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Review Article

Insights into the redox biology of *Trypanosoma cruzi*: Trypanothione metabolism and oxidant detoxification

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ABSTRACT

Trypanosoma cruzi is the etiologic agent of Chagas' disease, an infection that affects several million people in Latin America. With no immediate prospect of a vaccine and problems associated with current chemotherapies, the development of new treatments is an urgent priority. Several aspects of the redox metabolism of this parasite differ enough from those in the mammalian host to be considered targets for drug development. Here we review the information about a trypanosomatid-specific molecule centrally involved in redox metabolism, the dithiol trypanothione, and the main effectors of cellular antioxidant defense. We focus mainly on data from *T. cruzi*, making comparisons with other trypanosomatids whenever possible. In these parasites trypanothione participates in crucial thiol–disulfide exchange reactions and serves as electron donor in different metabolic pathways, from synthesis of DNA precursors to oxidant detoxification. Interestingly, the levels of several enzymes involved in trypanothione metabolism and oxidant detoxification increase during the transformation of *T. cruzi* to its mammalian-infective form and the overexpression of some of them has been associated with increased resistance to macrophage-dependent oxidative killing. Together, the evidence suggests a central role of the trypanothione-dependent antioxidant systems in the infection process.

Contents

Abbreviations: $T(SH)_2$, dihydrotrypanothione; TS_2, trypanothione disulfide; GSH, glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; TryR, trypanothione reductase; TXN, tryparedoxin; Gsp, glutathionylspermidine; GspS, glutathionylspermidine synthetase; dsRNAi, double-stranded RNA interference; RR, ribonucleotide reductase; GCS, γ-glutamylcysteine synthetase; BSO, buthionine sulfoximine; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; DFMA, difluoromethylarginine; H_2O_2, hydrogen peroxide; TXNPx, tryparedoxin peroxidase; GPx, glutathione peroxidase; APx, ascorbate-dependent hemoperoxidase; SOD, superoxide dismutase; $^{\bullet}NO$, nitric oxide; $O_2^{\bullet-}$, superoxide radical; Sec, selenocysteine.

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Introduction

Trypanosoma cruzi is a protozoan parasite from the order *Kinetoplastida* that causes Chagas' disease, an infection that affects 16–18 million people mostly in rural regions of Latin America, and belongs to the group of neglected diseases as defined by the World Health Organization (http://www.who.int/tdr/diseases/chagas/swg_chagas.pdf) [1]. The parasite life cycle involves an extracellular, proliferative stage (epimastigote) that resides in the insect vector, and two forms that occur in the mammalian host, a nonproliferating, infective form (trypomastigote) and an intracellular, proliferative form (amastigote). Only two drugs, nifurtimox and benznidazol, are currently available to treat this disease but these are unsatisfactory given their toxic side effects and limited efficacy in the chronic phase of the infection. With no immediate prospect of a vaccine, the search for parasite-specific traits exploitable in terms of new chemotherapies is an urgent priority (http://www.who.int/tdr/diseases/chagas/swg_chagas.pdf). Thus, basic research on metabolic pathways that are crucial for parasite viability or infectivity and distinct from those in the mammalian host may provide clues for rational drug design. In this regard, the thiol-based redox metabolism of *T. cruzi* and its close relatives, *T. brucei* and *Leishmania* spp. (recently reviewed in [2]), has long been considered a source of promising candidates. In contrast to their hosts, which rely on glutathione (GSH)/glutathione reductase (GR) and thioredoxin/thioredoxin reductase for maintaining the intracellular thiol redox homeostasis, trypanosomatids lack GR and thioredoxin reductase. Their redox metabolism depends on a particular dithiol, called trypanothione, and the corresponding reductase, trypanothione reductase (TryR). Here, we review the main features of the trypanothione-dependent metabolic pathways in *T. cruzi*, with special emphasis on trypanothione synthesis and on the proteins involved in antioxidant defense.

Trypanothione, the key player of redox metabolism in trypanosomatids

Trypanothione [N^1,N^8-bis(glutathionyl)spermidine] is a low-molecular-mass dithiol consisting of two GSH molecules covalently linked to spermidine (Fig. 1). Trypanothione was identified 23 years ago in trypanosomatids [3], where it replaces GSH in the majority of the thiol–disulfide exchange reactions of the cell (Fig. 2). Dihydrotrypanothione [$T(SH)_2$] is more reactive than GSH, a property explained by the pK_a of its cysteines, ~7.4, coincident with the intracellular pH, and its dithiol nature [4]. Being a dithiol favors the formation of an intramolecular disulfide bridge after one or two electron oxidations (Fig. 2), and, in the first case, prevents the formation of sulfinyl radicals ($RSOO^{\bullet}$), a species able to propagate oxidation to other molecules. In *T. cruzi*, $T(SH)_2$ levels vary between life stages, being about 1.5–2.1 mM in epimastigotes cultured in the presence of polyamines [5,6] and 0.5 and 0.12 mM in trypomastigotes and amastigotes, respectively [6,7]. These variations in $T(SH)_2$ concentration may be relevant for the functioning of pathways that will be discussed below, such as: (i) those that involve noncatalyzed reactions of $T(SH)_2$ with other molecules (Fig. 2), and (ii) tryparedoxin-dependent reactions (Fig. 2) in amastigotes only, as the K_m of tryparedoxin for $T(SH)_2$ is 40 μM, implying saturating conditions in the other life stages. Although $T(SH)_2$ biosynthesis is likely to take place in the cytosol [8], the presence of $T(SH)_2$ in other cellular compartments has been suggested based on the occurrence of enzymes that use it as electron donor, but this has not been experimentally verified.

Trypanothione is maintained in its reduced state by the activity of the NADPH-dependent flavoenzyme trypanothione reductase. TryR has sequence identity (35%) with human GR, with the two enzymes sharing many physical and chemical properties. The main difference lies in their disulfide specificity: TryR interacts with oxidized forms of positively charged glutathionyl–polyamine conjugates, such as trypanothione, glutathionylspermidine, and bis(glutathionyl)spermine [7] (the apparent second-order rate constant (k_{cat}/K_m) for the reduction of trypanothione disulfide [TS_2] is about $2–41\times10^6$ M^{-1} s^{-1} [9]) while GR only accepts negatively charged oxidized glutathione (GSSG). Specificity is largely determined by five amino acids residues at the substrate binding site, making the active site pocket of TryR wider, more hydrophobic, and negatively charged than that of GR [10]. Studies aimed at localizing TryR in *T. cruzi* have yielded conflicting results. Experiments using antisera raised against a TryR-derived peptide indicated that the enzyme is located in the cytosol and mitochondrion [11]. On the other hand, biochemical fractionation studies suggested that it is found mainly in the cytosol with a significant portion (15–25%) present in glycosomes [12,13] (Fig. 3). Moreover, in one of these studies traces of TryR activity was also

