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Forum Review

Peroxidases of Trypanosomatids

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Abstract

This article provides an overview about the recent advances in the dissection of the peroxide metabolism of Trypanosomatidae. This family of protozoan organisms comprises the medically relevant parasites *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp. Over the past 10 years, three major families of peroxidases have been identified in these organisms: (a) 2-cysteine peroxiredoxins, (b) nonselenium glutathione peroxidases, and (c) ascorbate peroxidases. In trypanosomatids, these enzymes display the unique feature of using reducing equivalents derived from trypanothione, a dithiol found exclusively in these protozoa. The electron transfer between trypanothione and the peroxidases is mediated by a redox shuttle, which can either be tryparedoxin, ascorbate, or even glutathione. The preference for the intermediate molecule differs among each peroxidase and so does the specificity for the peroxide substrate. These observations, added to the fact that these peroxidases are distributed throughout different subcellular compartments, point to the existence of an elaborate peroxide metabolism in trypanosomatids. With the completion of the trypanosomatids genome, other molecules displaying peroxidase activity might be added to this list in the future. *Antioxid. Redox Signal*. 10, 000–000.

Trypanosomatids

Kinetoplastida are primitive eukaryotic organisms that parasitize animals and plants, some of which are relevant due to the diseases they cause and the economic losses thereof associated. Among the Order Kinetoplastida, the Trypanosomatidae family acquires particular significance because it includes the human parasites *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp. These are the causative agents of sleeping sickness, Chagas' disease, and the three manifestations of leishmaniasis, which together cause 112,000 deaths per year (http://www.who.int/tdr/, last accessed May 2008). Apart from infecting humans, *T. brucei brucei*, *T. congolense*, and *T. vivax* also cause Nagana in cattle in Africa, and some species of *Leishmania* lead to viscerocutaneous leishmaniasis in dogs. The medically important trypanosomatids have digenic life cycles that alternate between a mammalian host and an insect vector, usually the responsible for disease transmission (Fig. 1). Another member of Trypanosomatidae is *Crithidia fasciculata*, a parasite of mosquitoes, noninfectious to animals and, for this reason, frequently used as model for the disease-causing species. Being phylogenetically close, trypanosomatids share many biochemical traits, among which a peroxide metabolism exclusively dependent on a unique dithiol, trypanothione. Throughout this review, the various trypanothione-dependent peroxidases of trypanosomatids will be presented from a comparative point of view, and their importance to the parasites analyzed.

Trypanothione

In nearly all living organisms, maintenance of an intracellular reducing milieu is possible by means of high concentrations of the sulfur-containing tripeptide, glutathione (GSH). In members of the order Kinetoplastida, however, most of their glutathione content is found in the form of a unique thiol, N^1,N^8-bis(glutathionyl)spermidine, also known as trypanothione (34).

Trypanothione is a conjugate of two glutathione molecules with one spermidine. In trypanosomatids, as in other organisms, glutathione is synthesized by the consecutive activity of the enzymes α-glutamylcysteine synthetase and glutathione synthetase (41, 51). This is not the case of spermidine, for which the biosynthetic pathway diverges according to the trypanosomatid species. While in *T. brucei*, *C. fasciculata*, and *Leishmania* spp. this polyamine is synthesized from or-

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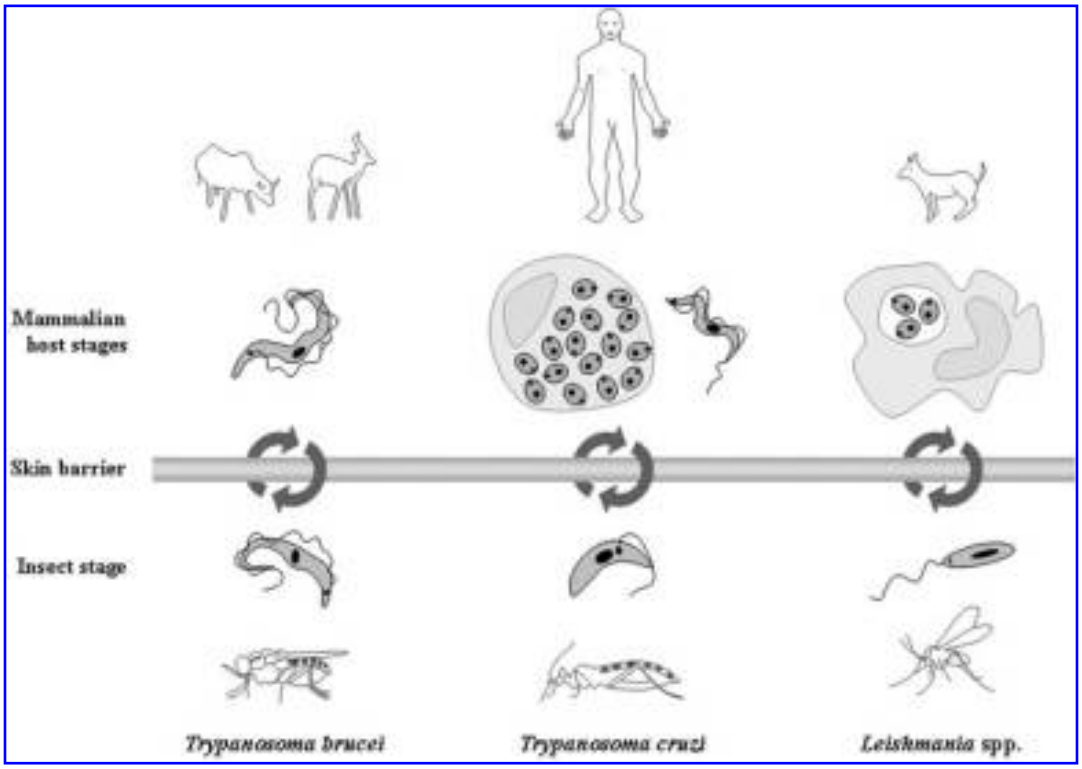

FIG. 1. Life cycles of *Trypanosoma brucei*, *T. cruzi*, and *Leishmania* spp. *T. brucei* is transmitted to man and to other mammals when *Glossina* spp. (tsetse fly) has a bloodmeal. This parasite is extracellular and flagellated during the complete life cycle. In the mammalian hosts, *T. brucei* is found in the circulatory and lymphatic systems. Both man and cattle are susceptible to disease. *T. cruzi* is transmitted to a wide range of mammals through the feces of infected triatomines, but only man is afflicted by the disease. In mammals, *T. cruzi* alternates between an extracellular flagellated infective stage and an intracellular aflagellated replicative form, the so-called amastigote. Almost any cell, phagocytic or nonphagocytic, can be infected, although the parasite has a tropism for muscle and nervous cells. *Leishmania* spp. are a complex of over 20 species. Infective flagellated forms, inoculated into different mammals by the bite of phlebotomine insects, are internalized by phagocytic cells, mainly macrophages. In phagolysosomes, those transform into amastigotes that replicate and infect new macrophages. Both man and dogs can develop leishmaniasis.

nithine and methionine via a ubiquitous pathway consisting of four enzymatic steps, in *T. cruzi* one of the enzymes, ornithine decarboxylase, is absent, and these organisms depend on efficient polyamine uptake by high-affinity transporters (46).

