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Redox Metabolism in Mitochondria of Trypanosomatids

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Abstract

Significance: In the single mitochondrion of protozoan trypanosomatid parasites there are several sites for the generation and elimination of reactive oxygen species (ROS), a class of molecules that exhibit a dual role in cells, either as regulatory mediators or as cytotoxic effectors. ***Recent Advances:*** Formation of ROS in trypanosomatid mitochondria can be induced by various drug compounds. Importantly, it can also be triggered by specific physiologic stimuli, indicating that this phenomenon may occur in living parasites as well. Elimination of ROS in these organelles is attributed to the activity of two iron-dependent superoxide dismutases (FeSODs) and up to three different peroxidases (a cytochrome *c* peroxidase and two thiol peroxidases). ***Critical Issues:*** Data regarding the formation of ROS in trypanosomatid mitochondria are limited and nonsystematic. Another critical issue refers to the exact contribution of mitochondrial FeSODs and peroxidases for ROS removal, given that their antioxidant activity is not essential when abrogated individually. This suggests some level of functional overlapping or that ROS produced in mitochondria under normal conditions can be removed noncatalytically. Also still unsolved is the mechanism by which mitochondrial thiol peroxidases are regenerated to their reduced (active) form. ***Future Directions:*** The production of intramitochondrial ROS under physiologic conditions and their implication in parasite biology should be further clarified. The relative importance of enzymatic *versus* nonenzymatic mechanisms for ROS elimination in trypanosomatid mitochondria also requires investigation. Simultaneous depletion of several redundant antioxidant enzymes and determination of noncatalytic antioxidants are possible ways to achieve this. *Antioxid. Redox Signal.* 19, 696–707.

Introduction

Mitochondria are organelles where essential physiologic processes take place. The hallmark of these is oxidative phosphorylation, which provides aerobic organisms the majority of their energy, but other important functions, namely the synthesis and catabolism of crucial amino acids, fatty acid oxidation, or iron–sulfur cluster biogenesis, are ascribed to these compartments. Mitochondria are also organelles where reactive oxygen species (ROS) (*i.e.,* free radicals and other molecules derived from the incomplete one-electron reduction of molecular oxygen) can be found (50, 51), either because they are generated there or because they diffuse into this organelle from other cell sites. Although fluctuations in the basal levels of ROS in response to certain stimuli do occur and are crucial for cell physiology (10), high concentrations induce oxidative stress and need to be removed in order to prevent toxicity. This review contemplates mitochondrial redox metabolism, focusing on the production of ROS and on their elimination in mitochondria of trypanosomatid parasites.

Trypanosomatids encompass a vast group of organisms included in the order Kinetoplastida, many of which are parasites of humans, animals, and plants. For simplicity, this review is restricted to the medically relevant *Leishmania* spp., the agents of human and canine leishmaniasis, to the *Trypanosoma brucei* complex, which causes sleeping sickness in humans and Nagana in cattle, and to *Trypanosoma cruzi*, responsible for Chagas' disease. The main life cycle stages of these parasites are depicted in Figure 1.

Several pieces of evidence, including recent data on the characterization of the mitochondria protein-import machinery (75), indicate that trypanosomatids are among the earliest diverging eukaryotes to have mitochondria (43). In these organisms, this organelle is a single structure (68) with a tubular form which extends throughout the cell. It contains an outer membrane, a dense matrix, and an inner membrane that can fold into thin and irregularly distributed cristae whose number varies widely (25). In fact, one characteristic of trypanosomatid mitochondria is their plasticity, with the exact morphology, volume, and activity depending on factors such

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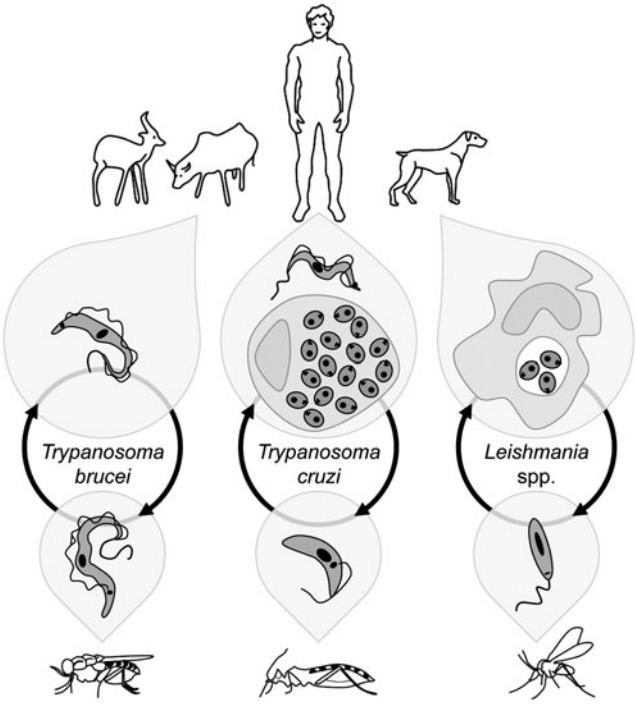

FIG. 1. Life cycles of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp. Trypanosomatids have digenic life cycles, which alternate between an insect vector, usually responsible for disease transmission, and a mammalian host. In the invertebrate hosts, trypanosomatids infect the gut, wherein they adopt a flagellated form and an extracellular life style. These insect vector stages are designated as procyclic (*T. brucei*), epimastigote (*T. cruzi*), or promastigote (*Leishmania* spp.). In the case of the mammalian stages of these organisms, their morphology and residence varies according to the parasite species. *Trypanosoma brucei* resides as a flagellated, extracellular cell (the trypomastigote or bloodstream form) in the circulatory and lymphatic systems of its vertebrate hosts. As for *T. cruzi*, it alternates between an extracellular, flagellated, and nonreplicative trypomastigote form, and an intracellular, aflagellated, and replicative amastigote stage. Almost any cell, phagocytic or nonphagocytic, can be infected by *T. cruzi*, although the parasite has a tropism for muscle and nervous cells. Finally, *Leishmania* spp. resides and replicates as nonmotile amastigotes inside the phagolysosomes of macrophages. Also depicted are a tse-tse fly, a triatomine bug, and a sandfly, the insect vectors for *T. brucei*, *T. cruzi*, and *Leishmania* spp., respectively. These organisms cause diseases in humans and also in cattle (*T. brucei*) and in dogs (*Leishmania* spp.).

