported the view that high affinity TfRs were evolutionary selected to enable the parasite to cope with the diversity of mammalian transferrins (Steverding, 2003, 2006).

In the aerobic and buffered milieu of the cytosol iron is never free, mostly because it can readily produce highly toxic oxygen species through Fenton chemistry (see section 3.2.2), but also because the ferric form tends to form insoluble hydroxides. The cellular labile or chelatable iron pool is defined as the intracellular pool of redox-active iron that is not associated with proteins. LIP comprises ~ 5 % of the total cellular iron and consist of Fe<sup>2+</sup> and Fe<sup>3+</sup> associated with a diverse population of low molecular mass ligands such as organic anions (phosphate, citrate, inositol phosphate, etc.), polypeptides such as glutathione (GSH) and/or components of membranes (phospholipids, etc; Kakhlon & Cabantchik, 2002). Whether this percentage represents the situation in trypanosomes is unknown. The nature of the ligand(s) can vary between cells and among different physiological states but recent reports support the notion that intracellular GSH (2-5 mM) is the most relevant low molecular mass complexing agent in most GSH-dependent organisms (Hider & Kong, 2011; Kumar *et al.*, 2011; Mühlenhoff *et al.*, 2010; Overath *et al.*, 1986). The nature of this LIP in trypanosomes was never assessed and it is worthy to speculate that trypanothione (T(SH)<sub>2</sub>, see below), and not GSH, will be the relevant physiological ligand. Beyond that T(SH)<sub>2</sub> is the most abundant intracellular low molecular mass thiol in trypanosomes (Krauth-Siegel & Comini, 2008), our working model is supported by experimental evidences showing the formation of T(SH)<sub>2</sub>-Fe and T(SH)<sub>2</sub>-Fe/S complexes *in vitro* (Ceylan *et al.* 2010; Manta *et al.*, unpublished, Fig. 2) and by the extensive documentation showing that most thiol-dependent functions in these organisms evolved to use T(SH)<sub>2</sub> and not GSH as cofactor (reviewed in Irigoín *et al.*, 2008; Krauth-Siegel and Comini, 2008). How iron enters and exits the LIP and which are the proteins involved in this process remain as open questions. Recent studies propose a link between the LIP and Fe/S protein metabolism by means of a mechanism that involves Fe/S clusters as signalling molecule and cytosolic monothiol glutaredoxins as mediators (see section 3.2.1; Kumar *et al.*, 2011; Mühlenhoff *et al.*, 2010). In fact, the nature of monothiol glutaredoxins (*i.e.* iron-sulfur proteins that use GSH as ligand

cofactor for Fe/S cluster assembly) make these proteins exceptional candidates for the integration of the cellular iron and Fe/S cluster status with thiol redox status, and as signal transducer regulating iron uptake and utilization (Mühlenhoff *et al.*, 2010; Rodriguez-Manzanaque *et al.*, 2002; Rouhier *et al.*, 2010; Ye *et al.*, 2010). Another important form of “low-molecular mass” iron species are the dinitrosyl iron complexes (DNIC, Bosworth *et al.*, 2009) formed between LIP and low molecular mass thiols when nitric oxide is present (Vanin, 2009). This point is addressed in the next section.

### 3. Cellular fates of Iron

Iron is an important redox or structural cofactor of several indispensable proteins of trypanosomes. For instance, DNA synthesis, protein translation, oxidant defense and cytochrome respiration are important cellular functions that involve the utilization of this metal (Taylor & Kelly, 2010).

In this section we will review the most relevant iron-related molecules and metabolic pathways of bloodstream *T. brucei*. Whenever possible, the components and mechanisms employed by the parasite to incorporate different forms of iron onto target molecules will be described. Taking advantage of the recent availability of genome sequencing data for the most representative species of *Trypanosoma* (Berriman *et al.*, 2005; El-Sayed *et al.*, 2005; Ivens *et al.*, 2005) and the current knowledge of the iron metabolism in model eukaryotes (Hentze *et al.*, 2010), we here provide a state-of-the-art view of iron metabolism in African trypanosomes.

#### 3.1 Dinitrosyl iron complexes

Parasites circulating in host's bloodstream or inside the phagolysosome of activated macrophages are exposed to reactive oxygen and nitrogen species. In both cases, the second messenger nitric oxide ( $\cdot\text{NO}$ ) is produced by endothelial or immune cells, which, if not neutralized rapidly, can lead to the formation of the highly reactive oxidant peroxynitrite (Girard *et al.*, 2005). The effect of  $\cdot\text{NO}$  and derivatives was studied in *T. brucei* (Lu *et al.*, 2011; Steverding *et al.*, 2009; Vincendeau *et al.*, 1992) and *T. cruzi* (Piacenza *et al.*, 2007; Alvarez *et al.*, 2011 and see papers quoted in Irigoín *et al.*, 2008). A recent work by Bocedi *et al.* (2010) demonstrated the formation *in vitro* of a DNIC involving the parasite specific dithiol T(SH)<sub>2</sub>. Based on the high intracellular concentration of T(SH)<sub>2</sub> the authors proposed that formation of this complex may play an important role in trapping  $\cdot\text{NO}$  and, thus, preventing the formation of dangerous oxidants. This work raises the possibility for a new potential link between iron and low-molecular mass thiols in trypanosomes.

#### 3.2 Mononuclear iron proteins

Mononuclear iron proteins can be classified according to their biological function, by Fe centre type, by type and number of prosthetic centres, and by sequence similarity. The iron atom is usually coordinated by thiolate groups (deprotonated form of cysteine), or the N $\delta$  atoms of histidines or the carboxylate anions of acidic residues present in the polypeptide. In these proteins the iron provides redox activity and the surrounding aminoacidic and structural environment confer the specificity for different substrates. The characteristics and functions of a number of iron-centered proteins from trypanosomatids are addressed below.

### 3.2.1 Energy metabolism: Alternative oxidase

The bloodstream form of the parasite lacks cytochrome activity yet they “respirate” at high rates (Priest & Hajduk, 1994). The molecular entity responsible for this is a plant-like mitochondrial ubiquinol oxidase (EC 1.10.3.10), known as trypanosomal alternative oxidase (TAO; Clarkson *et al.*, 1989). The enzyme is imported into the mitochondrion of bloodstream and procyclic parasites by distinct mechanisms involving external ATP supply and inner membrane potential, respectively (Williams *et al.*, 2008). It localizes at the outer membrane of the organelle where it transfers electrons from ubiquinol to oxygen without proton translocation or ATP generation (Chaudhuri *et al.*, 2006), resulting in the reoxidation of NADPH produced during glycolysis. TAO genes have been identified in the genome of all the subspecies forming the “*brucei*” clade but are absent in related trypanosomatids such as *Leishmania* spp. or *T. cruzi* (Chaudhuri *et al.*, 2006; Nakamura *et al.*, 2010). In agreement with its metabolic function (NADP/NAPDH-shunt during carbohydrate catabolism), TAO is developmentally regulated (Chaudhuri *et al.*, 2002) achieving ~100 times higher levels in bloodstream parasites than in procyclic cells (Tsuda *et al.*, 2005; Tyler *et al.*, 1997). The essential role of TAO in the physiology of infective *T. brucei* was recognized early using the iron chelator salicyl hydroxamic acid (2-hydroxybenzhydroxamic acid, SHAM) and glycerol to alter the metabolic output (Clarkson & Brohn, 1976; Grant & Sargent, 1960). On the other hand, recent studies show that TAO plays an important role in preventing oxidant-induced programmed cell death of long-slender bloodstream parasites (Tsuda *et al.*, 2005, 2006). This antioxidant function of TAO resembles that proposed for the orthologue enzyme from plants (Maxwell *et al.*, 1999). In trypanosomes, apoptosis is a highly regulated process deeply associated with the accumulation of quiescent parasite forms (short stumpy) in the preparation for transmission to the insect vector (Welburn *et al.*, 2006). It is therefore tempting to speculate that, at least in bloodstream trypanosomes, TAO might be an important checkpoint connecting metabolic status (NADP<sup>+</sup>/NAPDH ratio) with programmed cell death and differentiation. In procyclic forms, TAO has been shown to compensate for a depletion of complex III or IV activities in the mitochondrial electron transfer chain (Horváth *et al.*, 2005). Despite this backup role in respiration, the enzyme appears to fulfill a yet unknown but essential function in this parasite stage (Tsuda *et al.*, 2005; Tyler *et al.*, 1997).