The synthesis of trypanothione starts with formation of glutathionylspermidine, followed by insertion of a second glutathione molecule to yield trypanothione. These ATP-dependent reactions are catalyzed by one single enzyme, trypanothione synthetase (TRYS) (25, 26, 78, 79). Trypanothione biosynthsesis shows, nevertheless, some divergence among the individual trypanosomatids. Indeed, contrary to what was observed in *T. brucei* (25) and in some species of *Leishmania* (79), *C. fasciculata* harbors an additional enzyme capable of driving the first step of synthesis, glutathionylspermidine synthetase (GSPS) (26). Genes encoding for putative GSPSs are also found in the genomes of *T. cruzi* and *L. infantum* (http://www.genedb.org/, last accessed May 2008). *T. cruzi* TRYS is additionally capable of conjugating glutathione with other polyamines (5), a feature that may be advantageous during a potential shortage of polyamines in the surrounding environment.

Since its discovery in 1985, a myriad of biological functions have been directly or indirectly attributed to trypanothione, namely ascorbate homeostasis, synthesis of deoxyribonucleotides by ribonucleotide reductase, conjugation and export of metals and drugs, glyoxal removal. and peroxide metabolism (reviewed in ref. 60). These reactions consume reducing equivalents derived from dihydrotrypanothione [$T(SH)_2$], the reduced form of trypanothione, and generate trypanothione disulfide (TS_2). Regeneration of the dithiol is made possible by the activity of trypanothione reductase (TR) (Fig. 2), an enzyme present in all Kinetoplastida representatives (reviewed in ref. 36). TR belongs to the protein family of FAD disulfide oxidoreductases, that also comprises glutathione reductase, lipoamide dehydrogenase, thioredoxin reductase, and the flavoprotein AhpF of the alkylhydroperoxide reductase system of *S. typhimurium*. The catalytic mechanism of TR resembles that of glutathione reductase, the main difference being the high specificity of each enzyme towards their respective disulfide substrates (reviewed in ref. 59).

Trypanothione participation in numerous physiological pathways renders this thiol crucial for Kinetoplastida survival. Illustrating this, TRYS was found essential for *T. brucei* survival (6, 27) and TR was shown to be critical for survival and/or infectivity of *L. donovani* and *T. brucei* (reviewed in ref. 75).

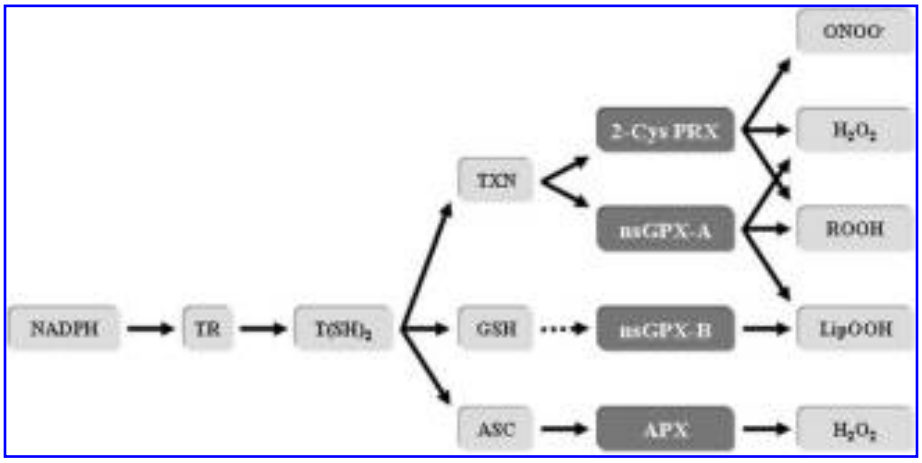

FIG. 2. Peroxidases of trypanosomatids. Hydroperoxide and peroxynitrite ($ONOO^-$) elimination in trypanosomatids is mainly carried out by peroxidases (*dark gray boxes*) belonging to three distinct families of enzymes: 2-Cys peroxiredoxins (2-Cys PRX), nonselenium glutathione peroxidase-like enzymes (nsGPX), and ascorbate peroxidases (APX). Trypanosomatidal nsGPXs can be further segregated into groups A and B, according to their amino acid sequences and their preference for the reductant. In these organisms, peroxidases are fueled by reducing equivalents derived from dihydrotrypanothione [$T(SH)_2$], which itself is kept reduced by the NADPH-dependent flavoenzyme trypanothione reductase (TR). Electron transfer between $T(SH)_2$ and the various peroxidases occurs via an intermediate molecule, either tryparedoxin (TXN), glutathione (GSH), or ascorbate (ASC). Molecules, such as thioredoxin, can also act as redox shuttles between trypanothione and the peroxidases, although less efficiently than TXN. The *broken arrow* was chosen to point out the high K_M of nsGPX-B for GSH. ROOH, small organic chain hydroperoxides (*e.g.*, *t*-butyl hydroperoxide and cumene hydroperoxide); LipOOH, lipid hydroperoxides (*e.g.*, linoleic acid hydroperoxide and phosphatidylcholin hydroperoxide).

Trypanothione-dependent Peroxidases

It has long been known that trypanosomatids lack catalase and classical selenium-containing glutathione peroxidases, two major hydroperoxide-eliminating enzymes generally present in eukaryotes (reviewed in ref. 36). Instead, these organisms' hydroperoxide metabolism was found to depend on trypanothione (16, 47, 82, 83), even though no trypanothione peroxidase molecule could ever be isolated. The enzymatic pathway linking trypanothione to hydroperoxide reduction was eventually solved in 1997 (76), as consisting of the concerted action of two proteins, a tryparedoxin and a tryparedoxin peroxidase belonging to the peroxiredoxin family of enzymes. This unique trypanothione-dependent antioxidant system, first described in *C. fasciculata* (76), is common to all pathogenic species of the Trypanosomatidae family, including *T. brucei rhodesiense* (32), *L. infantum* (18), *T. brucei brucei*, *T. cruzi*, *L. major*, and *L. donovani* (reviewed in ref. 60). Since the dissection of this enzymatic pathway, other trypanothione-dependent systems culminating in hydroperoxide reduction have been described, as detailed later in this review.