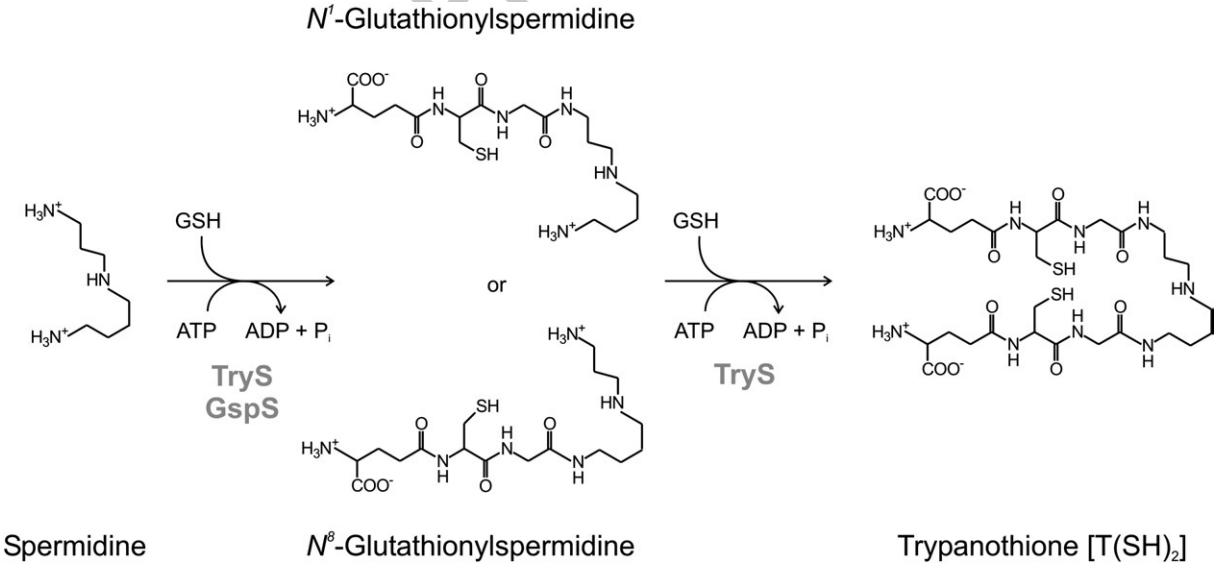

Fig. 1. Biosynthesis of trypanothione. The synthesis of trypanothione [$T(SH)_2$] occurs in two consecutive steps in which the glycine carboxylate groups of glutathione (GSH) are covalently linked to the terminal amino groups of spermidine. The whole process consumes two ATPs and is catalyzed by trypanothione synthetase (TryS). In *C. fasciculata*, glutathionylspermidine (Gsp) can also be synthesized by a separate enzyme, glutathionylspermidine synthetase (GspS).

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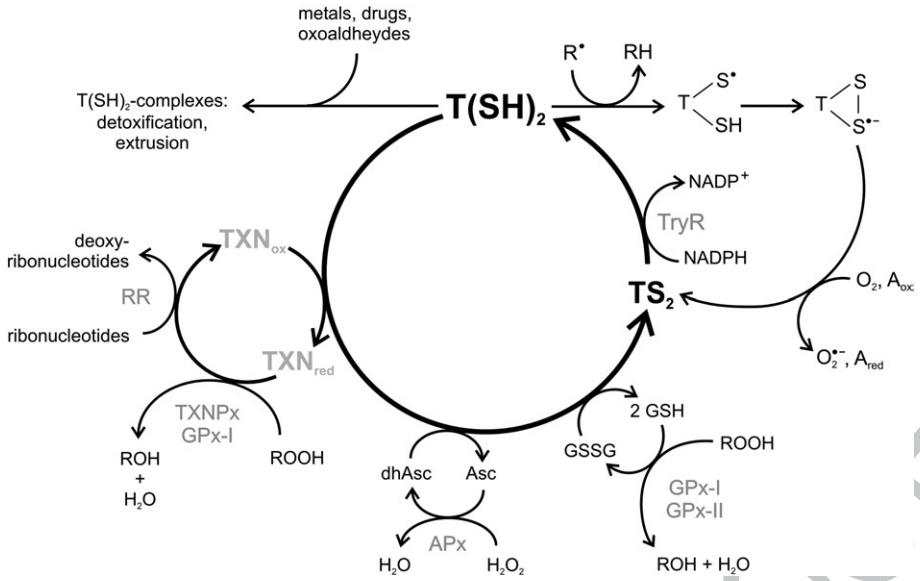

Fig. 2. Trypanothione-dependent reactions. Trypanothione is maintained in its reduced form at the expense of NADPH, in a reaction catalyzed by trypanothione reductase (TryR). $T(SH)_2$ then directly reduces tryparedoxin (TXN), dehydroascorbate (dhAsc) to ascorbate (Asc), glutathione disulfide (GSSG) to glutathione (GSH). Via these intermediaries $T(SH)_2$ participates in the synthesis of deoxyribonucleotides and decomposition of peroxides. $T(SH)_2$ can also interact directly with various electrophiles in detoxification of oxoaldehydes, metals, and drugs. Also, $T(SH)_2$ can react with radical species ($R^\bullet$) in scavenging and/or repair reactions that lead to the transient formation of trypanothione thiyl radical. This sulfur-centered radical is expected to readily combine with the vicinal thiol to yield a trypanothione disulfide anion radical, a good reductant, that evolves to the stable trypanothione disulfide (TS_2) with the concomitant formation of secondary radicals, including superoxide ($O_2^{\bullet-}$) that will be subsequently detoxified. RR, ribonucleotide reductase; ROOH, hydroperoxides; A, one-electron oxidant; TXNPx, tryparedoxin peroxidase; GPx-I, GPx-II, glutathione peroxidase-like tryparedoxin peroxidases I and II; APx, ascorbate-dependent peroxidase. Figure adapted from [80].

detected in mitochondrial fractions [12], but this was probably due to contamination from the cytosolic fraction (S. R. Wilkinson, unpublished). It has been noted that TryR from several trypanosomatids, including *T. cruzi* and *T. brucei*, contains a carboxyl-terminal extension with a weak glycosomal targeting tripeptide and it was thus postulated that it could have a dual cytosolic/glycosomal localization [14]. However, in African trypanosomes TryR has been detected exclusively in cytosolic fractions [15,16]. Intriguingly, in *L. amazonensis*, immunolocalization suggested the presence of TryR also in regions close to the flagellar pocket and other intracellular structure(s) in the posterior end of promastigotes cells [17]. Reverse genetic approaches in *T. brucei* [18] and *L. donovani* [19] have shown that TryR is essential for parasite viability thus validating it as a drug target. In the case of *T. brucei*, conditional gene deletion studies have revealed that parasites can survive with only 10% of their normal TryR activity [18], indicating that low levels of TryR are sufficient to maintain trypanothione in its reduced state under optimal growth conditions. However, TryR-depleted parasites grown in a thiol-free medium could not efficiently detoxify hydrogen peroxide (H_2O_2) and, moreover, showed a reduced capacity to infect mice when compared to wild types [18].