Although no structures are available for TAO or any other related alternative oxidase, current structural models (Moore & Albury, 2008) and proteomic data (Acestor *et al.*, 2009) indicate that it is an interfacial membrane protein that interacts with a single leaflet of the lipid bilayer, and contains a non-heme di-iron carboxylate center bounded by two highly conserved EXXH motifs (Kido *et al.*, 2010a, 2010b). The iron-dependence of TAO was established working *in vitro* with chelating agents and mutant forms of the protein (Ajayi *et al.*, 2002; Chaudhuri *et al.*, 1998).

### 3.2.2 Oxidant defense: Iron-dependent superoxide dismutases

Aerobic respiration produce partly reduced oxygen intermediates that leak from several protein complexes from the electron transport chain. The most important radical species formed is the anion superoxide (O<sub>2</sub><sup>•-</sup>), the one-electron reduction product of molecular oxygen. Superoxide can reduce or oxidize biological targets *in vivo*, or dismutate to the less reactive but highly diffusible oxidant hydrogen peroxide (Halliwell & Gutteridge, 1999). Despite hydrogen peroxide is biologically more toxic than O<sub>2</sub><sup>•-</sup>, active removal of this anion

radical is necessary to prevent formation of the most reactive and harmful radical product, hydroxyl radical (OH<sup>•</sup>), which originates from a physiological reaction involving iron and known as Fenton reaction<sup>5</sup>. To accomplish this, all living organisms contain enzymes devoted to the O<sub>2</sub><sup>•-</sup> dismutation, called superoxide dismutases (EC 1.15.1.1). There are three major families of SODs, depending on the metal cofactor. Most of the cytosolic eukaryotic SODs use a bimetallic active site with copper and zinc (CuZn-SODs) while the mitochondria harbor a bacterial-related Mg-dependent SOD. In prokaryotes and plastids most of the SODs are Fe-dependent (Abreu & Cabelli, 2010).

Trypanosomes express four different isoforms of SODs (Dufernez *et al.*, 2006; Kabiri & Steverding, 2001; Le Trant *et al.*, 1983; Wilkinson *et al.*, 2006) that, in contrast to homologues from prokaryotes and eukaryotes, show a restricted metal dependency all of them being Fe-dependent enzymes (Wilkinson *et al.*, 2006; Bachega *et al.*, 2009). In *T. brucei*, SOD-A and SOD-C localize at the mitochondrion whereas SOD-B1 and SOD-B2 are mostly compartmentalized within the glycosome and less abundantly at the cytosol (Dufernez *et al.*, 2006). A similar localization was reported for SOD-B enzymes from *L. chagasi* (Plewes *et al.*, 2003). RNAi-mediated knockdown of SOD-A and SOD-C revealed that under normal growth conditions the mitochondrial isoforms are dispensable for bloodstream *T. brucei* (Prathalingham *et al.*, 2007; Wilkinson *et al.*, 2006). However, the biological importance of SOD-A was put in evidence when parasites depleted in this isoform showed a higher sensitivity towards paraquat, an O<sub>2</sub><sup>•-</sup>-generating compound (Wilkinson *et al.*, 2006). Consistent with a role in oxidant defense for mitochondrial Fe-SOD, transgenic *T. cruzi* overexpressing a mitochondrial isoform was found to be more resistant to fresh human serum, a death stimuli mediated by oxidative stress (Piacenza *et al.*, 2007). In contrast, the SOD B-type enzymes are indispensable for infective *T. brucei* grown under normal culture conditions (Wilkinson *et al.*, 2006), which poses the question to the glycosomal source of O<sub>2</sub><sup>•-</sup>, a charged molecule that does not diffuse through lipid membranes. In this respect, unwanted O<sub>2</sub><sup>•-</sup> might leak as byproduct from a variety of metabolic activities occurring in this organelle, such as glycolysis, oxidation of fatty acids, lipid biosynthesis, and purine salvage (Michels *et al.*, 2006). Further dissection of the functional relevance of SOD-B isoforms was achieved by means of targeted gene replacement, which demonstrates that SOD-B1 and not SOD-B2 is critical to withstand exposure to nifurtimox and benznidazole (Prathalingham *et al.*, 2007), two anti-trypanosomal drugs whose mechanism of action involves the generation of reactive oxygen intermediates (Maya *et al.*, 2003 and 2007). Also the homologue isoforms present in *L. chagasi* and *L. tropica* were earlier reported to be important for parasite survival in mouse or human macrophages as well as under paraquat insult (Plewes *et al.*, 2003; Ghosh *et al.*, 2003). Interestingly, *T. brucei* SOD-B1 has been shown to be developmentally regulated with higher intracellular concentration in proliferating stages (Kabiri & Steverding, 2001). This particular expression pattern led the authors to propose that the role of SOD-B1 in dividing cells is to counteract the formation of superoxide radicals released during the generation of the iron-tyrosyl free-radical centre in the small subunit (R2) of ribonucleotide reductase, other iron-containing enzyme (see next section). Unexpectedly, a 5- to 8-fold increase in SOD-B1 activity in transgenic *T. cruzi* was accompanied by a significant sensitization of parasites against two pro-oxidant compounds namely gentian violet and benznidazole (Temperton *et al.*, 1998). This striking behavior was



interpreted as a consequence of an imbalance in the redox homeostasis of the parasite resulting from the overexpression of Fe-SOD, a hypothesis that deserves further investigation.

In summary, trypanosomal SODs diverged from their human homologues by using iron as cofactor. They apparently evolved to protect parasites against toxic  $O_2^{\cdot-}$  produced in the glycosomes and/or mitochondrion as a result of sudden changes in metabolism or originated from the different environment they live in (*e.g.*, insect midgut, macrophages, epithelium, mammal bloodstream).

### 3.2.3 Cell proliferation: Ribonucleotide reductase

Ribonucleotide reductase (RNR, EC 1.17.4.1-2) catalyses the reduction of ribonucleotides to deoxyribonucleotides needed for DNA synthesis. There are three different classes of RNR being class I the most abundant in eukaryote organisms. Class I RNR are heterotetrameric enzymes formed by the association of two related but not identical polypeptides known as subunit R1 and R2. The large R1 subunit binds substrates and allosteric effectors, conferring specificity and regulatory potential, while the small R2 subunit contains the catalytic center composed of two high spin  $Fe^{3+}$  atoms antiferromagnetically coupled to each other through a  $\mu$ -oxo bridge, a highly conserved tyrosine residue and two cysteines (Cotruvo & Stubbe, 2011). The di-iron center generates a free radical on this catalytic tyrosine through electron donation. In a reaction involving several intermediate states, the tyrosine radical attacks the nucleotide resulting in reduction of the 2'-OH group of ribonucleoside and the formation of a disulfide. Regeneration of active RNR is finally achieved *via* reduction of this disulfide mainly by thioredoxin (Trx), which is subsequently reduced at expenses of NADPH (Cotruvo & Stubbe, 2011).

The biochemical information about parasite RNR is rather limited. Both subunits of RNR from *T. brucei* were cloned, expressed (Dormeyer *et al.*, 1997; Hofer *et al.*, 1997) and kinetically characterized (Hofer *et al.*, 1998). The parasite-specific thioredoxin-like oxidoreductase tryparedoxin (TXN, Lüdemann *et al.*, 1998) and, in contrast to all other RNR from eukaryotes, also the low-molecular weight dithiol T(SH)<sub>2</sub> (Dormeyer *et al.*, 2001) but less likely glutaredoxins (Ceylan *et al.*, 2010) proved to be physiological reductants of recombinant RNR. The activity of the enzyme appears to be post-transcriptionally regulated by a redox mechanism (Dormeyer *et al.*, 2001) and by the selective expression of its catalytic subunit R2. For example, whereas the R1 protein is actively expressed throughout the whole life cycle of the parasite, the R2 protein is not detected in cell cycle-arrested short stumpy trypanosomes (Breibach *et al.*, 2000). How iron is incorporated into the R2 subunit is yet elusive (Cotruvo & Stubbe, 2011). Recent findings suggest that, at least in yeast, the di-iron non-heme incorporation into apo-proteins is tightly related to the Fe/S cluster biogenesis machinery both from the cytosol and mitochondria (see section 3.3) (Cotruvo & Stubbe, 2011; Mühlhoff *et al.*, 2010) and in particular to the cytosolic monothiol glutaredoxins (Grx3/4), discussed later in this review (see section 3.3.2). In trypanosomes, the only link between iron and RNR came from experiments in which parasites treated with the iron chelator deferoxamine (DFX) show an extremely reduced [<sup>3</sup>H]-thymidine incorporation, pointing to an essential role of iron in DNA synthesis (Breibach *et al.*, 2002).