Tryparedoxin/tryparedoxin peroxidase pathways

Tryparedoxins. Tryparedoxin (TXN) is the term coined to designate members of the thioredoxin superfamily that are found exclusively in Kinetoplastida (76). TXNs are trypanothione-dependent oxidoreductases that differ from typical thioredoxins in several aspects: (a) they share only 15% homology with thioredoxins, (b) are ~5 kDa larger than thioredoxins, and (c) the TXNs' active site motif is Trp–Cys–Pro–Pro–Cys–Arg, instead of Trp–Cys–(Gly/Ala)–Pro–Cys–Lys for most thioredoxins. In addition, unlike typical thioredoxins, TXNs are not directly reduced by a NADPH-dependent flavoprotein. Instead, the electron transfer between the flavoprotein trypanothione reductase (TR) and TXN is mediated by trypanothione. TXN can also be reduced by glutathione, although with a very low efficacy (19, 40, 65), unlikely to be physiologically relevant. Kinetically, TXN follows an enzyme substitution or ping-pong mechanism, whereby the reducing [$T(SH)_2$] and the oxidizing (peroxidase) substrates react with the enzyme in two independent steps. The rate limiting step of the catalysis is the reaction with trypanothione (19, 40, 109). TXNs are highly abundant proteins (28, 76, 109), representing 3–5% of the total soluble cell protein content of *C. fasciculata* (76) and *T. cruzi* (109). They are found in the cytosol and, at least in *Leishmania* (19), also in the parasite's single mitochondrion. Depending on their subcellular compartmentalization, TXNs react with different tryparedoxin peroxidases (76, 109–111) and also with other substrates, namely ribonucleotide reductase (31) and possibly the Universal Minicircle Sequence Binding Protein (UMSBP) (77), in this way being linked to diverse cell functions such as peroxide metabolism, synthesis of deoxynucleotides, and regulation of mitochondrial DNA replication, respectively.

Tryparedoxin peroxidases. The term "tryparedoxin peroxidase" designates all peroxidases that use TXN as source of reducing electrons during removal of peroxides. These include members of the peroxiredoxin and of the non-selenium glutathione peroxidase families (Fig. 2).

2-Cysteine peroxiredoxins (2-Cys PRXs). Peroxiredoxins (PRXs) represent a ubiquitous family of antioxidant enzymes with hydroperoxide and peroxynitrite reducing activity. Recently, these enzymes have also been attributed the function of chaperones (22, 52, 53, 71). Peroxiredoxins lack prosthetic groups or tightly bound metal ions and, instead, use redox active cysteines (Cys) to reduce their substrates. Depending on the number of Cys residues directly involved in catalysis and on the mechanism of reaction, peroxiredoxins fall into three categories: (a) typical 2-Cys peroxiredoxins, (b) atypi-

Table 1. Comparative Analysis of the Peroxidases of the Human Trypanosomatids *Trypanosoma brucei*, *T. cruzi*, and *Leishmania* spp.

Organism	*Peroxidase*		*Subcellular compartment*	*Reductant*	*Peroxide preference*	*k′ (M^{-1} s^{-1})*	*References*	*Author's designation*
T. brucei	2-Cys PRX	PRX1	Cytosol	TXN	H_2O_2/ROOH	1.7×10^7	14, 99	TRYP1 or *Tac*TXNPx
					$ONOO^-$	9×10^5	100	
		PRX2	Mitochondrion	TXN	H_2O_2/ROOH	ND	99	TRYP2
	nsGPX	nsGPX-A1/2	Cytosol	TXN	§	ND	49, 93	Px I and Px II
		nsGPX-A3	Mitochondrion and glycosomes	TXN	H_2O_2/ROOH/ThyOOH	1×10^5	24, 49, 93	Px III
		nsGPX-B	ND	ND	ND	ND	–	–
	APX	–	–	–	–	–	–	–
T. cruzi	2-Cys PRX	PRX1*	Cytosol	TXN	H_2O_2/ROOH	3.2×10^4	42, 108	TcCPX or *Tcc*TXNPx
					$ONOO^-$	7.2×10^5	100	
		PRX2	Mitochondrion	TXN	H_2O_2/ROOH	ND	108	TcMPX
	nsGPX	nsGPX-A1	Cytosol and glycosomes	TXN	ROOH/LipOOH	2.4×10^5	107, 109	TcGPXI
		nsGPX-A2/3	Cytosol‡	ND	ND	ND	–	–
		nsGPX-B	ER	GSH (K_M = 5 mm)	LipOOH	1.8×10^4	111	TcGPXII
	APX	APX	ER	Ascorbate	H_2O_2	3.5×10^6	110	TcAPX
Leishmania spp.	2-Cys PRX	PRX1/2	Cytosol	TXN	H_2O_2/ROOH	2.4×10^5	8, 9, 18, 37, 54, 61	LicTXNPx1/2, LcPxn2/1, LaPxn2/1, Lmf30 or LdTXNPx
					$ONOO^-$	1×10^6	[a]	
		PRX3	Glycosomes‡	ND	ND	ND	8	LcPxn3
					H_2O_2/ROOH	3.8×10^6	17, 18	LimTXNPx
					$ONOO^-$	1.6×10^6	[a]	
		PRX4	Mitochondrion	TXN	H_2O_2/ROOH	ND		
	nsGPX	nsGPX-A1	Mitochondrion and/or glycosomes‡	ND	ND	ND	–	–
		nsGPX-A2†	Mitochondrion‡	ND	ND	ND	–	–
		nsGPX-A3	Cytosol‡	ND	ND	ND	–	–
		nsGPX-B	ND	ND	ND	ND	–	–
	APX	APX	ND	Ascorbate	H_2O_2	ND	2	LmAPX

*In the *T. cruzi* genome, two additional open reading frames are annotated, which code for protein sequences carrying one amino substitution relative to PRX1; the substitutions are unlikely to be functionally relevant and for that reason the molecules are not listed here; †not annotated in the *L. infantum* genome project; ‡based on the presence or absence of an obvious organelle targeting signal and on bioinformatics analysis, but not experimentally demonstrated; §not determined, but possibly the same as the *T. brucei* nsGPX-A3. [a]S. Romao and Ana M. Tomás, unpublished.