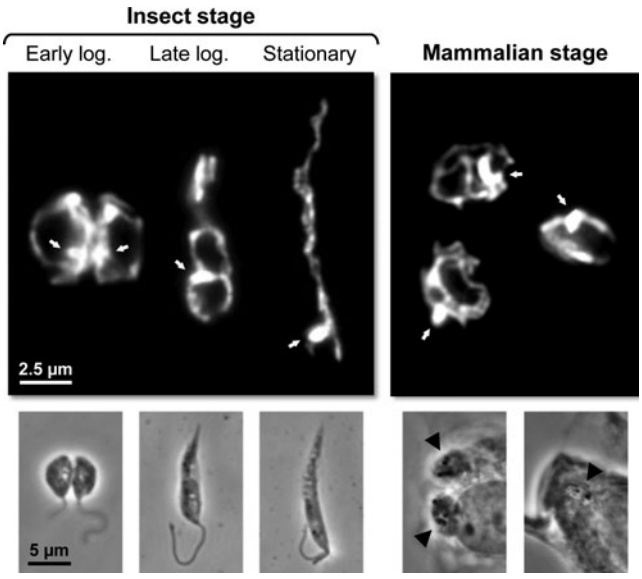

FIG. 2. Mitochondria plasticity along *Leishmania* development. Immunofluorescence micrographs showing the tubular mitochondrion of *Leishmania infantum* stained with an antibody against a mitochondrial protein (*Li*mTXNPx, see text) in different life cycle stages of the parasite (*upper panels*). Dividing promastigotes (insect stage) in the early and late logarithmic phases of growth present a circular and symmetric mitochondrion. A similar description applies to this organelle in the mammalian stage of *L. infantum*. This contrasts with the elongated mitochondrion of nondividing (stationary) insect stage forms. Also depicted are the phase contrast pictures of the parasites (*lower panels*). The mitochondrial DNA (kDNA) is indicated by *white arrows. Black arrowheads* point to intramacrophagic *L. infantum* amastigotes. (Castro and Tomás, unpublished work).

as the cell cycle phase, the stage, and the species, or the nutritional sources available (25). Figure 2 illustrates the alterations in the morphology of *Leishmania infantum* mitochondria along parasite development. The variability in trypanosomatid mitochondria is even more striking in *T. brucei*. While in procyclic parasites thriving at the expenses of amino acids such as proline, mitochondria are large and highly branched; in bloodstream forms, which obtain their energy solely from glycolysis, they are small and repressed. Within the mitochondrial matrix and located perpendicular to the axis of the flagellum, lies the kinetoplast, a disk-shaped structure that contains the mitochondrial DNA (the kinetoplast DNA or kDNA) which in trypanosomatids is composed of a dense network of two types of circular molecules, maxicircles and minicircles. The former encode some of the mitochondrial proteins while the second specify unique RNA molecules required for RNA editing, a distinctive process occurring within trypanosomatid mitochondria which converts aberrant RNA sequences into functional messages (7). Despite singularities such as the ones mentioned above, many of the physiologic processes taking place in mitochondria of eukaryotes are also present in trypanosomatids. Still, some of these may exhibit unique features as is the case of the mechanism for cytochrome *c* (cyt *c*) biogenesis (3).

ROS Formation Within Trypanosomatid Mitochondria

Physiologic generation of ROS is an unquestionable phenomenon in aerobic organisms, the mitochondrion being an important site for its occurrence. Although technical limitations have up to now precluded the accurate determination of the levels generated *in vivo*, studies with isolated mitochondria estimate that monoelectronic reduction of molecular oxygen (O_2) to superoxide anion ($O_2^{\bullet -}$) accounts for up to 0.1% of the total O_2 consumed by resting cells (37). In trypanosomatids, production of ROS within mitochondria is also documented. Evidence collected from a number of studies have established that generation of these molecules can be