### 3.2.4 Lipid biosynthesis: Stearoyl-CoA desaturase

Stearoyl-CoA desaturases (SCD) from eukaryotes are di-iron containing proteins responsible for the *de novo* synthesis of monounsaturated fatty acids from saturated fatty acids. The iron is usually coordinated by 8 histidine residues that form the active site (Man *et al.*, 2006). They are integral membrane proteins anchored in the endoplasmic reticulum. Recent genetic and chemical validation of the orthologue enzyme from *T. brucei* demonstrated its indispensability for *in vivo* survival of bloodstream forms, as well as for the procyclic stage (Alloati *et al.*, 2010, 2011).

### 3.3 Iron-sulfur cluster proteins<sup>6</sup>

The name iron-sulfur proteins refer to a broad group of proteins. A class of them contains mononuclear Fe centers coordinated directly by cysteine residues (e. g., rubredoxins and related proteins). A second class ligates a complex between iron and inorganic sulfur through side-chain atoms provided mainly by the amino acid cysteine, or eventually histidine or aspartate. These centers are known as Fe/S clusters (Lill & Mühlenhoff, 2008). Fe/S centers are ubiquitous inorganic cofactors present in all forms of life and are probably the most ancient cofactors and catalysts in the prebiotic world. Among them, the [2Fe-2S] is the simplest and most common cluster found *in vivo*, whereas Fe/S clusters of higher complexity require further “maturation” (Lill & Mühlenhoff, 2008; Lill, 2009; Py & Barras, 2010). In eukaryotes, the list of Fe/S dependent proteins has nearly one hundred members (Rouault & Tong, 2005; Ye & Rouault, 2010). In general, this cofactor enables electron transfer reactions due to the propensity of the iron atoms to switch between reduced (Fe<sup>2+</sup>) and oxidized (Fe<sup>3+</sup>) states. In addition, the redox potential of the cluster can be finely tuned by the protein environment covering a wide range of potentials, from very reducing (~-500 mV) to highly oxidizing (~+300 mV) (Lill, 2009; Xu & Møller, 2011). In consequence, Fe/S clusters are essential components of the most important biological electron transport chains, *i.e.* photosynthesis and mitochondrial respiration. But the role Fe/S clusters can play in proteins goes far beyond their redox properties. For instance, they act as important structural or regulatory moieties of some proteins from the DNA metabolism and as cofactors in enzymes of the amino acid biosynthesis or Krebs cycle (Lill & Mühlenhoff, 2006; Lill, 2009; Netz *et al.*, 2010; Py & Barras, 2010; Ye & Rouault, 2010a, 2010b).

#### 3.3.1 Biogenesis of iron-sulfur clusters and proteins

Despite the chemical simplicity of these cofactors, their biosynthesis and insertion into apoproteins within the cell requires devoted machineries that are highly conserved from bacteria to humans (Lill & Mühlenhoff, 2006, 2008; Xu & Møller, 2011). In bacteria, there are three different systems for the biogenesis of Fe/S proteins, all encoded in specific operons and tightly regulated. The “nitrogen fixation” (NIF) system was the first Fe/S cluster biogenesis mechanism described and is exclusively dedicated to the maturation of nitrogenase enzymes from certain bacterias. On the contrary, the “iron-sulfur cluster” (ISC)

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<sup>6</sup> Due to the high amount of literature on this topic, this section contains mainly quotations to the most recent reviews, facilitating further reading for interested lectors. We therefore apologize to the authors of the original contributions.

and “sulfur utilization factor” (SUF) systems are widely distributed among bacteria and are responsible for the biosynthesis of Fe/S proteins in basal and stressed conditions, respectively (Xu & Møller, 2011). The mitochondria and plastids of eukaryotes have inherited the ISC and SUF systems, respectively, from ancient symbionts (Balk & Lobréaux, 2005; Balk & Pilon, 2011; Xu & Møller, 2011). Additionally, maturation of Fe/S cluster proteins in eukaryotes can be accomplished in the cytosol by a specific set of proteins that constitute the cytosolic iron sulfur cluster assembly (CIA) machinery. In this section we will summarize the most relevant aspects of the mitochondrial machinery related to the bacterial ISC system, and will introduce the limited information available for the CIA system. Most of the reports published stem from studies with the budding yeast *Saccharomyces cerevisiae*, and we will refer to them before describing what is known for Fe/S protein biogenesis in trypanosomes (Section 3.3.2).

The building blocks for the biosynthesis of Fe/S clusters are: iron atoms (in the reduced form, Fe<sup>2+</sup>), sulfide (S<sup>2-</sup>), reducing power and proteins, which act as scaffolds to assemble the Fe/S cluster that is being formed (Py & Barras, 2010; Lill & Mühlhoff, 2006). Sulfide is generated from cysteine by the pyridoxal-5-phosphate-dependent cysteine desulfurase (eukaryotic Nfs1 or the bacterial NifS, IscS and SufS). This reaction produces alanine and leaves a protein-bound cysteine persulfide intermediate, which is presumably reduced by the ferredoxin system (NADPH-dependent ferredoxin reductase and ferredoxin) leading to release of S<sup>2-</sup>. Ferredoxin is a Fe/S protein that in mammals is also known as adrenodoxin, an essential component of the thyroid hormone production. Recent studies identified an accessory protein, Isd11, that is essential for *in vivo* Fe/S biogenesis in eukaryotes (Adam *et al.*, 2006; Shi *et al.*, 2009; Wiedemann *et al.*, 2006). Isd11 forms a tight complex with Nfs1 that renders the cysteine desulfurase active (Xu & Møller, 2011). The iron-carrying protein Yfh1, known in humans as frataxin or CyaY in bacteria, participates in the delivery of iron for Fe/S cluster biosynthesis but whether this protein is or not the direct iron-carrier is still a matter of debate. Frataxin dysfunction is responsible for the most common form of inherited ataxia in humans, namely “Friederich ataxia” (Py & Barras, 2010; Lill & Mühlhoff, 2006, 2008; Stemmler *et al.* 2010). The next step involves the transient assembly of the newly synthesized Fe/S cluster into “scaffold” proteins. These macromolecules are ubiquitous to most living organisms and contain conserved cysteine residues that bind Fe/S clusters in a labile manner, allowing the complex to be readily transferred to target proteins. The most conserved ones are the U-type scaffold proteins (*e.g.*, bacterial IscU and SufU and eukaryotic Isu1), and other include the bacterial NifU, plastid NFU and the A-type scaffolds (*e.g.*, IscA and SufA; Lill, 2009; Xu & Møller, 2011), which are supposed to work downstream the U-type ones or to subrogate their function in specific situations (*e.g.*, heme biosynthesis in mammals; Ye & Rouault, 2010b). Whereas the mitochondrial ISC system has more than 15 proteins identified (Lill & Mühlhoff, 2008), Nfs1-Ids11, Isu1/Isu2 and Yfh1 are the core elements for Fe/S cluster biogenesis in eukaryotic mitochondria (Fig 2; Lill & Mühlhoff, 2006).

The transference of the Fe/S cluster to the target apoprotein is assisted by a set of proteins and consumes energy (Amutha *et al.*, 2008; Subramanian *et al.*, 2011). The minimal components are: a dedicated chaperone from the HSP70 family termed Ssq1, the DnaJ-like chaperon Jac1, the nucleotide exchange factor Mge1 and a mitochondrial monothiol

glutaredoxin (Grx5 in humans and yeast; Fig. 2). The requirement for a glutaredoxin-like protein, formerly considered an oxidoreductase (Lillig *et al.*, 2008; Rouhler *et al.*, 2010), in Fe/S cluster biogenesis was initially suggested by studies in a yeast Grx5 deletion mutant. These cells displayed deficient cluster assembly for at least two Fe/S proteins (aconitase and succinate dehydrogenase), leading to impaired respiratory growth and increased sensitivity to oxidative stress with accumulation of free iron in the cell (Rodríguez-Manzanique *et al.*, 1999 and 2002). Although the precise function of monothiol glutaredoxins within the iron metabolism remain to be established, they are critical components of the ISC biosynthetic pathway in distantly related organisms such as bacteria (Fernandes *et al.*, 2005), protists (Comini *et al.*, 2008) and vertebrates (Xu & Møller, 2011; Ye *et al.*, 2010; Ye & Rouault, 2010b).