ND, not determined; ROOH, small chain organic hyroperoxides (*e.g.*, *t*-butyl and cumene hydroperoxides); ThyOOH, thymine hydroperoxide; LipOOH, lipid hydroperoxides (*e.g.*, linoleic acid and phosphatidylcholine hydroperoxides); Lm, *L. major*, Ld, *L. donovani*; Li, *L. infantum*; Lc, *L. chagasi*; La, *L. aethiopica.*

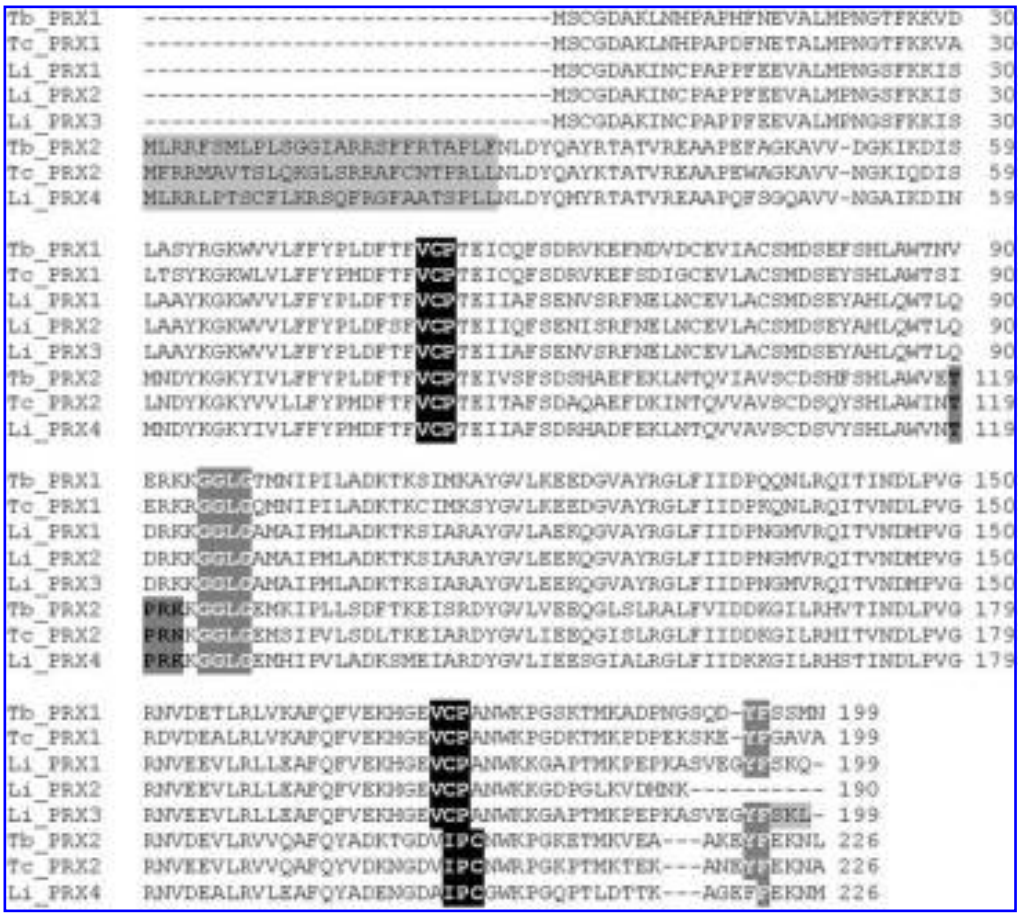

FIG. 3. Alignment of trypanosomatidal 2-Cys peroxiredoxins. Sequence accession numbers are the following: Tb_PRX1, AAG45225; Tc_PRX1, CAA09922; Li_PRX1: AY058210; Li_PRX2: AAG40074 (*Leishmania chagasi*); Li_PRX3, AAK69587 (*L. chagasi*); Tb_PRX2, Q9GU47; Tc_PRX2, CAA06923; Li_PRX4: AY058209. In the *T. cruzi* genome, two additional open reading frames are annotated, which code for protein sequences carrying one amino acid substitution relative to Tc_PRX1. These substitutions are unlikely to be functionally relevant and, for that reason, the sequences are not shown here. The VCP and IPC motifs directly involved in catalysis are highlighted in *white letters on black*. Residues implicated in 2-Cys PRX sensitivity to overoxidation by H_2O_2 are shown in *white letters on gray*. Amino-terminus mitochondrial and C-terminus glycosomal targeting signals are marked on *light gray background*. A sequence homologous to CDKs-mediated phosphorylation motifs is depicted in **bold** on *dark gray*. Tb, *T. brucei*; Tc, *T. cruzi*; Li, *L. infantum* (same as *L. chagasi*).

cal 2-Cys peroxiredoxins, and (c) 1-Cys peroxiredoxins (reviewed in ref. 86).

Peroxiredoxins were the first enzymes reported to display tryparedoxin peroxidase (TXNPx) activity (76). Peroxiredoxins of trypanosomatids are typical 2-Cys peroxiredoxins and can be grouped according to their cytosolic or mitochondrial compartmentalization (Table 1). Mitochondrial peroxiredoxins are unusual in having the second redox active Cys embedded in an Ile–Pro–Cys motif, instead of Val–Cys–Pro often found in 2-Cys peroxiredoxins (Fig. 3). Also, mitochondrial peroxiredoxins are encoded by single-copy genes, while the cytosolic enzymes may include closely related genes clustered within the same chromosomal *locus* (see Fig. 3. for accession numbers). In *Leishmania* spp., one of these genes carries a glycosomal targeting signal, suggesting that the enzyme may localize to this peroxisome-like organelle. Trypanosomatidal peroxiredoxins are broad-spectrum peroxidases acting on H_2O_2 and organic hydroperoxides. The crithidial enzyme also decomposes complex lipid hydroperoxides (76); however, the peroxiredoxins of *T. brucei* and *Leishmania* react poorly and are rapidly inactivated by these substrates (in particular, linoleic acid and phosphatidylcholin hydroperoxides) (14, 17, 37, 61). In addition, the Kinetoplastida enzymes act catalytically on peroxynitrite decomposition at considerably high rates (9, 100, Table 1). Evidences about the function of peroxiredoxins within the parasites was obtained from *Leishmania* and *T. cruzi* overexpression mutants, which are more resistant to hydroperoxides and peroxynitrite added exogenously (9, 18, 62, 108), and also from double-stranded RNA interference in *T. brucei* (112).

Typical 2-Cys peroxiredoxins are obligate homodimers, containing subunits of ~22 kDa and two identical reaction centres. According to the generally accepted mechanism for these enzymes, the proximal Cys of one enzyme subunit reduces the peroxide substrate being oxidized to a cysteine sulfenic acid (Cys-SOH). This residue is subsequently attacked by the C-terminal Cys of an inverted subunit to form a stable intersubunit disulfide bond, which is then resolved by a cell-specific disulfide oxidoreductase (reviewed in ref.