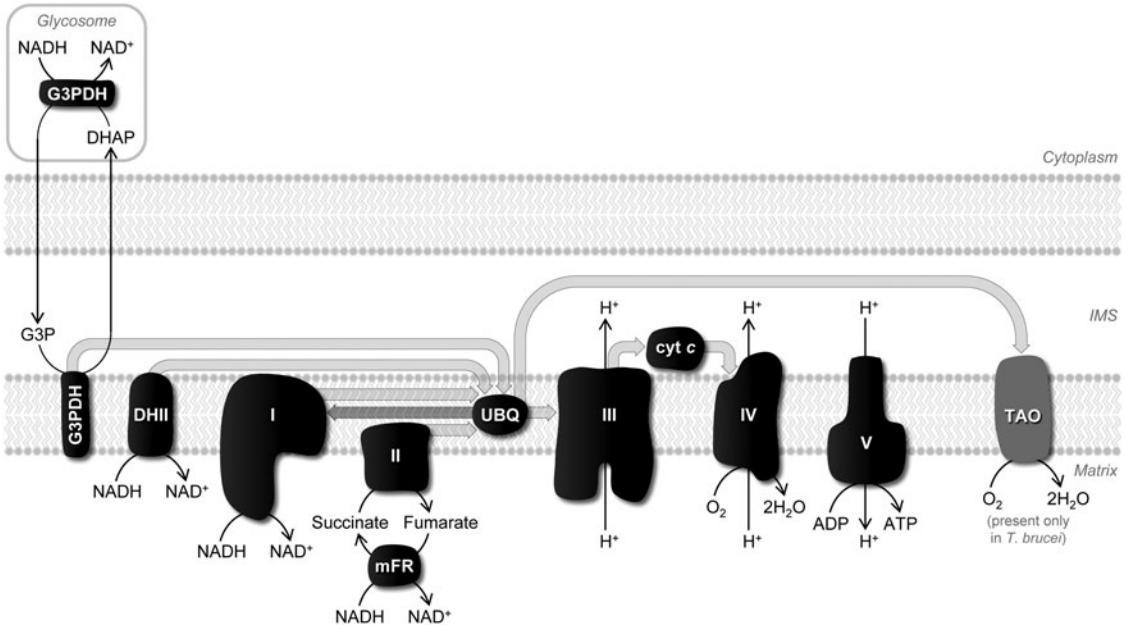

FIG. 3. Main features of the respiratory chain of trypanosomatids. Reducing equivalents produced during mitochondrial catabolic pathways enter the respiratory chain at complex I, type II NADH-dehydrogenase (DHII), and at complex II, and reduce the pool of ubiquinone (UBQ) in the inner membrane. Glycerol-3-phosphate dehydrogenase (G3PDH), which regenerates glycosomal glycerol-3-phosphate (G3P) back to dihydroxyacetone phosphate (DHAP), also serves as an electron entering point to the respiratory chain. Electrons from UBQ are then successively transported to complex III, to the intermembrane space (IMS) mobile carrier cytochrome *c* (cyt *c*) and to complex IV, where they reduce molecular oxygen (O_2) to water (H_2O). Electron transfer through complexes III and IV is coupled to proton (H^+) translocation from the matrix to the IMS. This induces the formation of an electrochemical gradient ($\Delta\Psi$) between both sides of the inner mitochondrial membrane, which is used to produce ATP from ADP and inorganic phosphate. In *T. brucei* bloodstream forms, the respiratory chain is considerably simpler and entails only G3PDH and the trypanosomatid alternative oxidase (TAO). The latter is also present in the *T. brucei* insect stage but not in *Leishmania* spp. and *T. cruzi*. In trypanosomatids, complexes I, II, and III, as well as DHII, are potential sites for superoxide anion generation, as is the matrix enzyme mitochondrial fumarate reductase (mFR). In this scheme, *block arrows in light gray* represent electron flux along the several components of the scheme. The reverse electron transfer between ubiquinone and complex I is depicted with a *dark gray block arrow*.

triggered by many drug compounds (14, 38), including some used in clinics as is the case of miltefosine (52), as well as by classic respiratory chain inhibitors (33, 56, 77). An important question, however, is whether ROS formation does occur along the parasites life cycle. In this regard, studies reporting production of mitochondrial ROS in response to physiologic stimuli, such as serum complement (72) and heat shock (4), and linking the formation of these molecules to induction of apoptotic-like death, are particularly important as they suggest that ROS generation is a phenomenon that can occur *in vivo* and have functional significance for trypanosomatids.

Although there are solid data associating ROS with trypanosomatid mitochondria, the exact site for their production has not been as thoroughly addressed as in other systems. Of relevance, the isolation of the single mitochondrion of trypanosomatids in an intact form is difficult. Such analyses are, thus, usually carried out either using mitochondrial enriched fractions (vesicles) displaying membrane potential or, more frequently, whole parasites selectively permeabilized with digitonin at concentrations that preserve the integrity of the organelle (85).

In most eukaryotes, the respiratory chain is the main site for ROS production within mitochondria. During transference of reducing equivalents along the several intermediates of the chain, some electrons may escape, allowing for the monovalent reduction of molecular oxygen to superoxide anion ($O_2^{\bullet -}$). This radical ion is the primary ROS formed in cells and the precursor for hydrogen peroxide (H_2O_2) and other species (48, 51). With the possible exception of *T. brucei* bloodstream forms, the respiratory chain might as well constitute a source of reactive oxygen species to trypanosomatids. In fact, in spite of differences relative to other eukaryotes, the metabolism of all these organisms also entails electron flow along the chain (11, 59, 62, 83).

The main features of the respiratory chain of trypanosomatids are depicted in Figure 3. Although there are species and stage differences in the chain, in general terms, electrons from NADH and succinate enter the chain at different points via the mobile carriers ubiquinone (UBQ) and cyt *c* and are transferred to a terminal oxidase (complex IV). The latter uses this reducing power to produce water from molecular oxygen. Electron transfer through complexes III and IV is coupled to proton translocation promoting the formation of an electrochemical gradient between both sides of the inner mitochondrial membrane that sustains ATP synthesis.

Complex I

In a typical eukaryote, one of the main sites for ROS generation within the respiratory chain is complex I, which

releases $O_2^{\bullet -}$ on the matrix side (48). In trypanosomatids, this complex shows some singularities, including the absence of proton extrusion activity, but it appears to conserve all subunits containing the redox centers required for UBQ reduction (1, 63, 86). Nevertheless, it is relatively consensual that in all these organisms complex I displays low NADH dehydrogenase activity (1, 13, 77), which could explain why $O_2^{\bullet -}$ originating from this complex was not detected neither in *T. brucei* procyclics (33, 86) nor in *T. cruzi* epimastigotes (13, 38). Superoxide formation from this *locus* was, however, reported in *Leishmania donovani* promastigotes upon stimulation with 100 μM rotenone (56).

Type II NADH dehydrogenase

In addition to complex I, a second inner membrane NADH dehydrogenase (DHII), with flavin as cofactor and also devoid of proton translocation activity, was characterized in *T. brucei* (35). Owing to the presence of orthologous sequences in the genomes of *T. cruzi* and *Leishmania* spp. (Tc00.1047053508717.20 and LinJ.36.5620; http://tritrypdb.org/tritrypdb/, last accessed on August 8, 2012), this molecule should constitute an alternative electron point entry to the respiratory chain of all these organisms. Type II NADH dehydrogenases have also been implicated in ROS production in *T. brucei* procyclics (33) and, very likely, in *T. cruzi* epimastigotes (13).

Mitochondrial glycerol 3-phosphate dehydrogenase

Another dehydrogenase present in the inner mitochondrial membrane of all trypanosomatids, but particularly important in bloodstream forms of *T. brucei*, is glycerol-3-phosphate dehydrogenase (G3PDH) (64). This FAD-dependent enzyme oxidizes glycolysis-derived glycerol-3-phosphate (G3P) forming dihydroxyacetone phosphate (DHAP), as part of a shuttle between the glycosome (the organelle where glycolysis takes place) and the mitochondrion that helps keeping redox balance within the glycosome (Fig. 3). Mitochondrial G3PDH also feeds electrons in the respiratory chain. Although not recognized as a source of ROS in trypanosomatids, analogous enzymes in other organisms generate $O_2^{\bullet -}$ to the intermembrane space (24).