The CIA machinery was first discovered in yeast when seeking for cytosolic proteins essential for Fe/S cluster assembly on aconitase (Fig. 2). Nowadays, several components are recognized: i) two P-loop NTPases (Cfd1 and Nbp35) that form a stable heterotetrameric complex with a [4Fe-4S] cluster bound to the C-terminal region, suggesting a scaffold role for cluster assembly; ii) Nar1, a Fe/S-dependent protein related to bacterial iron-containing hydrogenases, iii) WD40  $\beta$ -propeller protein Cia1, which preferentially localizes in the nucleus and assists cluster delivery to Nbp35 (Lill & Mühlhoff, 2008; Sharma *et al.*, 2010; Xu & Møller, 2011), and iv) redox components of the CIA machinery (Tah18, Dre2 and monothiol glutaredoxins 3 and 4). In yeast, intracellular iron level is sensed by the transcription factor Aft1 and requires the regulatory proteins Fra1-Fra2 that interact with the cytosolic-nuclear monothiol glutaredoxins Grx3 or Grx4 through the formation of heterodimers linked by a Fe/S cluster (Kumánovics *et al.*, 2008; Mühlhoff *et al.*, 2010; Pujol-Carrion *et al.*, 2006). Interestingly, besides the existence of specific and compartmentalized machineries, Fe/S cluster biogenesis in the cytosol has been confirmed to depend on the mitochondrial system. A still unknown essential compound (noted as "X" in Fig. 2) is exported from mitochondria *via* the inner mitochondrial membrane ATP-binding cassette (ABC) transporter Atm1 and the intermembrane space protein Erv1, in a process that requires GSH (Netz *et al.*, 2010; Sharma *et al.*, 2010; Xu & Møller, 2011). As pointed by Ye and Rouault (2010a, 2010b) the next frontier in Fe/S cluster biogenesis will consist in unraveling how the monothiol glutaredoxin 5 and ABCB7 transporter are involved in the mitochondrial synthesis and export to the cytosol/nucleus of a still unknown factor that connects these processes in both compartments. Moreover, it remains to be studied how the system operates as a whole (Mühlhoff *et al.*, 2010; Netz *et al.*, 2010; Zhang *et al.*, 2008).

### 3.3.2 Biogenesis of iron-sulfur clusters in trypanosomes

The characterization of the Fe/S biogenesis in trypanosomes was driven by the completion of the genome sequencing of three trypanosomatid species (Berriman *et al.*, 2005; El Sayed *et al.*, 2005; Ivens *et al.*, 2005). As noted previously, the mitochondrion is a key organelle in Fe/S cluster biosynthesis that undergoes important metabolic and morphological changes in trypanosomatids, especially in African trypanosomes, during life cycle. For instance, several mitochondrial respiratory-chain proteins that depend on these cofactors are developmentally synthesized in insect stage parasites (Alfonzo & Lukeš, 2011; Tyler *et al.*, 1997). Based on sequence comparison with identified Fe/S cluster biogenesis proteins from yeast, plants and humans, a number of members of the ISC system are conserved in

trypanosomatids (see Fig. 2). Smíd *et al.* (2006) characterized two major components of this machinery present in *T. brucei*: a cysteine desulfurase (*TbIscS2*, related to *Nfs1*) and one U-type scaffold protein (*TbIscU*, related to *Isu1*). Both proteins proved to be essential for the mitochondrial biogenesis of Fe/S proteins (Smíd *et al.*, 2006). In agreement with phenotypes observed in yeast mutants, down-regulation of *TbIscS2* in procyclic parasites by RNAi results in inhibition of Fe/S cluster dependent processes both in the mitochondrion and cytosol, with a concomitant impairment in ATP production, cellular respiration and growth (Smíd *et al.*, 2006; Paris *et al.*, 2009). The occurrence of this protein in the rudimentary mitochondrion of bloodstream parasites was still not addressed but appears reasonable considering that this stage relies on important Fe/S protein activities (Comini *et al.*, 2008). As mentioned above, a stable tricomponent system between *Nfs1*, *Isd11* and *IscU* have to be formed to enable the formation of Fe/S cluster on *IscU*. An *Isd11* homologue is actively expressed by both life stages of *T. brucei* and, as expected, is critical for cytosolic and mitochondrial Fe/S cluster biosynthesis in trypanosomes (Paris *et al.*, 2010). A second *Nfs*-like gene was identified in *T. brucei* (Smíd *et al.*, 2006) and characterized as a selenocysteine lyase (SLC; Poliak *et al.*, 2010), an enzyme that cleaves selenocysteine into alanine and selenium during selenoprotein metabolization. Supporting the experimental evidences on the dispensability of the selenoproteome for this parasite (Aeby *et al.*, 2009), down-regulation of SLC is not detrimental for *T. brucei*, at least under cultivation (Poliak *et al.*, 2010). A single-copy gene with a significant sequence similarity to eukaryotic frataxin was identified in *T. brucei* genome (*TbFXN*; Long *et al.*, 2008b). Abrogation of frataxin function in the mitochondrion of procyclic cells induces a growth-retardation phenotype with a marked inhibition of Fe/S-dependent processes, *e.g.*, aconitase activity (Long *et al.*, 2008a, 2008b). Interestingly, *TbFXN* was also identified in the bloodstream form of the parasite but its biological relevance was not addressed (Long *et al.*, 2008b). Works with the fission yeast indicate that thiolation of tRNA depends on components from the mitochondrial and cytosolic ISC systems (Lill & Mühlhoff, 2006, 2008). A similar link between both pathways has recently been disclosed for trypanosomes (Alfonzo & Lukeš, 2011; Bruske *et al.*, 2009). More recently, two A-type scaffold proteins from *T. brucei* were characterized and their function rated as essential for the growth and Fe/S cluster metabolism of procyclic but not of bloodstream parasites (Long *et al.*, 2011), suggesting specificity for Fe/S protein acceptors that are developmentally regulated. The final step in mitochondrial Fe/S cluster biogenesis consists in the delivery of the cluster to acceptor proteins, in an ATP-dependent process assisted by mitochondrial HSP70 and monothiol glutaredoxins, interestingly, the last being Fe/S proteins (Comini *et al.*, 2008; Manta *et al.*, unpublished). Three sequences for putative monothiol glutaredoxins, named 1-C-Grx1 to 3, are present in the genome of different trypanosomatids (Berriman *et al.*, 2005; El Sayed *et al.*, 2005; Ivens *et al.*, 2005). The proteins from *T. b. brucei* are the best studied so far. 1-C-Grx1 and 1-C-Grx2 localizes at the parasite mitochondrion (Filser *et al.*, 2007; Comini *et al.*, 2008; Manta *et al.* unpublished), whereas 1-C-Grx3, a hybrid protein containing an N-terminal Trx domain, is probably cytosolic. 1-C-Grx1 and 1-C-Grx3, but not 1-C-Grx2, are abundant proteins in bloodstream parasites and reach maximum levels in stationary phase cultures (Comini *et al.*, 2008). Strikingly, targeting these proteins to the mitochondria of *S. cerevisiae* defective in *Grx5* did not rescue the mutant phenotype, suggesting the existence of structural or biochemical differences between the trypanosomal and eukaryote/prokaryotes orthologues (Filser *et al.*, 2007). Indeed, *in vitro* all three proteins are capable to coordinate an Fe/S cluster at expenses of a protein thiol and low molecular mass thiols that are parasite-specific, namely T(SH)<sub>2</sub> and

monoglutathionylspermidine (Comini *et al.*, 2008; Manta *et al.* unpublished). We observed a similar behavior for *T. cruzi* 1-C-Grx1 (Fleitas *et al.*, unpublished). 1-C-Grx1 is indispensable for infective *T. brucei* with an important function in parasite iron homeostasis and no role in protection against oxidants (Comini *et al.*, 2008; Manta *et al.*, unpublished), as previously proposed for yeast Grx5 (Rodriguez-Manzaneque *et al.*, 1999). Overexpression of a functional apo-mutant of 1-C-Grx1 impairs parasite survival inside an animal host (Manta *et al.*, unpublished). Also overexpression of a wildtype form of 1-C-Grx1 was detrimental for parasite survival either *in vitro* (under iron deprivation or oxidative stress conditions) and *in vivo* (mice), indicating that iron homeostasis in African trypanosomes is tightly controlled with this protein playing an important role. Altogether, this provides the first evidence for a critical physiological role of iron and Fe/S cluster metabolism for *T. brucei* survival during infection. The information concerning Fe/S cluster biogenesis in the cytosol of trypanosomes is very scarce. So far, only a putative orthologue of ABCB7 was identified in *T. brucei* genome (Sauvage *et al.*, 2009) and, as outlined above, the occurrence of a putative cytosolic monothiol glutaredoxin orthologue of yeast Grx3/Grx4 was described (Smíd *et al.*, 2006; Filser *et al.*, 2007; Comini *et al.*, 2008). Further investigations are required to establish whether trypanosomal 1-C-Grx3 shares a regulatory function on iron- and iron-sulfur-homeostasis as their yeast counterparts.