86). In the case of trypanosomatids, this oxidoreductase is TXN. Such sequence of independent catalytic steps meets the definition of an enzyme substitution mechanism, typical for peroxidases in general. Surprisingly, however, deviations from this mechanism were observed upon kinetic analysis of the peroxiredoxin enzymes of *L. infantum* and *T. brucei* (14, 17), and also upon review of the original kinetic data of *C. fasciculata* and *T. cruzi* (14). The results revealed that the enzymatic activity slowed down as the hydroperoxide concentration increased. Although the reasons for this behavior remain undisclosed, Budde *et al*. (14) provided an interpretation based on the Changeux–Monod model for oligomeric proteins (70). The authors could apply this model to 2-Cys peroxiredoxins because they exist as decamers built up of five dimers arranged in a ring-like structure, the transition between the dimeric and the decameric states being redox-dependent (3, 14, 17, 116). According to the proposed model, oxidation of one reaction center within the decamer would induce conformational changes in remote dimers, ultimately impairing their reactivity with the hydroperoxide substrate (14).

In the majority of eukaryotic cells, peroxiredoxins are considered to act as regulators of H_2O_2-mediated intracellular signaling processes, such as cell differentiation, proliferation, and apoptosis, and not as general antioxidant devices. Such fine-tuning role is possible because these enzymes are sensitive to inactivation by relatively low amounts of H_2O_2, a phenomenon attributed to overoxidation of the proximal Cys by the hydroperoxide substrate (reviewed in refs. 91, 92, 115). The inactive sulfinic acid residue (-SO_2H) resulting from overoxidation can be reduced back to the catalytically active thiol form by a sulfiredoxin or a sestrin molecule (reviewed in ref. 55). In the case of trypanosomatids, it is, nevertheless, unclear whether peroxiredoxins are involved in regulation. Certainly, these parasites possess additional peroxidases (presented later in this review) capable of general antioxidant functions. Also, in trypanosomatids peroxiredoxins are likely present in large amounts (76), a feature that has been related to their regulatory role in other organisms (91, 115). Furthermore, the two motifs required for H_2O_2-sensitivity [*i.e*., the Gly–Gly–Leu–Gly motif and the C-terminal arm containing a Tyr-Phe sequence (115)], are conserved in almost all trypanosomatidal peroxiredoxin sequences (Fig. 3). Dissection of the crystal structure of the *T. cruzi* cytosolic enzyme strongly suggests that these motifs may modulate activity (85) and the kinetic behavior of these enzymes complies with sensitivity to hydroperoxide inactivation (14, 17). So far, however, the only evidence that trypanosomatidal peroxiredoxins can be overoxidized came from a study performed on $ONOO^-$-challenged *T. cruzi* (84). In addition, molecules capable of reverting the overoxidized state of peroxiredoxins are apparently missing in these parasites. Indeed, sulfiredoxin is not present in the trypanosomatid genome and, although all three medically relevant organisms possess a putative open reading frame containing a sestrin signature (accession numbers: LmjF18.0650, LinJ18_V3.0660, Tb10.05.0190, Tc00.1047053511277.130), the homology to sestrins of higher eukaryotes is too low to infer a function.

One consequence of inactivation by H_2O_2 is the aggregation of peroxiredoxins into complexes with molecular weight higher than the decamer, which is usually associated with loss of peroxidase activity and appearance of chaperone activity (22, 52). More recently, aggregation and functional switching of peroxiredoxins was described to also take place as a result of phosphorylation by cyclin-dependent kinases (CDKs) (53). Phosphorylation occurs at the threonine residue located 38 residues downstream of the proximal redox-active cysteine, sitting in a (Ser/Thr)–Pro–Xaa–(Lys/Arg) consensus sequence (20). Interestingly, in all mitochondrial peroxiredoxins of trypanosomatids this sequence is conserved (Fig. 3). However, their subcellular compartmentalization renders phosphorylation by CDKs unlikely.

Nonselenium glutathione peroxidase enzymes (nsGPX-As). Glutathione peroxidases (GPXs) comprise a group of enzymes that act as pivotal antioxidants by reducing H_2O_2 or organic hydroperoxides with high catalytic efficiency ($\sim 10^8$ M^{-1} s^{-1}) and in different cellular locations (reviewed in ref. 13). GPXs share a common catalytic core formed by selenocysteine (SeCys), tryptophan, and a glutamine residue. However, in some GPXs, the SeCys residue is replaced by a Cys. Such substitution, characteristic of the so-called "nonselenium GPX-like enzymes" (nsGPXs), confers to these proteins decreased peroxidase activity (reviewed in ref. 48).

Elucidation of the genomes of trypanosomatids revealed that these organisms possess a genomic *locus* comprising a cluster of two to three genes coding for closely related nsGPXs, that we classify here as nsGPX-A (Table 1). In *T. cruzi*, one of these proteins, nsGPX-A1, is glycosomal, a small fraction possibly localizing also to the cytosol (109). Regarding the other two, the absence of any obvious organelle endorsement signal and the detection of a substantial level of glutathione peroxidase activity in the parasite cytosol, suggests that they are cytosolic (109). In both *T. brucei* and *Leishmania* spp., the nsGPX-A molecules are almost identical except in the very N- and C-terminal regions, which may possess signal peptides for glycosomes and for the mitochondrion. The *T. brucei* nsGPX-A3 and the *Leishmania* spp. nsGPX-A1 molecules carry both peptide endorsement sequences. In the case of the *T. brucei* enzyme, experimental evidence points towards its dual localization in both organelles in the insect stage of the parasite (24, 93). As for the other two *T. brucei* isoforms (nsGPX-A1 and 2), they are cytosolic (93).

The highest degree of similarity of Kinetoplastida nsGPXs is found with GPXs proteins from plants, which belong to the phospholipid hydroperoxide GPX (PHGPX) clade. Trypanosomatidal nsGPXs, like PHGPXs, contain deletions in regions thought to mediate protein dimerization/tetramerization (12) (Fig. 4). Absence of such regions accounts for the monomeric nature of these enzymes and, as suggested for PHGPXs, may facilitate the access of complex hydroperoxides to the catalytic core. Accordingly, the *T. cruzi* nsGPX-A1 metabolizes fatty acid and phospholipids hydroperoxides (107). That is not the case, however, of the *T. brucei* nsGPX-A3, for which no activity towards lipid hydroperoxides was detected (93). Instead, this enzyme preferentially reacts with H_2O_2, additionally accepting linoleic acid, *t*-butyl, and thymine hydroperoxides as substrates. More recently, the monomeric nature of nsGPXs was postulated to be also a prerequisite for interaction with thioredoxin and related proteins containing a Cys–Xaa–Xaa–Cys motif (66).