$T(SH)_2$ serves as electron donor in a number of different pathways (Fig. 2). In one of them, it drives a series of two-step oxidation/reduction reactions that result in the decomposition of oxidants such as H_2O_2, peroxynitrite, and a range of short-chain organic, fatty acid and phospholipid hydroperoxides. In all cases $T(SH)_2$ delivers reducing equivalents to intermediary molecules, such as GSSG, dehydroascorbate, or the dithiol protein, tryparedoxin (TXN), which, in their reduced state, can then transfer electrons to the peroxidases. From a structural and functional point of view, TXN represents a distinct molecular clade within the thioredoxin superfamily of oxidoreductases characterized by a WCPPC motif at their catalytic center. Two genes coding for TXN have been identified in *T. cruzi*, though only one isoform, a cytosolic TXN (TXN-I), has been functionally characterized [12]. The other gene codes for a protein (TXN-II) that has around 34% identity with other TXNs, differing from them in having an insertion of 15 amino acids and a C-terminal tail of 26 amino acids. It is not known if TXN-II is a functional TXN and where it is localized. *L. infantum* is the only trypanosomatid where a TXN (TXN-II) has been experimentally detected in the mitochondrion [20]. However, this subcellular compartmentalization has also been suggested for TXN-II of *T. brucei* [21] and inferred for a TXN of *T. cruzi*, based on the existence of a TXN-dependent peroxidase activity in this organelle [22,23]. TXN is specifically reduced by $T(SH)_2$ and, in contrast to thioredoxin, is a poor substrate for other members of the disulfide reductase superfamily, such as TryR [24] or thioredoxin reductase (an enzyme absent in *Kinetoplastida*). The reaction of *T. cruzi* TXN-I with $T(SH)_2$ has a K_m around 40 μM and an apparent second-order rate constant of 8×10^3 M^{-1} s^{-1} [12].

$T(SH)_2$ also plays a role in DNA synthesis, providing reducing equivalents for the ribonucleotide reductase (RR)-catalyzed synthesis of DNA precursors [25]. At high concentrations, $T(SH)_2$ reacts directly with RR, being the only known low-molecular-mass thiol able to deliver reducing equivalents directly to this enzyme. However, in the presence of TXN electron flux is more efficient (similar values of V_{max} but K_m for TXN 560 times lower than for $T(SH)_2$) [25], suggesting that in vivo the dithiol protein is the reductant of RR. Interestingly, TS_2 has shown to be a potent inhibitor of TXN-mediated reduction of RR, thus providing a direct link between the cell redox state and its ability to proliferate [25]. More recently, another link between the $T(SH)_2$-dependent system and the cell division has been proposed. In this case, $T(SH)_2$ and mitochondrial TXN are believed to work in concert to reduce and activate the universal minicircle sequence-binding protein, an enzyme involved in mitochondrial-DNA replication [21]. Although the role of $T(SH)_2$ in cell proliferation has come mainly from work with *T. brucei*, the proteins involved are also present in *T. cruzi* [26], thus suggesting a common pathway.

Lastly, $T(SH)_2$ can react with a variety of electrophiles and as such has been implicated in detoxification of ketoaldehydes, heavy metals, and xenobiotics (reviewed in [27]). Reactive ketoaldehydes are decomposed by the glyoxalase system, whose enzymes, glyoxalase I and II, catalyze the dismutation of the 2-ketoaldehyde to the corresponding 2-hydroxy acid. In the majority of the organisms GSH

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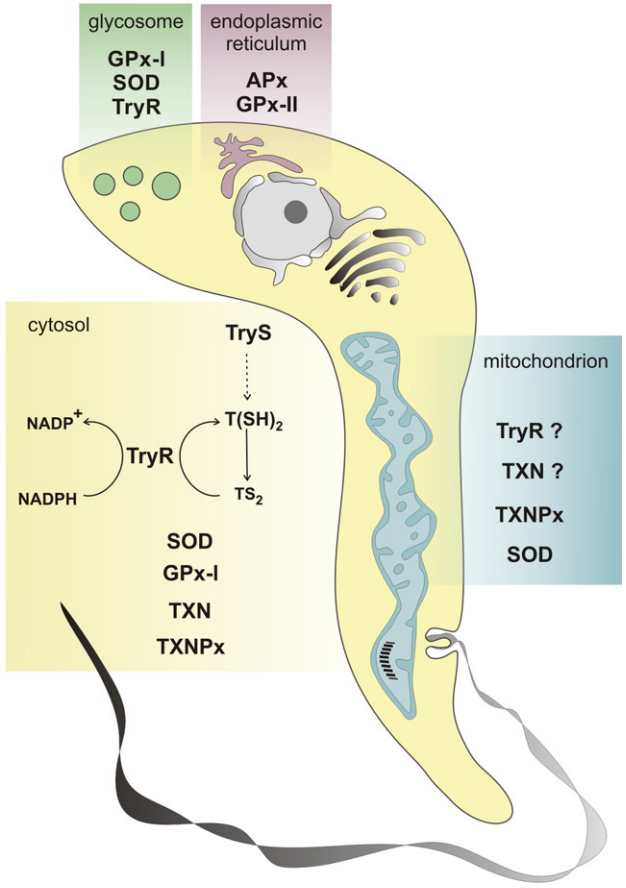

Fig. 3. Subcellular distribution of enzymes involved in redox metabolism. A schematic representation of a *T. cruzi* epimastigote showing the location of the different enzymes involved in redox metabolism. Yellow, cytosol; green, glycosome; purple, endoplasmic reticulum; light blue, mitochondrion. TryS, trypanothione synthetase; TryR, trypanothione reductase; TXN, tryparedoxin; TXNPx, tryparedoxin peroxidase; GPx-I; GPx-II, glutathione peroxidase-like tryparedoxin peroxidases I and II; APx, ascorbate-dependent peroxidase; SOD, superoxide dismutase.