As noted by Long *et al.* (2011), following the identification of several components of the ISC system in *T. brucei* and owing to the high degree of evolutionary conservation for this important pathway it is reasonable to assume a similar complexity for the Fe/S cluster assembly machinery in this unicellular eukaryote with respect to that of yeast or even human. Nevertheless, certain structural or biochemical specialization in some of its molecular components may be envisaged (Filser *et al.*, 2007; Comini *et al.*, 2008; Manta *et al.*, unpublished). The regulation of Fe/S biogenesis in trypanosomes is equally unexplored yet. Considering the Fe/S metabolic repertoire of each developmental stage of the parasite, it makes sense to consider multiple and inter-dependent mechanisms controlling substrate supply, Fe/S-biosynthesis and turnover, all important issues that await elucidation. Another challenging question raised by Smíd *et al.* (2006) deals with the identification of the factor(s) triggering Fe/S-cluster and -protein synthesis during differentiation.

### 3.3.3 Examples of iron-sulfur proteins in trypanosomes

#### 3.3.3.1 Aconitase

The enzyme aconitase (EC 4.2.1.3) is an essential component of the mitochondrial tricarboxylic acid cycle that catalyzes the reversible conversion of citrate to isocitrate through the intermediary formation of the tricarboxylic acid *cis*-aconitate. Aconitase contains an [2Fe-2S] center that acts both in substrate binding and catalytic addition or removal of H<sub>2</sub>O, but not in electron transfer reactions. In addition to this enzymatic activity, cytosolic isoforms of apo-aconitase participate in regulation of cellular iron homeostasis, therefore, receiving also the name of iron-regulatory proteins (IRP). In its apo-form (non-cluster bound) aconitase undergoes a conformational change that confers the protein with affinity to bind the 3'- or 5'- untranslated regions, known as IRE (iron-response elements), present in the mRNA encoding for several proteins related to iron uptake and metabolism. Under iron starvation the ratio apo:holo aconitase increases and, hence, IRP

activation takes over the post-translational response that triggers iron mobilization, uptake and utilization (Hentze *et al.*, 2010).

As mentioned previously, the bloodstream form of *T. brucei* possess a rudimentary mitochondrion with most enzymes from the Krebs cycle repressed (Saas *et al.*, 2000). In this parasite, aconitase is encoded by a single gene whose sequence resembles that of the cytosolic isoform of mammalian aconitase (Fast *et al.*, 1999; Saas *et al.*, 2000). The protein localizes within both the cytosol and mitochondrion (Saas *et al.*, 2000). While the mRNA abundance is almost unaffected during development, the protein content increases several times during transformation from dividing to arrested bloodstream form (Overath *et al.*, 1986), in concordance with the morphological and biochemical events that occur in the preparation of the parasite to differentiate to the insect stage (Saas *et al.*, 2000). The protein is not essential for infective or procyclic parasites (van Weelden *et al.*, 2003) neither involved in the regulation of the transferrin receptor (Fast *et al.*, 1999), as occurs in mammals. Moreover, genome survey indicated the absence of sequences with homology to IREs in trypanosomes (Berriman *et al.*, 2005; Ivens *et al.*, 2005; El-Sayed *et al.*, 2005), which in principle agrees with the low aconitase activity detected in bloodstream parasites and its dispensability for procyclic forms. The discovery that insect stage *T. brucei* does not rely on Krebs cycle for pyruvate metabolism has set a new metabolic paradigm for this parasite species. Despite *T. brucei* aconitase has never been shown *in vitro* to be a Fe/S-dependent protein, its high sequence conservation (Saas *et al.*, 2000) and several biological data supports its Fe/S nature. For instance, knockdown of IscS, IscU (Smíd *et al.*, 2006) and Isd11 (Paris *et al.*, 2010) was followed by down-regulation of aconitase activity both in the mitochondria and cytosol. The final answer for the physiological (in)dispensability of aconitase in infective parasites will have to await experiments in animal infection models.

### 3.3.3.2 Fumarate hydratases

Fumarases, also called fumarate hydratases (FHs, E.C. 4.2.1.2), are ubiquitous enzymes that catalyze the stereospecific reversible hydration of fumarate to malate. Most prokaryotes and eukaryotes express two isoforms of fumarases. In eukaryotes, the mitochondrial isoform is a component of the tricarboxylic acid cycle and, hence, central to aerobic respiration. The cytosolic isoform is probably involved in the metabolism of fumarate that stem from a number of reactions occurring in the cytosol. Two distinct classes of fumarases, class I and class II, have been identified so far. The proteins lack sequence homology but are functionally related. Class II fumarases (or *fumC* enzymes) are iron-independent enzymes. They occur in several bacteria and in eukaryotes, such as fungi, mammals, and higher plants. In contrast, class I fumarases are Fe/S-containing enzymes. Bacteria but also unicellular eukaryotes contain class I fumarases. The characterization of two class I fumarases in *T. brucei* revealed that two single copy genes code for mitochondrial and cytosolic isoforms that are expressed in procyclic but not in bloodstream parasites (Coustou *et al.*, 2006). Simultaneous downregulation of both transcripts was deleterious for the viability of procyclic trypanosomes, a phenotype that was counteracted by the addition of extracellular fumarate, highlighting the essential and stage-specific role of this metabolite (Coustou *et al.*, 2006). Taking into account that secretion of succinate has been described for the infective stage of *Leishmania* spp. and *T. cruzi* grown in glucose-supplemented medium

(Cazzulo, 1992), the authors proposed the occurrence of FHs in these trypanosomatids. Nevertheless, if any, their biochemical importance awaits elucidation.

#### 4. Heme uptake and utilization

Heme is an essential cofactor for various proteins. It can perform diverse functions such as oxygen transport and storage (hemoglobin and myoglobin), mitochondrial electron transport (Complex II–IV), xenobiotic detoxification and steroid metabolism (cytochromes), signal transduction (nitric oxide synthases, soluble guanylate cyclases), and regulation of antioxidant defense enzymes, since many enzymes like peroxidases, catalases, and the large group of cytochrome P450 (CYP450) rely on heme as a prosthetic group. Heme-proteins have also been implicated in microRNA processing and microbicide defense (Mochizuki *et al.*, 2010; Roberts & Montfort, 2007).

Heme consists of a cyclic tetrapyrrole ring structure (protoporphyrin IX) that coordinates an iron atom which can adopt Fe<sup>+3</sup> or Fe<sup>+2</sup> oxidation states. Different substitutions over the pyrrolic moieties originate the different types of heme (a, b and c; Severance & Hamza, 2009; Tripodi *et al.*, 2011). As stated earlier, African trypanosomes are extracellular parasites that depend on heme uptake to face their metabolic needs (Fig. 3), thus, the biosynthetic pathway operating in the host will be briefly outlined. The complete heme biosynthetic pathway involves eight-steps. Although the synthesis of  $\delta$ -aminolevulinic acid (ALA), the first heme precursor, may differ between organisms the remaining seven steps of the pathway (from ALA to heme b) are strictly conserved. In most heterotrophic eukaryotes the biosynthesis alternates between the mitochondria and the cytosol while in photosynthetic organisms heme is exclusively produced in the chloroplasts (Korený *et al.*, 2010). Despite its essentiality and versatility, iron and porphyrins are highly toxic to cells due to iron-induced pro-oxidant effect on DNA, proteins and membrane lipids. Therefore, its biosynthesis and storage is tightly regulated in order to reduce the level of free heme inside the cell.

Certain organisms lack some components of the biosynthetic pathway from ALA to protoporphyrin IX and have developed strategies to obtain heme from other sources. The tick *Boophilus microplus* (Braz *et al.*, 1999), the filarial nematode *Brugia malayi* (Wo *et al.*, 2009), the free-living nematode *Caenorhabditis elegans* (Rao *et al.*, 2005) and most kinetoplastid parasites are examples of eukaryotes that are partially or completely unable to synthesize heme despite some of their developmental stages depend on oxidative fosforilation (*i.e.*, cytochromes) to obtain energy. The order Kinetoplastida contains species where the complete biosynthetic pathway is absent (*e.g.* most members of the genus *Trypanosoma*), and others, such as *Leishmania* spp. or *Crithidia* spp., able to perform only the last three biosynthetic steps (Chang *et al.*, 1975; Salzman *et al.*, 1982). Although it is very likely that a kinetoplastid's ancestor harbored a complete set of eukaryotic genes for heme synthesis, during evolution these genes have been either lost (*e.g.* *Trypanosoma* genus) or subsequently rescued from a  $\gamma$ -proteobacterium endosymbiont by horizontal gene transfer, as proposed for the non-Trypanosoma trypanosomatids (Korený *et al.*, 2010). As a general consequence, trypanosomatids must scavenge heme or precursors from their hosts. At this point, it is important to note that there are several species of trypanosomatids that parasitize insects, including *Herpetomonas roitmani*, *Crithidia deanei*, *C. desouzai*, *C. oncopelti* and *Blastocrithidia*