One particularity of nsGPXs concerns their affinity for glutathione. In nsGPXs, similarly to that observed in PHGPXs, the residues involved in glutathione binding (33, Fig. 4) are

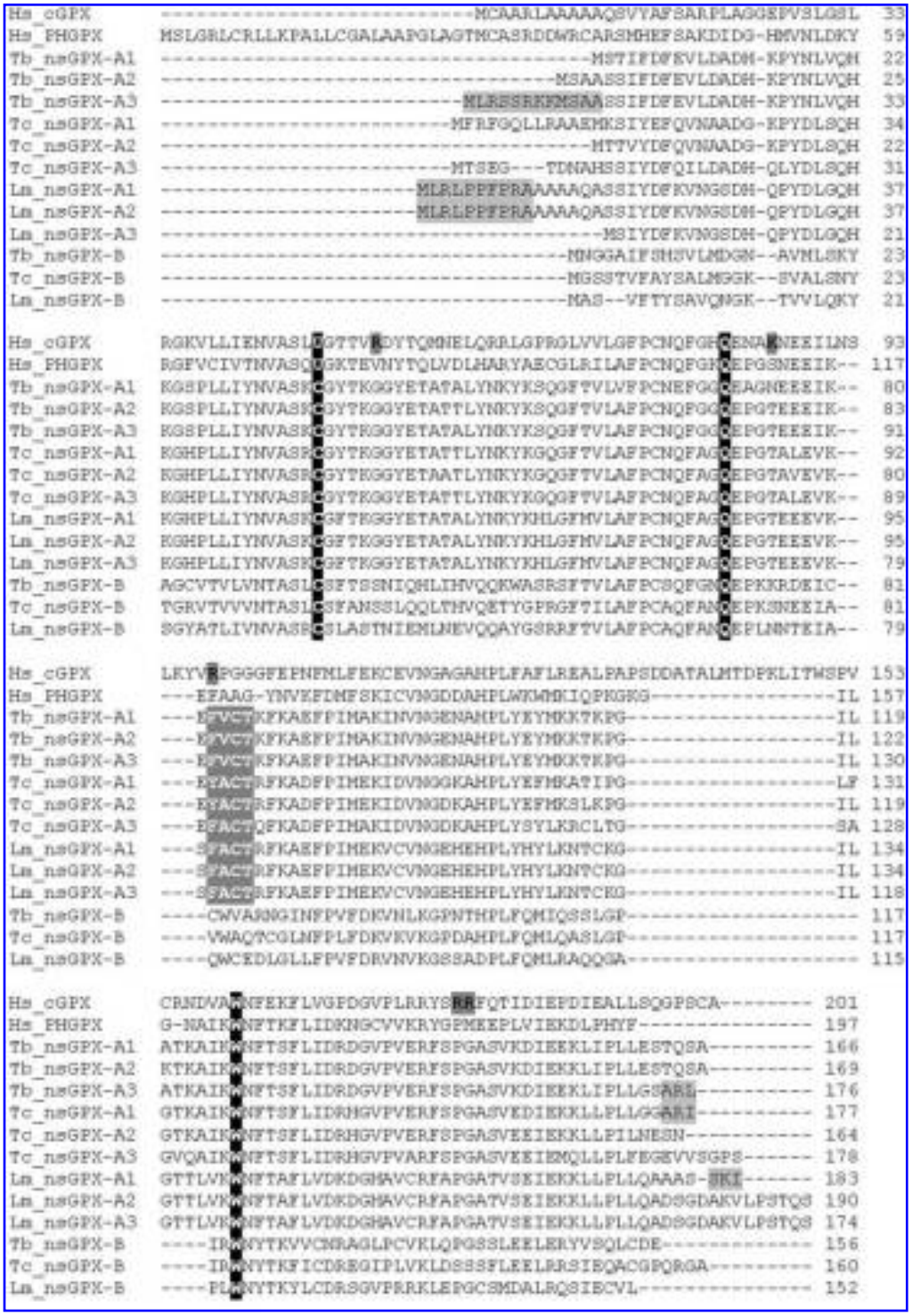

FIG. 4. Alignment of trypanosomatidal nonselenium glutathione peroxidases. The trypanosomatidal nonselenium GPXs are aligned with the human cytosolic GPX (Hs_cGPX, P07203) and the human phospholipid hydroperoxide GPX (Hs_PHGPX, NP_002076). Sequence accession numbers are the following: Tb_nsGPX-A1, Q869A7; Tb_nsGPX-A2, Q869A6; Tb_nsGPX-A3, Q869A5; Tc_nsGPX-A1, CAC85914; Lm_nsGPX-A1, Q4Q9B4; Lm_nsGPX-A2, Q4Q9B3; Lm_nsGPX-A3, Q4Q9B2; Tc_nsGPX-B, CAC85915; Lm_nsGPX-B, Q4Q1B8; Tc_nsGPX-A2, Tc_nsGPX-A3 and Tb_nsGPX-B sequences were derived from the genome projects. The conserved SeCys (represented by U), Gln and Trp residues characteristic of GPX are highlighted in *white letters on black*; in nsGPXs the SeCys is replaced by Cys. The consensus motif containing the distal redox active Cys of trypanosomatidal nsGPXs is shown in *white letters on gray*. Amino-terminus mitochondrial and C-terminus glycosomal targeting signals are marked on *light gray background*. Residues involved in glutathione binding are annotated in **bold** on *dark gray*. Tb, *T. brucei*; Tc, *T. cruzi*; Lm, *L. major*.

either mutated or deleted, rendering the affinity for this thiol weak (reviewed in ref. 48). This has led to the suggestion that glutathione is not their main reductant in the cell. In fact, some nsGPXs were shown to use thioredoxin as reducing substrate (reviewed in ref. 48). Trypanosomatidal nsGPXs also lack the glutathione-binding residues and, consequently, display extremely low activities with this thiol, which translate into high K_M values, within the milimolar range (49, 107, 111). In the case of nsGPX-As, they clearly prefer tryparedoxin as source of reducing equivalents (49, 109) and at least the *T. brucei* nsGPX-A3 can also use thioredoxin as reductant, although with less efficiency (49). The catalytic mechanism for the reaction of the *T. brucei* nsGPX-A3 with the Cys–Xaa–Xaa–Cys motif of tryparedoxin was recently disclosed (94) and shown to comply with that of other nsGPXs with thioredoxin (48, 66). Two cysteine residues are directly involved in catalysis: (a) Cys47 (*T. brucei* nsGPX-A3 numbering), which replaces the SeCys of classical GPXs, and (b) Cys95, which is part of the (Phe/Tyr)–(Ala/Val)–Cys–Thr consensus motif present in all trypanosomatidal nsGPX-As (Fig. 4). Cysteine47 is the residue that directly reacts with the hydroperoxide substrate, being oxidized to a sulfenic acid. This is then attacked by Cys95, the resulting intramolecular disulfide bridge being reduced by the Cys–Xaa–Xaa–Cys motif of the oxidoreductase. This mechanism differs from that of selenium-dependent GPXs and rather resembles that of atypical 2-Cys peroxiredoxins.