is used as cofactor but in trypanosomatids $T(SH)_2$ carries out this role. $T(SH)_2$ reacts directly with the ketoaldehyde and at the end of the catalytic cycle is recovered in reduced form. *T. cruzi* [28], *L. major* [29,30], and *L. donovani* [31,32] possess both glyoxalase I and glyoxalase II whereas *T. brucei* contains only glyoxalase II [33]. $T(SH)_2$ has also been implicated in the detoxification of heavy metal and drugs. Most evidence comes from the analysis of *Leishmania* species resistant to arsenite- or antimony-containing drugs. In vitro drug-selected resistant parasites have higher levels of $T(SH)_2$ than susceptible ones [34–36]. Additionally, some resistant parasites display an associated amplification of the *pgpa* (Gp-glycoprotein-like protein A) gene [35,36]. This codes for an intracellular ATP-binding cassette (ABC) transporter of the multidrug resistance proteins (MRP) subfamily, known to facilitate the efflux of metal–thiol conjugates [37]. Recently, these two features were also described in field isolates of *L. donovani* naturally resistant to antimony [38]. Moreover, it has been shown that $T(SH)_2$ can form adducts with arsenite in vitro and in vivo [34,39]. Taken together, these data suggest that resistance could be associated with the formation, followed by extrusion or sequestration of $T(SH)_2$–drug conjugates. In this scenario, the existence of a $T(SH)_2$–*S*-transferase activity had been proposed as another feature that could contribute to parasite resistance [40]. In fact, this activity has been described in several species of *Leishmania*, *T. brucei,* and *Crithidia fasciculata* but could not be detected in *T. cruzi* epimastigotes [41]. However, a recent study has shown that at least in *L. tarentolae*, $T(SH)_2$–*S*-transferase activity is not increased in antimony-resistant parasites [42]. In the case of *T. cruzi* the link between $T(SH)_2$ and drug detoxification is less clear. On the one hand, treatment of parasites with buthionine sulfoximine (BSO), a compound that reduces intracellular thiol pools by inhibiting GSH synthesis (see next section), resulted in increased sensitivity to nifurtimox and benznidazol of the three forms of *T. cruzi* [43,44]. On the other hand, it has been shown that exposure of *T. cruzi* to both drugs resulted in a decrease in intracellular thiols, specially $T(SH)_2$ [45]. As thiols could not be recovered by treatment of parasite extracts with TryR and NADPH, it was suggested that conjugation of the drugs to $T(SH)_2$ was the reason for the observed decrease in $T(SH)_2$ levels [45]. However, $T(SH)_2$–drug complexes have not been identified nor detected in the extracellular media.

In addition to $T(SH)_2$ and GSH, *T. cruzi* also contains several other low-molecular-mass thiols including glutathionylspermidine (Gsp) and ovothiol A [N^1-methyl-4-mercapthohistidine]. These, as well as GSH, are maintained in their reduced states by direct reaction with $T(SH)_2$ [6]. The concentrations of these thiols vary between parasite life stages and with the phase of growth, with $T(SH)_2$ being the most abundant in most conditions. Though several activities have been reported for Gsp [46,47] and ovothiol A [48] in vitro, specific in vivo functions remain unknown. This low-molecular-mass thiol pool, including $T(SH)_2$, may play an important and direct role in parasite antioxidant defense via scavenging of primary radicals (i.e., $^{\bullet}NO_2$ or $CO_3^{\bullet-}$) and repairing protein radicals (e.g., tyrosyl radical) (Fig. 2). Although these nonprotein thiols can also react with nonradical oxidants (i.e., H_2O_2 or peroxynitrite), their direct contribution to this pathway in vivo may be minor, taking into account that these two-electron oxidants are more efficiently decomposed by the enzymatic detoxification systems that are discussed later (e.g., apparent second-order rate constants for reaction of peroxynitrite with a cytosolic tryparedoxin peroxidase and $T(SH)_2$ are WW$1\times1\times10^6$ and 71×10^3 M^{-1} s^{-1}, respectively [49]).

Biosynthesis of trypanothione

Trypanothione synthesis results from the convergence of GSH and spermidine metabolic pathways, which are unconnected in mammalian cells. Trypanosomatids are able to synthesize GSH from its precursor amino acids. The first enzyme in this pathway, γ-glutamylcysteine synthetase (GCS), is rate limiting [50]. It is essential in *T. brucei* [51] and its activity can be inhibited by BSO, a compound that also blocks GSH transport [51] and cures or prolongs survival of *T. brucei*-infected mice [52]. GSH synthesis has not been extensively studied in *T. cruzi* but BSO has been used as an experimental tool for decreasing GSH and, to a lesser extent, $T(SH)_2$ and Gsp levels [43,44,53].

The way in which trypanosomatids obtain spermidine varies. *T. brucei* spp., *Leishmania* spp., and *C. fasciculata* are able to synthesize polyamines de novo from arginine, through the concerted action of arginase and ornithine decarboxylase (ODC). The resulting diamine, putrescine, is then converted to spermidine by spermidine synthase (reviewed in [54,55]). Spermidine has been identified as the crucial polyamine for parasite proliferation [56,57] and accordingly, the enzymes involved in its synthesis or that of its precursors have been shown to be essential (reviewed in [54]). In *T. cruzi* the scenario is rather different and polyamine metabolism has been a matter of controversy for almost 20 years [55]. It is certain that the three stages of the parasite are able to scavenge polyamines from the medium [58,59]. The transporters involved in diamine uptake have been functionally characterized in epimastigotes and they contain thiol residues that are critical for their activity [60]. A gene coding for a spermidine transporter has been expressed in an heterologous system where it was shown to be functional [61]. However, characterization of this transporter in *T. cruzi* is still lacking. On the other hand, the ability of *T. cruzi* to synthesize polyamines from arginine is a matter of