Complex II

Apart from NADH, electrons can be supplied to the electron transport chain from succinate to UBQ via complex II, or succinate dehydrogenase. In other eukaryotes, complex II is only seldom referred to as producing $O_2^{\bullet -}$ (90), perhaps because structural features of this complex limit accessibility of O_2 to FAD (48). ROS production from trypanosomatid complex II was reported in *T. cruzi* epimastigotes (38, 80) and in *L. donovani* promastigotes (56) stimulated with the complex II inhibitor thenoyltrifluoroacetone (TTFA).

Reverse electron transfer

Electrons derived from succinate are also often referred to give rise to $O_2^{\bullet -}$ through a different mechanism, reverse electron transfer (Fig. 3). In this case, reducing equivalents supplied via complex II to UBQ are transported back to complex I (and in trypanosomatids perhaps to type II NADH dehydrogenase) inducing ROS formation (48, 58). Reverse electron transfer, which is stimulated by a high inner membrane potential and a reduced UBQ pool, was credited as originating $O_2^{\bullet -}$ in *T. brucei* (33) and in *T. cruzi* (13), but it is debatable whether this mechanism occurs *in vivo*.

Complex III

Within the respiratory chain of different organisms, complex III (ubiquinol:cytochrome *c* oxidoreductase) is usually a major source of ROS. Interestingly, Fang and Beattie (33) did not rank this site as a potential local for $O_2^{\bullet -}$ generation in *T. brucei*. On the contrary, in *L. donovani* promastigotes treated with antimycin (56) or with the primaquine analogue tafenoquine (14), as well as in *T. cruzi* epimastigotes cultured with *Serratia marcescens* prodigiosin or stimulated with antimycin (38), $O_2^{\bullet -}$ formation was readily triggered at this complex. None of these studies has, however, addressed the submitochondrial compartmentalization of the produced ROS. In other systems, $O_2^{\bullet -}$ may be directed to the matrix or to the intermembrane space, depending on which of the two sites within mammalian complex III (designated as Q_i and Q_o) generates this radical species.

Complex IV and the alternative terminal oxidase of T. brucei

Complex IV, or cytochrome *c* oxidase, is not regarded as a source of ROS, neither in trypanosomatids nor in other eukaryotes. Nevertheless, a diminished oxidase function of complex IV, either inherent to the parasite or caused by drugs, obviously induces mitochondrial ROS production as any constraint to electron flow favors their escape to O_2. A lower activity of complex IV, together with an increased activity of complexes II and III, was referred to as the reason why *T. cruzi* trypomastigotes generated higher levels of ROS than epimastigotes (41).

When discussing ROS production in trypanosomatid mitochondria, it is important to mention trypanosomatid alternative oxidase (TAO) (Fig. 3). This protein, which is restricted to the inner mitochondrial membrane of both bloodstream and procyclic forms of *T. brucei*, does not exhibit proton translocation capacity and, therefore, cannot contribute to the proton gradient that drives ATP formation (23), but functions as an electron acceptor. In the parasite mammalian forms, TAO completely replaces complex IV, its function being to oxidize UBQ formed in consequence of G3PDH activity. In the insect stage, TAO coexists with complex IV and, albeit less active than in bloodstream forms (23), it is also functional. In fact, TAO could substitute for complex IV when this was silenced by RNAi (46). Interestingly, it was suggested that, by assisting complex IV in removing excess reducing equivalents and using these to reduce O_2 to water, TAO could function in procyclics to decrease mitochondrial $O_2^{\bullet -}$ (34).

Fumarate reductase as a potential site for ROS generation

In addition to the classic respiratory chain components, other mitochondrial NADH-dehydrogenases may leak electrons directly to O_2 and, in consequence, give rise to $O_2^{\bullet -}$ (48). In trypanosomatids one of these is mitochondrial fumarate