Nonselenium glutathione peroxidases not dependent on tryparedoxin (nsGPX-B)

Apart from the three identical clustered nsGPX-A genes, at least one additionally open reading frame, encoding a different nsGPX enzyme, can be found in a separate *locus* in the genomes of trypanosomatids (Fig. 4). These nsGPXs, which are classified here as nsGPX-B, share only 30% identity with nsGPX-As. One of these gene products was characterized in *T. cruzi* and assigned to the ER (111). As expected for a nsGPX, the *T. cruzi* enzyme metabolizes fatty acid and phospholipid hydroperoxides and reacts poorly with GSH. However, unlike trypanosomatidal nsGPX-As, it does not accept TXN as reductant. This may, at least in part, be explained by the fact that the consensus motif containing the C-terminal Cys residue implicated in nsGPX-A catalysis with TXN (Cys95 of *T. brucei* nsGPX-A3, 94) is missing in trypanosomatidal nsGPX-B (Fig. 4). Indeed, replacement of this Cys by a serine residue was shown to abolish the peroxidase activity of the *T. brucei* nsGPX-A3 in the presence of TXN (94). The possibility exists that other molecules, more efficient than GSH, react physiologically with nsGPX-Bs.

The activity of all trypanosomatidal nsGPXs is likely dependent on trypanothione, even in the case of enzymes for which thioredoxin (49) or glutathione (111) might be the source of reducing electrons. In fact, in trypanosomatids, lacking thioredoxin reductase and glutathione reductase, the pool of reduced thioredoxin and glutathione is maintained by the TR/trypanothione system (95). In the case of glutathione, reduction may occur either via the direct interaction with the dithiol (56) or via a reaction catalyzed by a trypanothione–glutathione thioltransferase, such as Tc52 in *T. cruzi* (73, 74).

Ascorbate peroxidase

Ascorbic acid (or vitamin C) is a cofactor for a range of enzymes involved in diverse metabolic pathways, such as protein folding, iron absorption, and the synthesis of collagen, neurotransmitters, peptide hormones, and carnitine (reviewed in refs. 7 and 44). However, its major function is as a powerful antioxidant, either by reducing free radicals (*e.g.*, RO·, RO_2·, HO·, NO_2·), scavenging several nonradical reactive species (*e.g.*, hypochlorous acid, ozone, and nitrating agents derived from peroxynitrite), maintaining α-tocopherol (vitamin E) in its reduced form, or by acting as electron donor of the ascorbate peroxidase (APX) family of enzymes (44). Detection of ascorbate in trypanosomatids dates back to 1994 (23), but only recently has the capacity of synthesizing vitamin C been demonstrated in *T. brucei* and *T. cruzi*, the enzyme responsible for the terminal step of ascorbate biosynthesis having been characterized in both organisms (64, 113). Contrary to that observed for *T. cruzi*, which acquires ascorbate solely by *de novo* synthesis (64), the *T. brucei* mammalian stage can additionally take up this vitamin from the surrounding environment (113). Ascorbate biosynthesis is distinct in mammals, plants, and yeast. In *T. cruzi* and *T. brucei*, the synthesis of this vitamin likely resembles that of plants, as deduced from genome analysis (64, 113). In the case of *Leishmania* spp., the enzyme responsible for the last catalytic step in the biosynthetic pathway is also present, thus ascorbate metabolism should not significantly differ from that of the other trypanosomatids.

Ascorbate peroxidases (APXs) are class I heme-containing peroxidase enzymes, which catalyze the H_2O_2-dependent oxidation of ascorbate in photosynthetic organisms (reviewed in ref. 89). It was known since 1980 that *T. cruzi* epimastigote extracts exhibit ascorbate-dependent peroxidase activity (11, 30). Recently, this activity was assigned to the unusual plant-like APX enzyme (110). This protein has a counterpart in *Leishmania* spp. (2), but interestingly, not in *T. brucei*. As inferred from their amino acid sequences (2, 110) and/or gel filtration analysis (2), APXs from *T. cruzi* and *L. major* are monomers within the 30 kDa range. In addition, the *T. cruzi* APX kinetic behavior complies with the monomeric nature of the enzyme (80, 110). Trypanosomatidal APXs differ from those of plants in possessing, near the C-terminus, a positively charged insert of unknown function. Also, they carry an N-terminal amino acid extension which is unique in coding for an amino acid topology that is reminiscent of signal sequences targeting proteins to the ER or plasma membrane. In the case of *T. cruzi*, the enzyme locates to the ER (110). The most significant difference to plant APXs, however, is that a residue crucial for ascorbate binding and oxidation, Arg172 (soya-bean cytosolic APX numbering; accession number T07056), is missing in the trypanosomatidal enzymes. This may explain the fact that trypanosomatidal APXs exhibit relatively low ascorbate utilization and also the nonsaturation kinetics observed for the *L. major* enzyme (2). Still, the fact that the affinity of both enzymes for ascorbate is within the micromolar range has led to the suggestion that an alternative ascorbate-binding mechanism could be operating in these molecules (2).

The catalytic mechanism of trypanosomatidal APXs likely conforms to that of plant APXs (2, 96). This involves the very fast second-order reaction of ferric heme with H_2O_2, con-

sisting in the heterolytic cleavage of the O–O peroxide bond and release of a water molecule. The catalytic intermediate resulting from this step, known as Compound I, is subsequently reduced by ascorbate in two sequential single electron transfer reactions, initially to a second intermediate, Compound II, and then back to ferric heme, regenerating the ground state enzyme. Two monodehydroascorbate radicals result from these reactions, which under physiological conditions, are reduced back to ascorbate either (a) by the activity of a NADH-dependent reductase, or (b) by the rapid disproportionation to dehydroascorbic acid and subsequent recycling by glutathione or by glutathione-dependent enzymes (reviewed in ref. 43). Dehydroascorbate can also decompose irreversibly, yielding a range of products, such as oxalate and threonate (43). In Kinetoplastida, regeneration of ascorbate seems to be achieved nonenzymatically via the spontaneous reduction of dehydroascorbate by trypanothione (58, 110). Even though TXN can also reduce dehydroascorbate, there is no obvious advantage in this reaction (90).

With respect to the hydroperoxide substrates, the *T. cruzi* enzyme specifically reduces H_2O_2 but not the organic hydroperoxides *t*-butyl and cumene hydroperoxides. Studies with mutant parasites overexpressing this molecule demonstrated that it can shield cells from exogenously added H_2O_2 (110). In the case of the *L. major* APX, it was demonstrated that, besides serving as substrate, H_2O_2 is also capable of acting as an inhibitor of this enzyme when the concentration of ascorbate is low (2). H_2O_2-mediated inactivation has also been described in another class I heme-peroxidases (50).