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debate. The idea that *T. cruzi* is auxotrophic for polyamines was based on evidence derived mainly from work on epimastigotes. One of the main arguments was that the parasite, though possessing an active spermidine synthase, lacks ODC [26,62]. Moreover, when epimastigotes were grown in polyamine-deficient medium, proliferation was arrested after 3 to 4 days and GSH levels increased while Gsp and $T(SH)_2$ were almost undetectable. When medium was supplemented with putrescine or spermidine proliferation was restored together with Gsp and $T(SH)_2$ levels, the latter becoming the predominant low-molecular-weight thiol. This recovery was not observed when the culture was supplemented only with arginine [58].[1] However, other results support the idea that *T. cruzi* synthesizes putrescine through a pathway different from the one used by the other trypanosomatids and similar to the one used by prokaryotes, plants, and the protozoan parasite *Cryptosporidium parvum*. This pathway involves the enzymes arginine decarboxylase (ADC), that forms agmatine from arginine, and agmatinase, that converts agmatine into putrescine (reviewed in [63]). The first evidence of the existence of this pathway in *T. cruzi* came from the observation that inhibitors of ADC, difluoromethylarginine (DFMA) among others, inhibit trypomastigote infectivity and amastigote proliferation in mammalian cells [64,65]. DFMA inhibition was reverted in the presence of agmatine or putrescine, indicating that DFMA was acting on parasite polyamine metabolism. Later, a modest DFMA-inhibitable ADC activity was measured in extracts of trypomastigotes by the detection of $^{14}CO_2$ released from L-[U-^{14}C]arginine and the production of [^{3}H]agmatine and [^{3}H]putrescine from L-[2,3,4,5-^{3}H]arginine [66]. Finally, an effect of DFMA was also observed in epimastigotes. This form of the parasite dies by programmed cell death when exposed to fresh human serum [67,68]. Parasite death was partially inhibited by arginine as well as by several polyamines, including agmatine [68]. In this model, DFMA abrogated the protective effects of arginine, suggesting that at least part of it depends on endogenous polyamine synthesis [68]. Until now no ADC or agmatinase genes have been identified in the *T. cruzi* genome [26]. However, it is important to take into account that ADCs are evolutionary highly divergent enzymes [69], and therefore, identification of the corresponding genes may require strategies more refined than normal BLAST searches based on full-length sequences. Given that trypanosomatids have developed a special molecule to govern the thiol metabolism of the cell, it would be surprising if *T. cruzi* had lost the capacity to synthesize one of the precursors of this molecule. The fact that this parasite resides in environments rich in polyamines would not by itself explain such a loss, as other parasites, which take up polyamines from the host, have conserved the machinery to synthesize them [70,71]. In our opinion, although *T. cruzi* undoubtedly depends on exogenous polyamines in many conditions, the question about the contribution of endogenous polyamine synthesis is not completely settled.

Trypanothione is synthesized by the sequential covalent binding of two GSH molecules to terminal NH_2 groups of spermidine, in a process that consumes two ATPs (Fig. 1). Both steps are catalyzed by a single enzyme, called trypanothione synthetase (TryS), that has been characterized from *T. cruzi* [72], *T. brucei* [73,74], *L. major* [8], and *C. fasciculata* [75]. The *T. cruzi* TryS (*tctrys*) gene is present as a single copy per haploid genome and the encoded protein shows 74–81% similarity with the amino acid sequence of the corresponding trypanosomatids homologs. Also, it shows homology with Gsp synthetase (GspS) from *C. fasciculata* and *Escherichia coli* (46 and 51% similarity, respectively) and it does not have homologs in mammals. The catalytic properties of *Tc*TryS have been addressed using purified recombinant enzyme [72]. The reported apparent K_m for GSH was 570 and 190 μM with spermidine and Gsp, respectively, and 625 μM for spermidine and 66 μM for Gsp. The enzyme showed ratios of k_{cat}/K_m of 6×10^3 M^{-1} s^{-1} for spermidine and 151×10^3 M^{-1} s^{-1} for Gsp as substrates. In the presence of equal amounts of GSH and spermidine, $T(SH)_2$ is formed with no apparent accumulation of Gsp [72]. The synthetase activity of TryS resides in its C-terminal domain. The proposed catalytic mechanism involves the formation of a ternary complex among GSH, Mg^{2+}-ATP, and the enzyme, leading to the activation of GSH (probably by phosphorylation of the glycine carboxyl group) for the glutathionylation of the terminal amino group of spermidine or Gsp [75]. Apart from its synthetase activity, all TryS and GspS so far studied have a $T(SH)_2$/Gsp-amidase activity located in their N-terminal domains. In recombinant *Tc*TryS and *Tb*TryS this represents about 1% of the synthetase activity [72,74]. For *T. cruzi* and *C. fasciculata*, the opposing synthetic and hydrolytic functions of TryS and GspS were proposed to regulate polyamine levels in response to polyamine availability and growth phase [72,76]. The 3D crystal structure of *L. major* TryS has been determined recently [77]. It was noted that the last 20 amino acids at its C-terminus contribute to the amidase domain and, in particular, block the accessibility to the active site [77]. A similar peptide segment (20- to 26-residues long) is present in TryS from *C. fasciculata*, other *Leishmania* spp., and *T. cruzi* but the amino acids responsible for placing this C-terminal lid in the amidase active site are conserved only in the enzymes of the first two trypanosomatid genus [77]. Interestingly, *Tb*TryS and *E. coli* GspS lack this C-terminal extension and interdomain communication has nevertheless been demonstrated to negatively regulate the amidase activity of the bacterial enzyme [78]. Evidently, further studies will be required to dissect the different molecular mechanisms controlling the opposite activities in these enzymes as well as to elucidate whether this bifunctional enzyme assemble represents a selective advantage for the parasites.

*Tc*TryS has broad substrate specificity; it can accept polyamines other than spermidine, such as aminopropylcadaverine or spermine [72]. The respective glutathionylated products, namely homotrypanothione and mono- or bis(glutathionyl)spermine are also substrates for TryR [7]. However, these products have been detected in vivo only when epimastigotes cultures were specifically supplemented with cadaverine or spermine. Indeed, in epimastigotes containing similar amounts of spermine and spermidine the derivatives of the latter [Gsp and $T(SH)_2$] are preferentially synthesized over the spermine derivatives. This broad substrate specificity has been proposed as an advantage for *T. cruzi* in the case of spermidine shortage and in view of its apparent inability to synthesize polyamines de novo.

Inhibition of *trys* expression in bloodstream and procyclic forms of *T. brucei* by double-stranded RNA interference (dsRNAi) showed that this enzyme is essential for parasite viability [79,80]. On induction of dsRNAi, $T(SH)_2$ levels rapidly decreased, and this was followed by growth arrest and cell death. Moreover, the decrease in $T(SH)_2$ also resulted in increased susceptibility to oxidants [80]. In procyclics, the decrease in $T(SH)_2$ is paralleled by a marked increase in free GSH (4.8 times), a moderate increase in total GSH (1.3 times), and a decrease in ODC activity [79]. There are no studies about the essentiality of TryS in other species of trypanosomatids, but it is reasonable to think that the situation in *T. cruzi* will be similar to the one described in *T. brucei*.

In addition to TryS, *T. cruzi* contains a gene coding for GspS [26]. The homolog in *C. fasciculata* encodes for an enzyme that has a K_m for spermidine 15-fold lower than *Cf*TryS [76]. For this reason, GspS has been proposed as the first enzyme of the $T(SH)_2$ biosynthetic pathway in this organism [76]. *Tc*GspS has 63% similarity with *Cf*GspS and contains the conserved arginine residues shown to be required for substrate binding in related enzymes [75,81]. However, there is no direct evidence that the enzyme may represent an additional source of Gsp in vivo. Actually, the level of Gsp in epimastigotes grown in the presence of polyamines is five times lower than in *C. fasciculata,* and similar to the amount found in promastigotes of *T. brucei* [6], where there is no evidence for GspS [82].