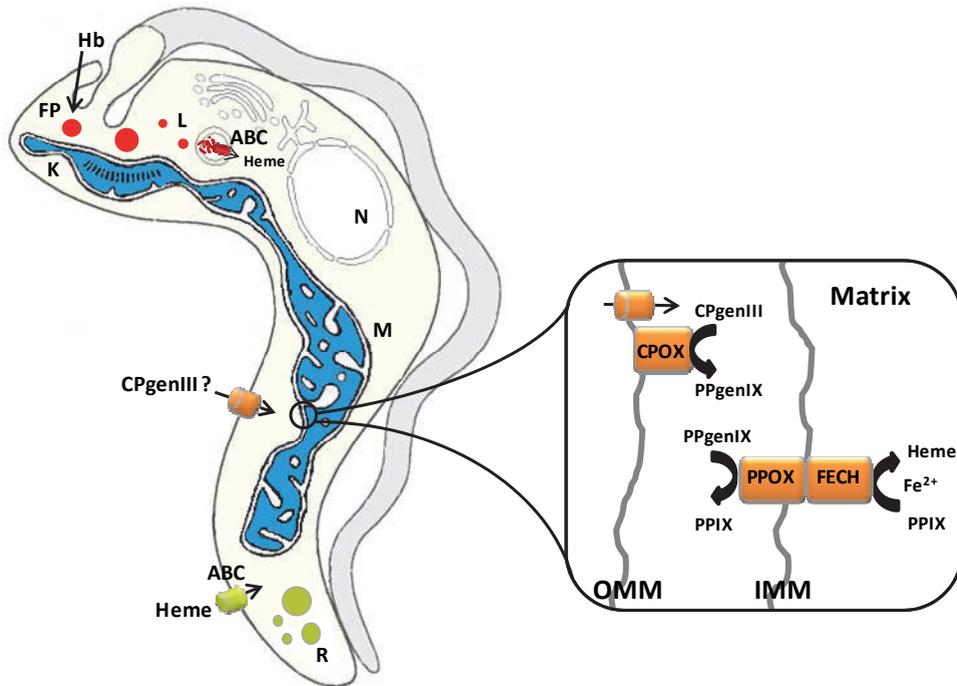


Fig. 3. Pathways for heme incorporation and biosynthesis in trypanosomes. Although two possible routes for heme internalization have been described in *T. cruzi*, so far none of the putative transporters has been characterized. In *T. brucei*, hemoglobin (Hb, upper left) endocytosis was shown to depend on a haptoglobin-hemoglobin receptor (HpHbR) localized at the flagellar pocket (FP) and expressed exclusively in the bloodstream form of the parasite. An ABC transporter (ABCG5 in *L. major*) has been shown to rescue heme after lysosomal (L) degradation of Hb. On the other hand, in *T. cruzi* a non-endocytic route was described for free forms of heme (lower part, in green). This is an active process mediated by ABC transporters that leads to accumulation of heme inside storage organelles, the reservosomes (R). Specific inhibitors for ABC transporters (cyclosporin A, verapamil and indomethacin) reduce significantly heme uptake (Peixoto Cupelloa *et al.*, 2011). Intracellular parasites endowed with the last three enzymes of heme biosynthesis, such as *Leishmania* spp., are capable to incorporate heme precursors, such as coproporphobilinogen III (CPgenIII), which upon transport to the mitochondrial intermembrane space can undergo a series of oxidative reactions leading to the formation of protoporphyrin IX (PPIX) that is finally converted into heme by addition of iron through a ferrochelatase (FECH) facing the mitochondrial matrix (middle part and insert, orange). Additional subcellular structures and biosynthetic components are abbreviated as follows: nucleus (N), kinetoplast (K), mitochondrion (M), outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), coproporphyrinogen oxidase (CPOX), protoporphyrinogen IX (PPgenIX), protoporphyrinogen oxidase (PPOX).

*culicis*, which have been found to contain symbiotic bacteria that may supply the protists with heme and other essential nutrients (Chang & Trager, 1974). The bacterial symbiotes would be subject of regulation by their hosts, hence, functioning as cell organelles rather than as independent living entities (Chang *et al.*, 1975).

Heme's transport and distribution in trypanosomatids remain elusive. Diffusion across the lipid bilayer is hampered for heme due to its anionic carboxylate side-chains. Instead, high affinity heme-binding proteins in the cell surface of *T. cruzi* (Lara *et al.*, 2007), procyclic forms of *T. brucei* (Vanhollebeke *et al.*, 2008) and *L. donovani* (Campos-Salinas *et al.*, 2011) allows for active heme uptake. In *T. cruzi* epimastigotes, both endocytotic and non-endocytotic mechanisms of heme internalization have been confirmed. *L. donovani* can also incorporate heme by receptor-mediated endocytosis followed by lysosomal degradation of hemoglobin. An ABC transporter localized in membranes of multivesicular structures is probably related to intracellular heme trafficking after hemoglobin degradation. Porphyrin trafficking in trypanosomatids is yet an uncovered subject but intracellular transport appears to involve membrane-bound vesicles as carriers (Severance & Hamza, 1982; Tripodi *et al.*, 2011).

In summary, pathogenic trypanosomatids are auxotrophic for heme and have to adapt their heme-dependent metabolic pathways (*i.e.* biosynthesis of sterols and polyunsaturated fatty acids, respiration, oxidative stress response and detoxification) to fluctuations in nutrient availability across their life cycle. The most relevant cellular functions requiring heme in trypanosomatids are discussed in the next sections.

#### 4.1 Heme in oxidative defense

Trypanosomes lack catalase and selenium-dependent glutathione peroxidases but instead express two types of trypanothione/tryparedoxin dependent peroxidases to decompose endogenous and exogenously produced oxidants (reviewed in Schlecker *et al.*, 2007 and Krauth-Siegel & Comini, 2008) and some species have an additional plant-like ascorbate-dependent hemoperoxidase (APX; Adak & Datta, 2005; Wilkinson *et al.*, 2002a). Interestingly, APX is present in intracellular trypanosomatids (*T. cruzi* and *Leishmania* spp.) but absent in parasites with an extracellular life style in the mammal host, namely *T. brucei*. This feature has been attributed to the major demand for anti-oxidant capacity that intracellular parasites need during infection (Wilkinson *et al.*, 2005; Dolai *et al.*, 2009). APX localizes to the endoplasmic reticulum (ER; Wilkinson *et al.*, 2002a) and cell membrane (M. Hugo *et al.*, personal communication) of *T. cruzi* and in the intermembrane space of the mitochondrial inner membrane in the case of *L. major* (Dolai *et al.*, 2008). Both enzymes are reduced by ascorbate or, alternatively, cytochrome c (Wilkinson *et al.*, 2002a; Dolai *et al.*, 2008; M. Hugo *et al.*, personal communication). The regeneration of reduced ascorbate occurs upon spontaneous reaction of dehydroascorbate with T(SH)<sub>2</sub> (Krauth-Siegel & Lüdemann, 1996). With respect to the source of ascorbate, in contrast to humans, African and American trypanosomes can synthesize this metabolite *de novo*, a process that takes place in the glycosome (Wilkinson *et al.*, 2005). *T. cruzi* cannot take up ascorbate from the environment, as *T. brucei* does, and therefore relies entirely on its biosynthesis to fuel other unknown cellular functions, in addition to oxidant detoxication (Logan *et al.*, 2007).

The biological role of APX is consistently related to oxidant defense. Overexpression of APX in *T. cruzi* and *L. major* enhances tolerance against oxidative stress induced by exogenous

hydrogen peroxide (Wilkinson *et al.*, 2002a and 2005) and mitochondrial cardiolipin oxidation, providing thus protection towards programmed cell death and protein damage (Dolai *et al.*, 2008, 2009). This function appears to be redundant with that performed by the ER glutathione-like trypanothione-dependent peroxidase of *T. cruzi* (GPxII; Wilkinson *et al.*, 2002a) and the mitochondrial trypanothione-dependent peroxiredoxin (mTXNPx) from *Leishmania* spp. However, an explanation for this may lie on different substrate specificities (*e.g.*, GpxII reduces fatty acid and phospholipid hydroperoxides; Wilkinson *et al.*, 2002b) or the macromolecular targets these enzymes have.

#### 4.2 Heme in polyunsaturated fatty acids biogenesis

Fatty acid biosynthesis in trypanosomatids seems to be an essential pathway for the parasite life cycle. As stated earlier, differentiation involves dramatic morphological changes in the cell and its organelles that certainly require membrane fluidity. The biosynthesis of unsaturated fatty acids is another plant-like pathway inherited by trypanosomatids. In this respect, lipid desaturation occurs at the methyl end of the molecule, in contrast to mammals, in which a double bond is generated at the carboxy end of the molecule.