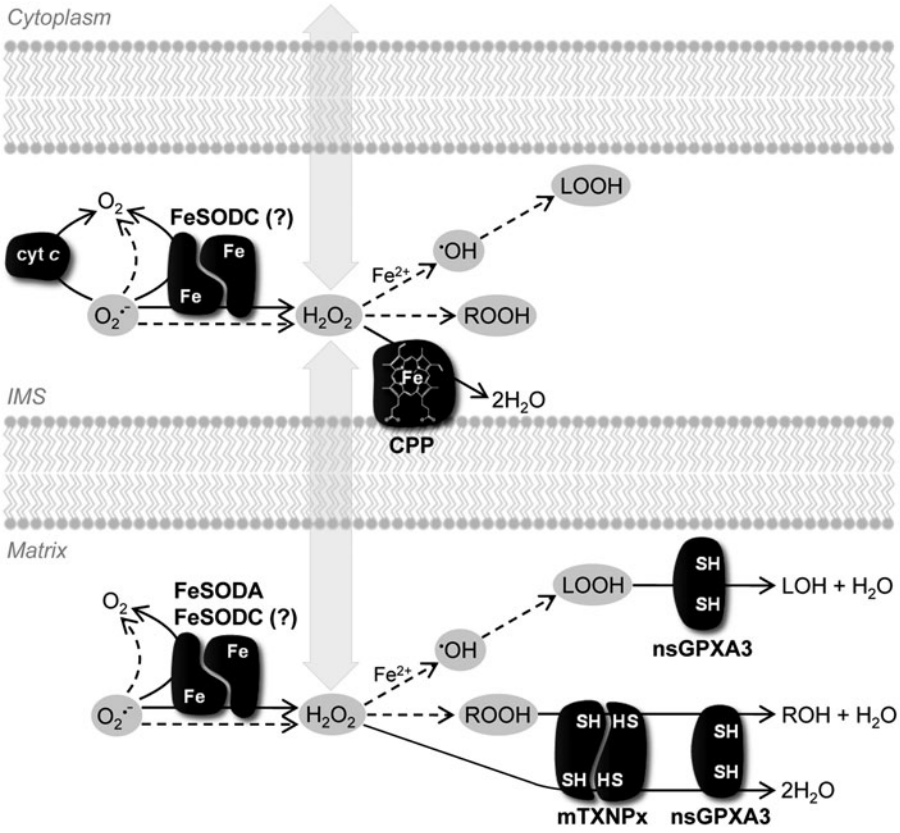

FIG. 4. Routes for formation and elimination of ROS in trypanosomatid mitochondria. This scheme shows the main routes for ROS generation from superoxide anion, as well as the antioxidant enzymes described to be present in trypanosomatid mitochondria. Superoxide anion ($O_2^{\bullet -}$), derived from the single-electron reduction of molecular oxygen (O_2) either in the matrix or in the intermembrane space (IMS), is the precursor of most ROS. It may be eliminated via cytochrome *c* (cyt *c*)-driven oxidation to O_2 in the IMS. Alternatively, it may be dismutated to O_2 and hydrogen peroxide (H_2O_2), either spontaneously or catalytically by FeSODA and FeSODC [the uncertain localization of the latter molecule, either to the IMS or to the matrix (see main text), is indicated by a *question mark*]. Hydrogen peroxide is the oxidizing substrate of CCP in the IMS, as well as of mTXNPx and nsGPXA3 in the matrix. If not eliminated by these enzymes, H_2O_2 can diffuse across membranes, participate in signaling pathways, or react with biological molecules to generate organic hydroperoxides (ROOH). Importantly, H_2O_2 can originate hydroxyl radical ($^{\bullet}OH$) via oxidation of a transition metal ion, such as Fe^{2+} (the "Fenton reaction"). The hydroxyl radical is a powerful oxidant, reacting directly with nucleic acids and proteins, and initiating lipid peroxidation (LOOH). Some of the products resulting from hydrogen peroxide- and hydroxyl radical-driven reactions, namely ROOH and LOOH, can be reduced to the corresponding alcohols (ROH and LOH) by mTXNPx and/or nsGPXA3. In this scheme, ROS are highlighted in *light gray ovals* and the various ROS-eliminating enzymes are represented in *dark gray shapes*. Noncatalyzed and catalyzed reactions are distinguished by *dashed and filled arrows*, respectively. Trypanosomatid FeSODs and mTXNPx are depicted as homodimeric enzymes.

reductase (mFR), a Krebs cycle enzyme that regenerates succinate from fumarate at the expenses of NADH in what constitutes a peculiarity of these parasites (Fig. 3). Mitochondrial fumarate reductase was demonstrated to be a potential site for $O_2^{\bullet -}$ formation in *T. brucei* and *T. cruzi* insect stages (13, 26, 33, 84).

ROS Elimination in Trypanosomatid Mitochondria

Superoxide anion formed in mitochondria may give rise to other ROS (Fig. 4). Among these, H_2O_2 may cross biological membranes (8) and exert its effects in other subcellular compartments. Apart from participating in physiologic processes (*e.g.*, by regulating signaling pathways) (10), H_2O_2 may have deleterious consequences on cells owing to its capacity to react with biological molecules as well as to originate the highly reactive hydroxyl radical ($^{\bullet}OH$). The fine regulation of ROS levels in mitochondria is thus required, so that the cytotoxic impact of these molecules is circumvented without compromising their physiologic functions. Enzymes with ROS-eliminating activity as well as nonenzymatic mechanisms are implicated in such control. Next, we will review the available information on the antioxidant enzymes present in trypanosomatid mitochondria (Fig. 5).

Superoxide dismutases

Elimination of $O_2^{\bullet -}$ is an adequate means to regulate the formation of other ROS. Even though this may occur by the spontaneous dismutation of superoxide to H_2O_2 and O_2, this reaction is accelerated by the activity of superoxide dismutases (SODs) (55). These enzymes dismutate $O_2^{\bullet -}$