[1] F. Irigoín, G. Peluffo, L. Piacenza, and R. Radi, unpublished.

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Effector proteins of the antioxidant defense: Old and new actors

Initial studies on *T. cruzi* antioxidant defenses suggested that the absence of classical vertebrate-detoxifying enzymes such as catalase and glutathione peroxidases rendered the parasite highly sensitive to oxidative stress [83]. Over the subsequent 28 years, this picture has been changing continuously and a whole battery of different detoxifying enzymes has been characterized. The antioxidant armamentarium of *T. cruzi* is distributed among different cellular compartments (Fig. 3) and is active against a wide range of oxidants. It comprises two peroxiredoxins, at least two peroxidases that share sequence homology with glutathione peroxidases (GPx), an ascorbate-dependent hemoperoxidase, and four iron-containing superoxide dismutases. Interestingly, transformation of the parasite from the epimastigote to the infective form is accompanied by an increase in the expression of several antioxidant enzymes [84,85]. This can be interpreted as a preadaptation of the parasite prior to invasion of the vertebrate host in readiness to environments where it may be exposed to reactive oxygen and nitrogen species such as those generated by immune cells. In this respect, it has been well established that macrophages activated by proinflammatory cytokines (i.e., IFN-γ, TNF-α) represent a first line of defense against *T. cruzi* infection [86]. Under these conditions macrophages produce high levels of nitric oxide ($^{\bullet}NO$) due to the activity of the inducible nitric oxide synthase. Moreover, on infection with trypomastigotes, the assembly of NADPH oxidase leads to the production of superoxide radical ($O_2^{\bullet -}$) [87] that in the presence of $^{\bullet}NO$ forms peroxynitrite, a highly toxic molecule that kills *T. cruzi* in a dose-dependent manner [88]. As discussed below, the antioxidant enzymes of the parasite are able to decompose the main toxic diffusible species (i.e., H_2O_2, peroxynitrite) produced by immune cells. Nitric oxide is the only diffusible species that cannot be enzymatically decomposed by any of the previously described enzymes. In prokaryotes, nitric oxide dioxygenases convert $^{\bullet}NO$ to NO_3^- [89]. Taking into account that *T. cruzi* has several enzymes that are present in bacteria and not in other eukaryotes (for example, GspS, Fe-containing superoxide dismutases [see below]), the existence of $^{\bullet}NO$ dioxygenases is an issue that deserves to be explored.

T. cruzi possess several distinct peroxidases, five of which have been characterized. Two of these are typical 2-Cys peroxiredoxins and are called tryparedoxin peroxidases (TXNPx) because they are reduced by TXN [22,24] (Fig. 2). The two isoforms, one cytosolic (cTXNPx) and the other present in the mitochondrion (mTXNPx) (Fig. 3), decompose H_2O_2, peroxynitrite, and, with lower efficiency, organic hydroperoxides. Kinetic parameters have been determined for recombinant cTXNPx, which has second-order rate constants of 51×10^6, 11×10^6, and 31×10^4 M^{-1} s^{-1}, for the reduction of the above-noted species, respectively[2] [49,90], values comparable with those determined for peroxiredoxins from other organisms (reviewed in [91]). Regeneration of the reduced enzyme by TXN occurs with an apparent second-order rate constant of 11×10^5 M^{-1} s^{-1} [90]. When looking at the catalytic efficiency of the individual steps of the electron flow from $T(SH)_2$ to the hydroperoxide, it seems that peroxide detoxification is dependent on $T(SH)_2$ concentration, as regeneration of reduced TXN by $T(SH)_2$ is the rate-limiting step in this pathway (81×10^3 M^{-1} s^{-1} [12]). In this regard, it is again surprising that *T. cruzi* depends entirely on host polyamine availability so as to maintain the concentration of such a central molecule.

The second class of trypanosomal peroxidase has similarity with mammalian phospholipid hydroperoxide GPxs. Four GPx homologs have been identified in the *T. cruzi* genome, two of which, *Tc*GPx-I and *Tc*GPx-II, have been characterized [12,92,93]. *Tc*GPx-I is found in both the cytosol and the glycosomes (organelles where most of the glycolysis takes place in trypanosomatids [94]) while *Tc*GPx-II is present in the endoplasmic reticulum (Fig. 3). Both decompose organic peroxides, especially those derived from phospholipids and fatty acids, but are unable to decompose H_2O_2 [92]. This substrate specificity has led to the suggestion that their main function may be to prevent cellular damage due to lipid peroxidation. Trypanosomal GPxs differ from their mammalian counterparts in that they have cysteine instead of selenocysteine (Sec) as their catalytic residue and they lack critical residues involved in GSH binding. Consequently they are not as efficient at reducing oxidants and their affinity for GSH is extremely low (K_m about 6 mM) [92,93]. The search for alternative electron donors showed that *Tc*GPx-I is actually another type of TXN-dependent peroxidase. Enzyme activity with TXN-I as reductant is 8- to 15-fold higher than with GSH [12]. The difference between these two substrates is even more dramatic in the case of the homologous enzyme of *T. brucei*, in which the activity of the recombinant enzyme is 5 orders of magnitude higher with the $T(SH)_2$/TXN system when compared with GSH [95]. These observations are compatible with the emerging idea that most cysteine-containing GPxs use thioredoxin or thioredoxin-like proteins as reductant instead of GSH [96]. Contrarily, oxidized *Tc*GPx-II cannot be regenerated by TXN-I, GSH being the only known electron donor identified to date. The catalytic efficiency of *Tc*GPx-II reduction by GSH is low (21×10^3 M^{-1} s^{-1}) [93], thus questioning the relevance of the *Tc*GPx-II function in vivo in a GSH-dependent fashion. In this regard, one can envisage functions for the trypanosomal GPxs different from or in addition to the detoxification of hydroperoxides. For instance, it has been suggested that they might act in hydroperoxide signaling/transduction [97] as it has been demonstrated for the yeast homolog GPx3 [98]. In summary, similar to the TXNPx described above, *Tc*GPxs activity is linked to the oxidation of $T(SH)_2$, through GSH, or in the case of *Tc*GPx-I, more efficiently through TXN (Fig. 2).