*T. cruzi* and both life stages of *T. brucei* are able to synthesize fatty acids, although *T. brucei* bloodstream form was formerly thought to depend on lipid uptake from their host. Short and medium chain fatty acids are usually synthesized by means of the soluble fatty-acid synthetase system and subsequently elongated by the elongase system that resides in the ER. *T. brucei* can elongate fatty acids up to stearate (C18:0). *T. cruzi* can further elongate C18 to C24 and C26 fatty acids required in the synthesis of anchors for surface macromolecules. The stearate is mainly converted into linoleate (C18:2), which represents near 30% and 40% of total fatty acids in *T. brucei* and *T. cruzi*, respectively. Moreover, unsaturated fatty acids can represent up to 70% of total fatty acid content depending on the parasite species and life cycle stage (Alloatti *et al.*, 2010).

*T. brucei* oleate desaturase shares high similarity with  $\Delta 12$  desaturases and, to a lesser extent, with  $\omega 3$  desaturases although the parasite enzyme lacks  $\omega 3$  desaturase activity. Fatty acid desaturases require an electron donor, that can be either ferredoxin in plastids and bacteria or cytochrome *b5* (*cytb5*) in the ER, as it is the case for the orthologue from *T. brucei* (Uttaro, 2006). Since *T. brucei* oleate desaturase lacks a consensus sequence for the covalent binding of *cytb*, it has been suggested that electron donation occurs *via* complex formation with another desaturase capable to bind *cytb5* (Petrini *et al.*, 2004). The drastic growth defect observed upon partial ablation of enzyme activity (even an 8% reduction) by RNAi is a strong indication of the essentiality of this pathway for infective parasites (see also section 3.2.4; Alloatti *et al.*, 2010)

#### 4.3 Trypanosomes contain unusual single-cys cytochromes

Cytochromes are heme-containing complexes that participate in many electron transfer reactions including oxidative phosphorylation in the respiratory chain (cytochromes type *c*, *cytc* and cytochromes type *c1*, *cytc1*), desaturation of fatty acids or xenobiotic detoxification (Comini *et al.*, 2011). In *cytc* heme is covalently attached through thioether bonds between the vinyl groups of the heme and the thiols of two cysteine residues in a conserved CXXCH motif, in which the histidine functions as an axial ligand for heme iron. Mitochondrial *cyt c*

is the best known example of this type of protein. It is located in the intermembrane space and transfers electrons from the cytochrome *bc1* complex to the cytochrome *aa3* oxidase. Cytochrome *c1* of the *bc1* complex (cytochrome reductase) is also a *c*-type cytochrome. Heme coordination by *cytc* and *cytc1* from euglenoids (e.g. the green flagellated algae *Euglena gracilis*) and trypanosomatids occurs through a single thioether bond involving the single thiol group from their binding motifs AAQCH and FAPCH, respectively (Priest & Hajduk, 1994). As another example of a metabolic trait that trypanosomatids share with higher plants, plastids contain *b6f*-type cytochromes with heme covalently attached through a single thioether bond (Allen *et al.*, 2004; Tripodi *et al.*, 2011). The biogenesis of holo cytochrome *c* is a catalyzed process that involves different mechanisms and enzymatic components that can be grouped into five major systems (Tripodi *et al.*, 2011). The cytochrome *c* maturation machinery employed by kinetoplastids remains unidentified and is possibly unique to this and the entire euglenid taxon (Allen *et al.*, 2004; Tripodi *et al.*, 2011). Based on the metabolic repertoire of each developmental stage, the relevance of cytochrome activity for energy production appears to be restricted to the procyclic form of the parasite.

#### 4.4 Heme in sterol biogenesis

Cholesterol, ergosterol and sitosterol are essential structural components of plasma membranes from mammals, fungi and plants, respectively. They stabilize membranes, determine their fluidity and permeability, and modulate the activity of membrane-bound enzymes and ion channels. *T. cruzi* and *T. brucei* encode all sterol biosynthetic enzymes, including sterol 14- $\alpha$ -demethylase (CYP51), a member of the CYP450 superfamily that operates at the initial phase (post-squalene stage) of the pathway that drives the oxidative removal of the 14- $\alpha$ -methyl group from the newly cyclized sterol precursors. Azole-based compounds, common in antifungal therapies (e.g., posaconazole) proved to be potent inhibitors of CYP51 by interacting with the enzyme active site and its prosthetic group (Chen *et al.*, 2010). Inhibition of CYP51 leads to accumulation of 14 $\alpha$ -methylated sterols, which are unable to replace ergosterol in the membrane because of steric hindrance, followed by growth arrest and cell death (Lepsheva *et al.*, 2007; Vanden Bossche *et al.*, 1995). In contrast to *T. cruzi* and *Leishmania* spp., ergosterol has been thought to be dispensable for *T. brucei*. However, the observation that antifungals are also detrimental for this parasite lead to the proposal that sterol derivatives may additionally serve as precursors for bioactive molecules that act as regulators of cell cycle and development (Lepsheva *et al.*, 2007).

Other CYP450s possess a monooxygenase activity that, upon oxygen activation, catalyzes the addition of an oxygen atom into an organic substrate and the reduction of the second one to water. After each catalytic cycle, CYPs must be restored to their reduced state by a CYP450 reductase (CPR), a NADPH-dependant diflavin (FAD/FMN) enzyme that couple a two-electron donor (NADPH) with one-electron acceptors, supplying the electrons one at a time. CYPs and CPR are involved in the metabolism of a wide range of endogenous compounds and xenobiotics including fatty acids, steroids, drugs, alkanes, polycyclic hydrocarbons, insecticides and other environmental contaminants (Murataliev *et al.*, 2004). In mammals, there is only one CPR gene. However, *T. cruzi* and *L. major* encode three different CPR genes (CPR-A, CPR-B and CPR-C), while *T. brucei* possesses four sequences, all of them sharing a high sequence identity. Similar to some higher plant species

(Koopmann & Hahlbrock, 1997; Ro *et al.*, 2002; Urban *et al.*, 1997), *T. cruzi* CPRs are encoded by a small gene family, with differential regulation and subcellular localization (Portal *et al.*, 2008). *TcCPR-B* is localized mainly in reservosomes, *TcCPR-C* is mostly found in the ER while *TcCPR-A* displays a more ubiquitous distribution pattern (De Vas *et al.*, 2011). The presence of multiple isoforms argues for a well-supported P450-mediated molecular system. Portal *et al.* (2008) showed that CPR-B overexpression in *T. cruzi* confers increased resistance to nifurtimox and, more significantly, to benznidazole. In addition, increased levels of CPRs are observed in trypomastigotes, a stage that is particularly exposed to stress during cell invasion supporting a role for CPRs in oxidative stress defense (Portal *et al.*, 2008). Moreover, CPR-B and CPR-C overexpression increases the synthesis of ergosterol, with the concomitant augmentation in membrane rigidity and decrease in endocytic activity. Modification in cell membrane fluidity could result in alteration of drug uptake and, as a consequence, in the observed drug resistance (De Vas *et al.*, 2011).

## 5. Iron and infection

As noted in the introductory section, African trypanosomiasis is a parasitic disease of medical and veterinary importance that has adverse impact in the health and economical development of the sub-Saharan region of Africa (Maudlin, 2006; Stijlemans *et al.*, 2008, 2010a). Since African trypanosomes multiply predominantly in the bloodstream as extra-cellular parasites, they are continuously dependent on host nutrient supply and exposed to host immune attack. As will be addressed next, the response mounted by the mammalian host towards infection includes the activation of both arms of the immune system.