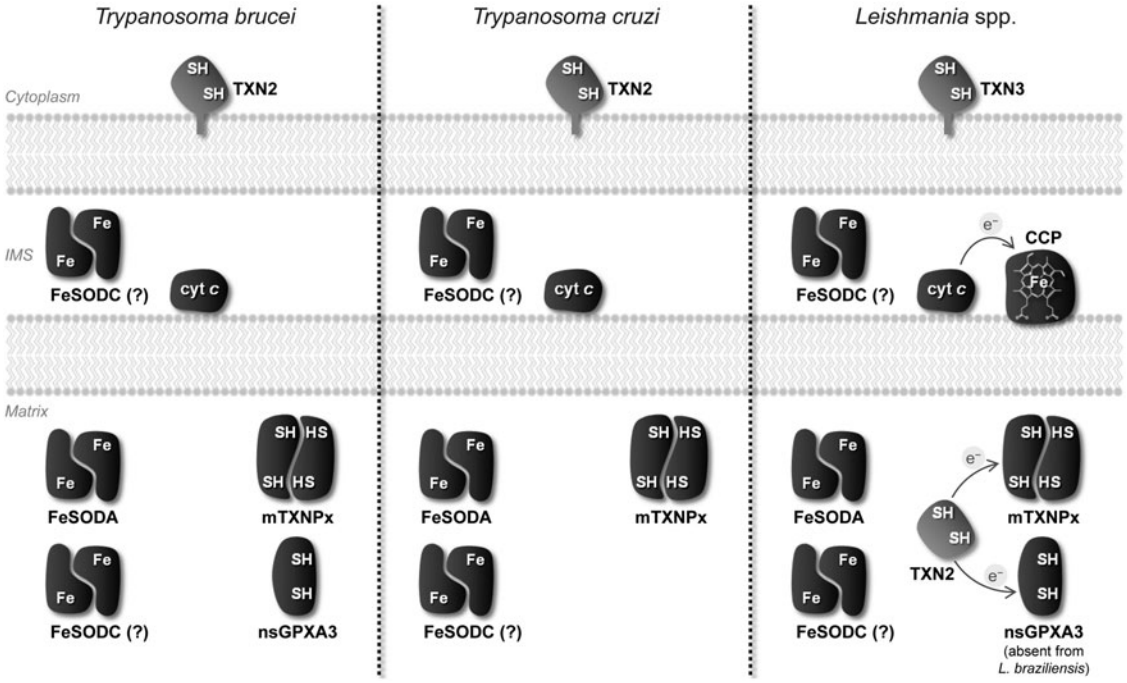

FIG. 5. Comparison of the mitochondrial antioxidant enzymes in *T. brucei*, *T. cruzi*, and *Leishmania* spp. This diagram shows the various mitochondrial ROS-eliminating enzymes in trypanosomatids. Depicted are the enzymes FeSODA, mTXNPx, and nsGPXA3, most likely located in the matrix. The latter enzyme has no obvious counterpart in *T. cruzi* and *L. braziliensis*. Also in the mitochondrial matrix is the TXN2 molecule of *Leishmania* spp., which supplies reducing equivalents (represented with an "ē") to mTXNPx and nsGPXA3. All trypanosomatids harbor a second TXN molecule in their mitochondrion, TXN2 in *T. brucei* and *T. cruzi*, and TXN3 in *Leishmania* spp. This protein is anchored to the outer mitochondrial membrane by a hydrophobic C-tail, with the redox active site facing the cytosol. The intermembrane space (IMS) is equipped with cytochrome *c* (cyt *c*) and presumably with FeSODC (albeit this enzyme may alternatively be located in the matrix). *Leishmania* spp., but not the other trypanosomatids, harbor one CCP enzyme in the IMS, which is reduced by cyt *c*.

following a mechanism that involves a metal (M) co-factor, as detailed next:

$$M^{(n+1)+} + O_2^{\bullet -} \rightarrow M^{n+} + O_2$$

$$M^{n+} + O_2^{\bullet -} + 2H^+ \rightarrow M^{(n+1)+} + H_2O_2$$

Trypanosomatid mitochondria are equipped with two SODs, FeSODA and FeSODC (30, 89) (Fig. 4). Noticeably, these two enzymes utilize iron as metal co-factor and not manganese as mitochondrial SODs of other eukaryotes. This dependence on iron is reminiscent of SODs of prokaryotes and plant chloroplasts. The submitochondrial compartmentalization of FeSODA and FeSODC has never been experimentally addressed, but, at least in the case of the former enzyme, bioinformatic analysis supports its residence in the matrix (9). As for FeSODC, its unusually large N-terminal signal peptide was suggested by Dufernez *et al.* to target the enzyme to the intermembrane space (30) and by Bodył *et al.* (9) to the matrix, its final destination remaining doubtful. As a note, one alternative means to remove $O_2^{\bullet -}$ in this specific compartment is via its cyt *c*-driven oxidation to O_2 (Fig. 4), in analogy to what is described for other systems (70). Additionally, it is conceivable that, as reported for other organisms, $O_2^{\bullet -}$ moves through anion channels into the cytosol (44) where it can be eliminated by local antioxidants.

The role of both FeSODA and FeSODC as antioxidants has been evidenced by forward and reverse genetics. Illustrating this, overexpression of FeSODA was reported to protect *Leishmania* from the free radical generating agents paraquat, nitroprusside (67), and antimycin (39), whereas down-regulation of FeSODC by antisense RNA rendered *Leishmania tropica* promastigotes more sensitive to menadione (40), a known $O_2^{\bullet -}$ inducer. Likewise, knocking down of *FeSODA* by RNAi had a negative impact on the resistance of *T. brucei* bloodstream form parasites to paraquat, a phenotype that was not reproduced by FeSODC-deficient parasites (89). Even though FeSODA and FeSODC are apparently active as antioxidants in the parasite context, neither enzyme is crucial for survival of bloodstream *T. brucei* parasites (89). This fact may result from a mutual compensation of function or it may reflect an insufficient generation of $O_2^{\bullet -}$ by the rudimentary mitochondrion of *T. brucei* at this life stage. In *Leishmania* the impact of mitochondrial SODs on parasite survival appears more prominent, as lack of FeSODC resulted in reduced survival of *L. donovani* amastigotes in mouse macrophages (40). In the case of *T. cruzi*, the report that FeSODA expression is upregulated in infective metacyclic trypomastigotes relative to noninfective epimastigotes (6) suggests that this enzyme may also play a role in parasite adaptation to the mammalian host. This observation may, nevertheless, apply only to some parasite strains, as it was reported that FeSODA expression may vary with the *T. cruzi*