A third type of peroxidase, related to ascorbate-dependent hemoperoxidases (APx) found in plants, has been detected in the endoplasmic reticulum of *T. cruzi* (Fig. 3) [99]. Sequences related to *Tc*APx have been identified in other intracellular *Kinetoplastida*, such as *Leishmania* species, but are apparently absent in the blood dwelling *T. brucei*. *Tc*APx decomposes H_2O_2 but not organic hydroperoxides, and uses ascorbate as a source of reducing equivalents; the resultant dehydroascorbate is reduced nonenzymatically by $T(SH)_2$ (Fig. 2) [100]. Ascorbate can be synthesized by the parasite itself. The enzyme that catalyzes the last step of ascorbate synthesis has been characterized in *T. brucei* and *T. cruzi* and is functionally equivalent to the plant ortholog galactonolactone dehydrogenase [101,102]. Unlike plants, where the enzyme has a mitochondrial localization, in *Trypanosoma* the enzyme is present in the glycosomes [102]. Interestingly, whereas both *T. brucei* and *T. cruzi* appeared to be unable to take up ascorbate or dehydroascorbate from the medium, the enzyme responsible for ascorbate synthesis seems to be essential only in *T. cruzi* [102]. This correlates with the presence of APx in *T. cruzi*, but not in *T. brucei*, suggesting that this branch of oxidant detoxification is important in *T. cruzi* biology and possibly related to its intracellular lifestyle in the mammalian host.

As discussed, all trypanosomal peroxidases depend on the $T(SH)_2$/TryR system for their activity. However, when looking at Fig. 3 it is clear that the complete regeneration pathway is not always present in the compartments where peroxidases are found. This poses a central question: How do electrons actually reach the different cellular compartments? For example, assuming that there is a mitochondrial TXN, as is the case in *L. infantum*, it is not known how trypanothione reaches this organelle. Are there specific trypanothione transporters? Is TS_2 reduced in the cytosol and subsequently transported? Is there a pool of $T(SH)_2$ within the mitochondrion that is maintained in this form by TryR present in the organelle [11]? The situation is even more complicated if we wish to explain GPx-I function in the glycosome, where we should add to the previous questions how does TXN reach this compartment. In the case of APx it has been suggested that

[2] T. Arcari, D. Pyñeiro, C. Robello, R. Radi, and M. Trujillo, M., unpublished.

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dehydroascorbate is reduced by $T(SH)_2$ in the cytosol and that ascorbate then enters the endoplasmic reticulum through specific transporters [13]. Thus, there is still a considerable amount of work to do to understand fully the functioning of the antioxidant system in trypanosomatids.

Apart from the hydroperoxide-detoxifying enzymes *T. cruzi* expresses several superoxide dismutase (SOD) isoforms able to metabolize $O_2^{\bullet-}$. Due to the very limited diffusion capacity of $O_2^{\bullet-}$ SODs are expected to decompose endogenously produced $O_2^{\bullet-}$only. With the exception of the bloodstream form of *T. brucei*, that has a nonfunctional mitochondrial electron transport chain (reviewed in [103]), the main source of $O_2^{\bullet-}$ is probably the mitochondrion. Interestingly, all trypanosomatid SODs are of the Fe class, a group restricted to other protozoans, prokaryotes, and chloroplasts. There are four SOD genes in the *T. cruzi* genome, though only two of them have been cloned and the recombinant proteins characterized [104,105]. These are a mitochondrial (SODA) [106] isoform and another isoform (SODB1) whose compartmentalization, cytosolic/glycosomal, has been inferred from localization studies of the *T. brucei* ortholog protein (Fig. 3). In *T. brucei*, two other proteins have been studied, SODB2, which is located mainly in glycosomes, and SODC, a second mitochondrial SOD isoform [107,108].

In order to probe the in vivo role of antioxidant enzymes, the general strategy has been to manipulate cells genetically to specifically increase or decrease their expression. Parasites thus transformed have been studied with regard to their ability to cope with oxidants and trypanocidal compounds or to infect cells or animals (Table 1). In the case of *T. cruzi*, experiments have predominantly involved overexpression of the enzyme under study; null mutants are difficult to obtain and dsRNAi cannot be employed as *T. cruzi* lacks the necessary machinery [109]. In general, elevated levels of TXNPxs, GPx-I, and APx rendered parasites more resistant to H_2O_2 [12,22,99] and *t*-butyl hydroperoxide [12,22]. Recently, it has been shown that cTXNPx-overexpressing cells were also resistant to exogenous and endogenous peroxynitrite [23]. Interestingly, these parasites were more infectious than controls to peroxynitrite-producing macrophages and mice.[3] Similar results were obtained with *L. donovani* cTXNPx-overexpressing cells, that were more resistant to oxidants and more virulent to macrophages than control cells [110]. The relevance of the $T(SH)_2$-dependent antioxidant metabolism is further supported by experiments in *T. brucei*, in which dsRNAi technology can be used. Decreased levels of many enzymes involved in $T(SH)_2$ metabolism and $T(SH)_2$-dependent peroxide detoxification correlated with impaired resistance to oxidants (Table 1) with GCS, TryS, TryR, TXN-I, cTXNPx, and GPx-I shown to be essential to the parasite in unstressed conditions (Table 1). Regarding $O_2^{\bullet-}$ detoxification, overexpression of cytosolic/glycosomal SOD (SODB1) in *T. cruzi* caused an increased susceptibility to some $O_2^{\bullet-}$generating drugs, such as benznidazol and gentian violet, but had no effect on the action of others, such as nifurtimox and paraquat [105]. The higher susceptibility of the overexpressing cells was interpreted in terms of increased levels of H_2O_2 under conditions where peroxide metabolism is rate limiting. Moreover, the unaltered susceptibility of overexpressing cells toward nifurtimox and paraquat was explained by different compartmentalization of the enzyme and the production of $O_2^{\bullet-}$. Overexpression of the mitochondrial isoform (SODA) rendered epimastigotes more resistant to complement-dependent programmed cell death, a stimulus that alters mitochondrial function with a concomitant increase in $O_2^{\bullet-}$ production [5]. In *T. brucei*, the simultaneous knockdown of SODB1 and SODB2 by dsRNAi resulted in growth arrest and cell death, whereas decrease in mitochondrial SOD isoforms did not affect cell proliferation [108]. Further dissection of the SODB function by using null mutants revealed that individually neither enzyme was essential to the parasite, indicating a degree of functional redundancy in this arm of the oxidative defense pathway

Table 1
Phenotypes associated with increased or decreased levels of enzymes involved in redox metabolism in *T. cruzi* and *T. brucei*