Beyond the aforementioned clinical outcomes for HAT (Section 1), the most prominent immunopathological disease-related feature of trypanosomiasis is anemia. In fact, anemia is the major cause of death in bovine trypanosomiasis and an important debilitating symptom in HAT (Stijlemans *et al.*, 2008). Anemia of inflammation, also termed anemia of chronic disease (ACD), is frequent among patients suffering chronic inflammatory disorders (*e. g.*, autoimmune diseases) or chronic infections (Camaschella & Strati, 2010). ACD is the consequence of a persistent activated inflammatory immune response and is considered to be a defense mechanism where the host establishes an iron “withholding” strategy to limit the availability of essential iron for the pathogens (Lalonde & Holbein, 1984; Stijlemans *et al.*, 2008; Sutak *et al.*, 2008). Stijlemans and coworkers (Stijlemans *et al.*, 2008, 2010a, 2010b; Vankrunkelsven *et al.*, 2010) studied the immunological and hematological development of *T. brucei* infection using animal models (cattle and mice). Despite mouse models show limitations and artifacts, like the extremely high parasitemia levels reached compared to infection in the natural host, they have contributed significantly to our current understanding of trypanosomiasis patho-physiology (Magez & Caljon, 2011). The molecular mechanism underlying the development of ACD during trypanosomiasis resembles that observed in other infectious diseases. Briefly, during the early stage of the infection trypanosomes stimulate T-cells to secrete interferon- $\gamma$  resulting in the activation of macrophages (type I cellular immune response) that liberate pro-inflammatory mediators like tumor necrosis factor, interleukin (IL) -1, IL-6 and \*NO (Stijlemans *et al.*, 2010b). This response leads to parasite clearance but also enhances erythrophagocytosis by activated macrophages within the spleen and liver (Kupffer cells). At this point it is important to recall that under physiological conditions Kupffer cells recycle iron from engulfed senescent

erythrocytes (see Section 2; Hentze *et al.*, 2010). Thus, enhanced removal of red blood cells from systemic fluids by the reticuloendothelial system will contribute to deprive host of iron. In addition, hepcidin (Section 2) is secreted in response to pro-inflammatory mediators, blocking iron release from macrophages and iron intake by enterocytes (Nishimura *et al.*, 2011), which decreases plasma iron levels and limits host and pathogen accessibility to the metal (Taylor & Kelly, 2010). The upregulation of several iron-related molecules (e.g. hemoxygenase 1, ferritin and ferroportin) provides additional evidence for the strong type I immune response, augmented liver iron-metabolism and accelerated senescence of erythrocytes that takes place during trypanosomal infection (Nishimura *et al.*, 2011; Omotainse & Anosa, 1992; Stijlemans *et al.*, 2010a; see Section 2). The class of anemia originated during the acute phase of trypanosomiasis is a typical non-haematopoietic anemia. However, if at the long term the type I response persists, then iron will be sequestered intracellularly, resulting in a marked iron deprivation with compromise of erythropoiesis and development of ACD (Mwangi *et al.*, 1995; Omotainse & Anosa, 1992; Stijlemans *et al.*, 2010b). Despite its adverse effects, if sustained on time, limited iron bioavailability can be considered a reasonable host response to control parasitemia (Ganz, 2009). In the last years, several evidences point to the possibility that the parasite deliberately manipulates the mammalian host immune response. Activation of a strong type I immune response in early stages of the infection promotes erythrophagocytosis (Igbokwe *et al.*, 1994), a strategy that yields abundant iron and lipids (e.g. myristic acid), the last needed for GPI biosynthesis and VSG coating (Stijlemans *et al.*, 2010a, 2010b). It is important to stress that the trypanosome body is totally covered by a dense GPI-anchored coat of VSGs that plays an important protective (physical and immunological barrier) function in bloodstream parasites (Comini *et al.*, 2011).

A large number of microbial pathogens have been reported to affect the morbidity and mortality of patients with high iron content, a physiopathological state characterized by the presence of non-transferrin bound iron in plasma. Moreover, iron overload due to genetic predisposition, therapeutic intervention or nutritional status is known to increase the risk of several infections (Kontoghiorghes *et al.*, 2010). It is worth to note that human iron overload is common in rural populations inhabiting sub-Saharan regions of Africa, the "tsetse belt", with a high incidence of HAT (Gordeuk, 2002; McNamara *et al.*, 1999). Originally, this chronic condition was supposed to be an exclusive consequence of an iron-rich diet based on the large consumption of homebrewed beers, but recent studies of pedigrees suggest the presence of genetic components (Gordeuk, 2002; Moyo *et al.*, 1998). It remains to be investigated whether there is a co-evolutionary development of iron overload and HAT, as it is the case for glucose-6-phosphate dehydrogenase deficiency and malaria.

Considering the well-known role of iron in *T. brucei* infection and the fact that *in vitro* iron deprivation with chelating agents cause growth retardation of bloodstream *T. brucei* (Fast *et al.*, 1999; Breidbach *et al.*, 2002; Comini *et al.*, 2008), it is surprising the absence of works studying the therapeutic potential of iron chelation against *T. brucei* (Taylor & Kelly, 2010). This is not the case for *T. cruzi*, for which the chelating agent deferoxamine (DFX) proved its efficacy to halt replication of intra-macrophage amastigotes (Arantes *et al.*, 2011; Lalonde & Holbein, 1984; Jones *et al.*, 1996), and to decrease parasitemia and mortality of infected mice (Loo & Lalonde, 1984). However, in contrast to extracellular *T. brucei*, the possible success of chelating therapies against *T. cruzi* infection are hampered by the intracellular nature of the

infective stage of this parasite, which, if not specifically targeted to the pathogen, will imply a high dosage or a long-term treatment, both deleterious for the host. Based on the demonstration that iron metabolism is crucial for parasite survival in the host (Manta *et al.*, unpublished; see section 3.3.2), our group has recently evaluated the therapeutic effect of intraperitoneal (i.p.) and oral administration of DFX to *T. brucei* infected mice. Preliminary results show a marked increase in life span of the animal group receiving i.p. treatment (200 mg/Kg for ~10 days) and almost no side effects (red blood cell and reticulocyte counts) on animals treated but non-infected (Manta *et al.*, unpublished). However, DFX displayed a cytostatic effect against acute infection by *T. brucei*, which implies that a successful therapeutic regimen will require drug association.

## 6. Summary and outlook

As any other living organism on earth, trypanosomes depend on iron to fulfill critical cellular functions (DNA and lipid biosynthesis, oxidant protection and energy generation). Their complex life cycle has added an additional problem in the struggle for the supply of (micro)nutrients. However, these organisms have developed different strategies and mechanisms in order to ensure a physiological iron status within the distinct hosts. Moreover, host-parasite co-evolution has resulted in the development of well-balanced growth regulation systems, allowing the parasite to survive sufficiently long without killing its mammalian host and ensuring an efficient transmission cycle. Interruption of this cycle is necessary to abolish the devastating disease these parasites produce to humans and livestock. In this respect, the iron metabolism of trypanosomatids should be considered as a promising target for effective antiparasitic treatments. Although metal-chelating compounds are, obviously, not innocuous to the host, in general they are less toxic than the drugs actually used for the treatment of HAT and their side effect is fully reversible upon dietary supplementation. In addition, several chelating agents are approved for the treatment of human pathologies, some of them are relatively cheap and available for oral administration (Sharma & Pancholi, 2010). The risk/benefit assessment for the use of iron chelating drugs as antiparasitic therapeutic depends on many factors not addressed yet.

Moreover, beyond the relevance of iron homeostasis for parasite survival, some components of this metabolism and/or iron-, Fe/S cluster- or heme-proteins offer excellent opportunities for the design of more specific and efficacious drugs. For instance, the enzymes involved in fatty acids biogenesis (oleate desaturase and stearoyl-CoA-desaturase) and sterol biosynthesis (CYP51); in defense against oxidants (the plant-like ascorbate peroxidase) and xenobiotics (CYP450 reductases); in cell proliferation (ribonucleotide reductase); and in Fe/S cluster biogenesis. Alternatively, chemical downregulation of the functions these proteins fulfill in the cell can be complemented by the action of first line drugs currently used in chemotherapy. A combined therapy would have the advantage of reducing dosage, shortening treatment and lowering the possibility for development of drug resistance. The specificity of the transferrin receptor is another target to be exploited as an efficient mechanism to deliver toxic molecules inside the parasite

The future prospects look promising in terms of finding novel therapeutic options to fight trypanosomiasis. However as depicted in Fig. 2 and Fig. 3, there are still several open questions that deserve to be investigated in detail to disclose atomic and functional divergences within the components of the parasite and host iron metabolism that can lead to the development of safer and efficacious drugs.

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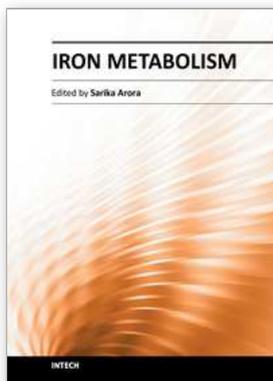
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## **Iron Metabolism**

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Iron has various functions in the body, including the metabolism of oxygen in a variety of biochemical processes. Iron, as either heme or in its "nonheme" form, plays an important role in key reactions of DNA synthesis and energy production. However, low solubility of iron in body fluids and the ability to form toxic hydroxyl radicals in presence of oxygen make iron uptake, use and storage a serious challenge. The discovery of new metal transporters, receptors and peptides and as well as the discovery of new cross-interactions between known proteins are now leading to a breakthrough in the understanding of systemic iron metabolism. The objective of this book is to review and summarize recent developments in our understanding of iron transport and storage in living systems.

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