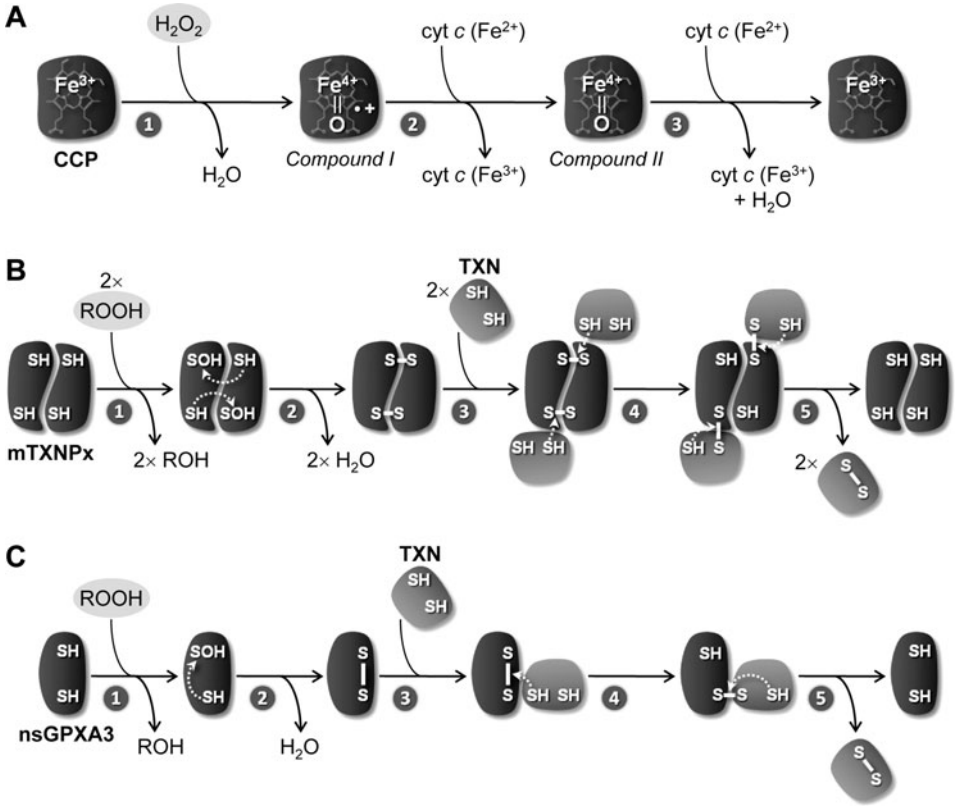

FIG. 6. Catalytic mechanism of cytochrome *c* peroxidase and of thiol peroxidases. (A) 1. In the first step of cytochrome *c* peroxidase (CCP) catalysis, the enzyme reacts with H_2O_2 in a two-electron redox reaction that leads to the heterolytic cleavage of the hydroperoxide and the release of a molecule of water. In the resulting oxidized enzyme (Compound I), the second H_2O_2-derived oxygen atom oxidizes the heme iron to a Fe^{4+}=O oxyferryl group and a nearby tryptophan residue to a cationic radical (•+). 2. This radical is subsequently reduced by one molecule of ferrous cyt *c* [cyt *c* (Fe^{2+})] to generate Compound II, which maintains the Fe^{4+}=O center. 3. The enzyme is reduced back to the resting ferric state by a second one-electron transfer from another cyt *c* (Fe^{2+}) molecule, with release of water. **(B)** and **(C)** The thiol peroxidases mTXNPx and nsGPXA3 share the same steps of catalysis, with the difference that the former reacts as a dimer and the latter as a monomer. 1. First, the proximal Cys of the peroxidase reacts with ROOH to yield ROH and sulfenic acid (-SOH). 2. A disulfide bond is then formed inter- (in the case of mTXNPx) or intramolecularly (for nsGPXA3) between the oxidized Cys and a distal thiol group, with concomitant release of water. 3. The disulfide is subsequently attacked by a Cys thiol group from tryparedoxin (TXN). 4. A catalytic intermediate is generated, in which the proximal Cys of the peroxidase is restored to its reduced (-SH) state and the distal Cys is covalently bound to TXN via an S-S bridge. 5. The reaction cycle is completed by a thiol-disulfide exchange reaction, in which the mixed disulfide is resolved by the second Cys residue of TXN, thus releasing the fully reduced peroxidase and the reducing substrate in its oxidized (S-S) form.

lineage (69). One final note to mention that the antioxidant activity of FeSODA may also account for its involvement in the regulation of apoptotic-like death, as suggested for both *T. cruzi* (72) and *L. donovani* (39).

Cytochrome c peroxidase

Within trypanosomatid mitochondria H_2O_2, the product of $O_2^{\bullet -}$ dismutation, can be removed by different peroxidases. One of these is cytochrome *c* peroxidase (CCP), a heme-containing enzyme that is present in *Leishmania* spp. Originally described in *Leishmania major* (*Lm*CCP) as an ascorbate-dependent peroxidase (2), this enzyme is now known to be preferentially reduced by ferrous cyt *c* (47) following the mechanism depicted in Figure 6A. *Lm*CCP is localized in the inner mitochondrial membrane with its catalytic domain facing the intermembrane space (28). The enzyme plays an antioxidant role (28, 29, 66), but it is redundant throughout the *L. major* life cycle (66). This observation is not entirely surprising if one takes into consideration that *Lm*CCP has no obvious representative in *T. cruzi* and *T. brucei* (Fig. 5). A specific review on *Lm*CCP is presented in this Forum Issue by Adak and Pal.

Thiol peroxidases

The other hydroperoxide-reducing enzymes operating within trypanosomatid mitochondria are two thiol peroxidases, namely a 2-Cys-peroxiredoxin enzyme, designated mTXNPx (16, 82, 87) [or, according to the recent nomenclature proposed by Gretes *et al.* (42), Prx1m], and a nonselenium glutathione peroxidase, referred to as nsGPXA3 (45, 79). The activity of these molecules does not require any cofactor or prosthetic group and, instead, depends on the reactivity of two redox active cysteines. The mechanisms of reaction of these two classes of enzymes, originally dissected by

Rhee (22), Poole (31), and Maiorino (53), are depicted in Figure 6B and 6C.

Both mTXNPx and nsGPXA3 are assumed to be present in the mitochondrial matrix, where they can exert their activity as H_2O_2-reducing enzymes (16, 74, 78) (Fig. 4). In addition to H_2O_2, these two enzymes accept small organic hydroperoxides (18, 27, 78) and, in the case of nsGPXA3, lipid hydroperoxides (27, 78) as oxidizing substrates.

Consistent with the *in vitro* activity of mTXNPx as a peroxidase, its ectopic overexpression was shown to confer parasites resistance towards H_2O_2 of exogenous origins (16, 49, 87). Based on these observations, the main physiologic function of mTXNPx was for a long time regarded to be that of an antioxidant. This concept was, nevertheless, challenged by a recent report demonstrating that the peroxidase function of mTXNPx is not crucial, even though the protein itself is essential during the mammalian stage of *L. infantum* (20). Instead, it is possibly the chaperone-like activity of mTXNPx that is required for amastigote replication (20). In *T. cruzi*, mTXNPx is also expected to be relevant for parasite survival in the vertebrate host, as suggested by the observations that this enzyme is upregulated in virulent *T. cruzi* strains (71) or when these parasites differentiate into metacyclic forms (6). Moreover, mTXNPx overexpression was found to enhance *T. cruzi* survival in mammalian cells (73). For the *T. cruzi* mTXNPx molecule, however, the actual contribution of its peroxidase activity for parasite survival remains to be investigated, following on the abovementioned report that its *L. infantum* counterpart displays an alternative function (20). In what concerns the mitochondrial nsGPXA3, functional studies in *T. brucei* revealed that this enzyme is not vital, its depletion having resulted in cardiolipin peroxidation and in a transient retardation of growth (27). That finding agrees with the fact that other trypanosomatids, namely *T. cruzi* and *Leishmania braziliensis*, can thrive in the absence of a nsGPXA3 orthologue (Fig. 5). Altogether, these observations suggest that when eliminated individually the mitochondrial thiol peroxidases, or more precisely their peroxidase activity, are not vital to trypanosomatids. One possible explanation for this redundancy could be that one molecule might overcome the absence of the other, even though they have different preferences for hydroperoxides. A definitive conclusion about the relevance of both mitochondrial thiol peroxidases as antioxidant devices would, hence, require their simultaneous down regulation. Another unsolved issue regarding mitochondrial thiol peroxidases is their physiologic source of reducing equivalents, as explained next.