Enzyme[a]	Species	Method	Phenotype
$T(SH)_2$ metabolism			
GCS	*T. brucei*	dsRNAi	Impaired cell growth and viability [51]
Spermidine synthetase	*T. brucei*	dsRNAi	Growth arrest [57]
TryS	*T. brucei*	dsRNAi	Impaired growth and viability, increased sensitivity to H_2O_2 and *t*-butyl hydroperoxide [79,80]
TryR	*T. cruzi*	Overexpression	Unchanged susceptibiliy to H_2O_2, nifurtimox, and gentian violet [116]
	T. brucei	Conditional null-mutants	Impaired growth and viability, increased sensitivity to H_2O_2, less virulent in mice [18].
$T(SH)_2$-dependent oxidant detoxification			
TXN-I	*T. brucei*	dsRNAi	Impaired cell growth and viability [117,118], enhanced sensitivity to H_2O_2 [118]
TXN-II	*T. brucei*	dsRNAi	None [117]
cTXNPx	*T. cruzi*	Overexpression	Enhanced resistance to H_2O_2, *t*-butyl hydroperoxide [22], and peroxynitrite [23]
	T. brucei	dsRNAi	Impaired cell growth and viability, increased sensitivity to H_2O_2, and *t*-butyl hydroperoxide [117]
mTXNPx	*T. cruzi*	Overexpression	Enhanced resistance to H_2O_2, *t*-butyl hydroperoxide [22], and peroxynitrite [23]
	T. cruzi	Gene deletion	Impaired cell growth (S.R. Wilkinson, unpublished data)
	T. brucei	Overexpression	Loss of kinetoplast DNA [21]
	T. brucei	dsRNAi	None [117]
APx	*T. cruzi*	Overexpression	Enhanced resistance against H_2O_2 [99]
GPx-I	*T. cruzi*	Overexpression	Enhanced resistance to H_2O_2 and *t*-butyl hydroperoxide [12]
	T. brucei	dsRNAi	Impaired growth and viability, increased susceptibility to H_2O_2 [117]
GPx-II	*T. cruzi*	Overexpression	None (S.R. Wilkinson. unpublished data)
	T. brucei	dsRNAi	None (S.R. Wilkinson, unpublished data)
$O_2^{\bullet-}$ detoxification			
SODA	*T. cruzi*	Overexpression	Increased resistance to complement-dependent programmed cell death [5]. Unchanged susceptibility to benznidazol (M.C. Taylor, personal communication)
	T. brucei	dsRNAi	No effect on parasite growth. Increased sensitivity to paraquat [108]
SODB1	*T.cruzi*	Overexpression	Enhanced sensitivity against benznidazol and gentian violet [105]
	T. brucei	Gene deletion	Increased sensitivity to nifurtimox and benznidazol [111]
SODB1/SODB2	*T. brucei*	dsRNAi	Impaired growth and viability [108]
SODB2	*T. brucei*	Gene deletion	None [111]

[a] GCS, γ-glutamylcysteine synthetase; TryS, trypanothione synthetase; TryR, trypanothione reductase; TXN, tryparedoxin; cTXNPx and mTXNPx, cytosolic and mitochondrial tryparedoxin peroxidase, respectively; APX, ascorbate-dependent hemoperoxidase; GPx, glutathione peroxidase-like tryparedoxin peroxidase; SODB1, SODB2, SODA, and SODC, cytosolic/glycosomal, glycosomal, and mitochondrial superoxide dismutases, respectively.

[3] M.N. Alvarez, G. Peluffo, L. Piacenza, S. Wilkinson, and R. Radi, unpublished.

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[111]. Intriguingly, cells deficient in SODB1 have increased sensitivity to nifurtimox and benznidazol, a phenotype not shared by the SODB2 null mutant lines. The interplay between the different SOD isoforms and trypanocidal drugs will be better understood when the mode of action of these drugs is clearly established. In the case of nifurtimox (a nitrofuran derivative) and benznidazol (a nitroimidazol derivative) it has been proposed that the nitro group of both compounds is reduced to an amino group by nitroreductases, with the formation of free radical intermediates and electrophilic metabolites. It is generally accepted that nifurtimox acts mainly as a redox cycling agent, producing $O_2^{\bullet-}$, whereas benznidazol acts mainly through the formation of drug–protein/drug–thiol conjugates [112]. However, it becomes now critically important to reassess these concepts and make direct measurements of rates and localization of $O_2^{\bullet-}$ production when these drugs are present, in view of the new available methodologies for oxidant detection and our current knowledge of T. cruzi redox biology.

The sequencing of trypanosomatid genomes has provided new tools for the identification of putative new proteins involved in redox metabolism. For instance, genes coding for the machinery of selenocysteine incorporation have been recently identified in these organisms. These include a putative selenophosphate synthetase (the enzyme that generates selenophosphate, the selenium donor for selenoprotein biosynthesis [113]), a Sec-tRNA, and a Sec-tRNA-specific elongation factor [114]. Moreover, genes coding for three selenoproteins have also been identified in the trypanosomatid genomes [114]. Two of them are distant homologs of the mammalian SelK and SelT, whereas the third one, SelTryp, has neither Sec- nor cysteine-bearing homologs in other organisms, suggesting that it is a *trypanosomatidae*-specific protein. Transcription of Sel-K and Sel-T was detected in *T. brucei* and a selenoprotein with the predicted molecular weight of SelK was identified in *T. cruzi* epimastigotes by metabolic labeling with ^{75}Se [114]. The function of these proteins is unknown; however, considering that all selenoproteins of known function participate in redox catalysis [115], this generalization could be extended to the ones found in trypanosomatids.

Conclusions

In the present work we have tried to summarize what is known about the redox biology of *T. cruzi* in the context of trypanothione metabolism and oxidant detoxification, the molecules that orchestrate these processes and their role in different aspects of parasite life. Also, we have pointed out some puzzling facts that need to be answered in the future to understand fully the functioning of the system.

The pleiotropic role of $T(SH)_2$ and the unique nature of the whole system in trypanosomatids make it an ideal target for pharmacological intervention. In this respect, it appears advisable to block the system in its initial steps, i.e., the synthesis of T(SH)2 and/or its reduction. TryR is the most studied enzyme of the $T(SH)_2$ system and numerous studies have looked for selective inhibitors (reviewed in [27]). However, the fact that its activity has to be reduced more than 90% to affect parasite viability probably underlies the poor efficacy demonstrated by TryR inhibitors in vivo. On the other hand, TryS appears as a promising target. Preliminary studies trying to inhibit its activity using substrate analogues have given some positive results with *T. brucei* although not yet with *T. cruzi*.[4] The three-dimensional structure of *L. major* TryS has been recently described [77] and this will be a major input for the design of structure-based inhibitors. Other molecules that may be envisaged as central for parasite life are the putative transporters that mediate $T(SH)_2$ movement among different cellular compartments, as well as the transporters involved in polyamine uptake. However, this is a field in which everything is still to be done.

Finally, the antioxidant enzymes have a key role in the detoxification of macrophage-derived oxidants and the success of the infection [23]. Preliminary data suggest that virulence of *T. cruzi* strains correlates positively with higher levels of several of these enzymes,[5] which may therefore constitute virulence factors whose inhibition could result in infection control.

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