Thiol peroxidases are typically reduced by CxxC-containing oxidoreductases of the thioredoxin family (which includes thioredoxins and glutaredoxins) or by proteins possessing thioredoxin-like active sites (Fig. 6B and 6C). Trypanosomatids harbor a specific thioredoxin-like subfamily of proteins, known as tryparedoxins (TXNs) (60), which are the preferred reducing substrates for thiol peroxidases in these organisms. Tryparedoxins are nonenzymatically reduced by trypanothione [$T(SH)_2$] (32), a conjugate of glutathione and spermidine that largely replaces glutathione functions in trypanosomatids and that is itself maintained in the reduced state by the flavoenzyme trypanothione reductase (TR) at the expenses of NADPH. Whereas in biochemical assays, both mTXNPx and nsGPXA3 are reduced by the TR/$T(SH)_2$/TXN system (18, 74, 78), it is not clear how this reaction proceeds in the parasite context. This doubt stems from three different reasons. First, the evidence for the presence of TR in mitochondria is conflicting. In an immunolocalization study in *T. cruzi* (45), TR was reported to partially localize to the mitochondrion (57), but this observation was not corroborated by subsequent studies (15, 78, 81, 88) based on subcellular fractionations of *T. brucei*, *T. cruzi,* and *L. infantum* (13, 58, 61, 67). Second, it is not known whether $T(SH)_2$ is present in mitochondria. Since trypanothione synthetase appears to be restricted to the cytosol (65), the thiol would have to be imported into the organelle by means of carriers either specific or not. Up to now, no such transporters have been identified. Third, the requirement for a mitochondrial TXN is also questionable as trypanosomatids can thrive without a TXN molecule in this cell compartment (17). These organisms do possess a mitochondrial TXN-like enzyme (TXN3 in *Leishmania* spp. or TXN2 in *T. cruzi* and *T. brucei*), but this is a mitochondrial outer membrane protein that has no possibility to interact directly with, hence reduce, mTXNPx and nsGPXA3 (Fig. 5). *Leishmania* spp., but not *T. cruzi* and *T. brucei*, harbor an additional mitochondrial TXN molecule (the *Leishmania* TXN2) (19) that is, nevertheless, redundant (17). In this context, it is worth mentioning that the nonessentiality of a mitochondrial TXN also impacts on other proposed TXN-dependent mechanisms taking place in this organelle, namely the reduction of the universal minicircle sequence binding protein (UMSBP) that is required for kDNA replication (61).

In the absence of a mitochondrial TXN, reduction of thiol peroxidases might be carried out by other oxidoreductases containing both a CxxC motif and a thioredoxin domain. Among these, thioredoxins and glutaredoxins are unlikely to exhibit such activity. The latter, despite having representatives in the mitochondrion of these parasites (21, 36), are unable to replace TXN as reductants of thiol peroxidases (21, 54). As for thioredoxin, although the *T. brucei* enzyme was shown to reduce TXNPx (79) and nsGPXA3 (45) *in vitro*, its presence in the mitochondrion was never demonstrated. In what concerns other putative dithiol-containing oxidoreductases predicted in the genome of trypanosomatids, it is difficult to infer about their role as reductants for thiol peroxidases without experimental evidence.

Nonenzymatic scavengers of hydroperoxides in trypanosomatid mitochondria

In trypanosomatid mitochondria hydroperoxides may also be eliminated nonenzymatically. Since $T(SH)_2$ is capable of scavenging H_2O_2 directly (12, 45), this thiol comes out as an attractive candidate to perform such a role, providing its presence in the organelle is ascertained. Apart from trypanothione, other low molecular mass thiols [*e.g.*, glutathione, ovothiol (5), or free cysteine] as well as thiols exposed on protein surfaces (76) and other antioxidants (*e.g.*, ascorbate) might contribute to mitochondrial antioxidant function. Determination of the concentration of trypanothione and of other thiols in the mitochondria of the several trypanosomatids will help elucidating the importance of nonenzymatic mechanisms on hydroperoxide removal in this organelle.

Conclusion

In spite of species and life cycle stage differences, mitochondria of trypanosomatids have the potential to generate ROS, either in the respiratory chain or in other sites. These organelles are equipped with several antioxidant enzymes, two of which dismutate $O_2^{\bullet -}$ (FeSODA and FeSODC) and three that reduce H_2O_2 and other hydroperoxides (CCP, mTXNPx and nsGPXA3). Interestingly, all five enzymes are only preserved in *Leishmania* spp., with *T. brucei* and *T. cruzi* missing one or two of these molecules (Fig. 5). This fact, added to the findings that the antioxidant activity of these enzymes is not crucial for parasite survival, suggests that their functions may overlap and/or may be efficiently replaced by nonenzymatic scavengers of ROS. The redundant role of mTXNPx and nsGPXA3 as peroxidases is in agreement with the nonessential character of their preferred reducing agent (TXN) within mitochondria. In the future, the simultaneous depletion of several redundant antioxidant enzymes will certainly shed light on the relative importance of catalytic elimination of ROS in trypanosomatids.

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Abbreviations Used

CCP = cytochrome *c* peroxidase
Cys = cysteine
cyt *c* = cytochrome *c*
DHAP = dihydroxyacetone phosphate
DHII = type II NADH dehydrogenase
FeSOD = iron superoxide dismutase
FR = fumarate reductase
G3P = glycerol-3-phosphate
G3PDH = glycerol-3-phosphate dehydrogenase
IMS = intermembrane space
kDNA - kinetoplast DNA
LOOH = lipid hydroperoxides
mTXNPx = mitochondrial tryparedoxin peroxidase
nsGPXA3 = mitochondrial nonselenium glutathione peroxidase
RNAi = RNA interference
ROOH = small organic hydroperoxides
ROS = reactive oxygen species
TAO = trypanosomatid alternative oxidase
TR = trypanothione reductase
$T(SH)_2$ = trypanothione (reduced form)
TXN = tryparedoxin
UBQ = ubiquinone