Other trypanothione-dependent pathways implicated in antioxidant function

Enzymes of the glutathione S-transferase class are important for the metabolism of phospholipid hydroperoxides in higher eukaryotes. They act by catalyzing the nucleophilic attack of GSH on a wide variety of hydrophobic substrates. In trypanosomatids, no such activity is present and, instead, these organisms possess trypanothione S-transferase activity associated to the eukaryotic translation elongation factor 1B (eEF1B) (102). The *L. major* eEF1B displays trypanothione-dependent peroxidase activity, reacting preferentially with linoleic acid hydroperoxide, but not with H_2O_2 (103). This, added to the fact that eEF1B localizes to the ER, has led to the suggestion that this molecule may be involved in the elimination of oxidized lipids within this organelle.

Trypanothione is also an efficient reductant of ovothiol A (4). This mercaptohistidine, present in several trypanosomatids, can act as a nonenzymatic scavenger of free-radicals (reviewed in ref. 97) and can catalyze the decomposition of nitrosothiols (105).

Finally, apart from supplying reducing equivalents to enzymatic cascades that culminate in peroxide elimination, trypanothione can spontaneously reduce H_2O_2 (4) and $ONOO^-$ (100). However, the second order rate constants estimated for these nonenzymatic reactions are low (5.37 and 7200 M^{-1} s^{-1} for H_2O_2 and $ONOO^-$, respectively), when compared to that of the peroxidases (Table 1), and are thus negligible under physiological conditions.

Complementary and Redundant Roles of Trypanosomatidal Peroxidases

In cells, peroxide metabolism is largely ensured by peroxidases, and trypanosomatids are no exception to this. Peroxides are generated internally or derived from exogenous sources. The effect they produce in cells greatly depends on their nature and concentration. They can either cause oxidative damage on a range of biomolecules or act as important effectors in cell signaling pathways.

Within the cytosol of all three clinically relevant trypanosomatids, 2-Cys peroxiredoxins, and another less abundant class of tryparedoxin-dependent peroxidases, the nsGPX-A, are likely in charge of peroxide elimination. There are little doubts about the essentiality of these two sets of enzymes for trypanosomatids' survival, as directly demonstrated for *T. brucei* by RNA interference (93, 112). Still, in the case of nsGPX-As, the possibility that the observed defects are due to loss of functionality of the noncytosolic isoform cannot be discarded, due to similarity between all three sequences. On the whole, the emerging picture points to the existence of specific, nonredundant functions for each class of cytosolic peroxidases in trypanosomatids. This may be explained by the peculiarity of peroxiredoxins to metabolize $ONOO^-$ (Table 1).

The cytosolic location of an effective antioxidant system is most favorable to confer protection against exogenous sources of oxidants. Upon invasion of the mammalian host, the intramacrophagic pathogens *Leishmania* spp. and *T. cruzi* are challenged with reactive oxygen and nitrogen species derived from the immune response (10, 15, 63). The capacity of 2-Cys peroxiredoxins to protect trypanosomatids from externally derived oxidants does not seem to be compensated by any other hydroperoxide detoxifying pathway. Accordingly, the *T. brucei* ability to cope with H_2O_2 added exogenously was impaired upon downregulation of the cytosolic peroxiredoxin, but not of nsGPX-A (112). The combined hydroperoxide and $ONOO^-$ metabolizing activities of 2-Cys peroxiredoxins are also likely to account for the increased infectivity of *Leishmania* mutants overexpressing a cytosolic peroxiredoxin, in an *ex vivo* model of infection (9). The significance of cytosolic peroxiredoxins for parasite pathogenecity was further proposed for *L. (Viannia) guyanensis*, wherein the parasite ability to disseminate from cutaneous lesions to the nasopharyngeal mucosa was related to upregulation of these enzymes (1, 106).

Additional sources of exogenous oxidative species may be provided by some of the drugs used clinically to treat trypanosomiasis and leishmaniasis (67, 88, 104, 117). Peroxiredoxin association with drug resistance was described for *L. amazonensis*, wherein a strain selected for arsenite resistance displayed upregulation of either the cytosolic or the mitochondrial enzyme (62). This likely happens to counteract an oxidative stress that occurs as a response to this metalloid drug (68).

Apart from functioning as antioxidant devices, 2-Cys peroxiredoxins and nsGPXs may also be implicated in cell signaling pathways, as described for other organisms (reviewed in refs. 48, 91, 92). In 2-Cys peroxiredoxins, this function has been attributed to their propensity to undergo overoxidation by H_2O_2. This results in inactivation of the peroxidase and subsequent accumulation of the hydroperoxide, a require-

ment for signal propagation. As discussed previously, a number of features in trypanosomatidal peroxiredoxins suggests that they might also be sensitive to hyperoxidation. Another peroxide that is being increasingly accepted as a redox-mediated intracellular messenger is $ONOO^-$ (reviewed in refs. 57, 101). At present, there is no direct evidence for $ONOO^-$ formation in trypanosomatids. However, its two precursors, superoxide anion and nitric oxide, may be generated in these cells (38, 39, 81, 98, 114). Thus, although rather speculative, the possibility remains that trypanosomatidal peroxiredoxins may also participate in $ONOO^-$-mediated regulation. Whether in Kinetoplastida 2-Cys peroxiredoxins act as regulators of signaling pathways is to be elucidated. The current belief is that they constitute the parasites' major antioxidant apparatus.

In eukaryotes, the ER is the main site for synthesis and post-translational modification of secretory and membrane proteins, a process that requires an oxidizing millieu. It is also in this organelle where fatty acid and phospholipids are synthesized and metabolized. Within the ER of *T. cruzi* and *Leishmania* spp., at least two enzymes are likely in charge of dealing with the hydroperoxide challenge that may arise therein, namely nsGPX-B and APX (110, 111). These peroxidases differ in terms of electron donor and substrate specificity (Table 1) and their combined activities may account for the parasite ability to metabolize a wide range of hydroperoxides. In this regard, APX may act by eliminating H_2O_2, while nsGPX-B may be crucial at repairing the oxidative damage of newly synthesized lipids. Interestingly, no APX coding sequence is annotated in the *T. brucei* genome. Wilkinson *et al*. (113) suggested that *T. brucei* may not require ascorbate-based antioxidant protection because, being an extracellular parasite, it is not exposed to the oxidative challenge produced by host immune cells in response to infection as do *T. cruzi* and *Leishmania* spp. Another molecule possibly in charge of eliminating oxidized lipid hydroperoxides in the endoplasmic reticulum is eEFB1 (102, 103).

Peroxisomes are major sources of reactive oxygen species (reviewed in ref. 29). In trypanosomatids these cell compartments are replaced by related organelles, the glycosomes, in which a number of biochemical processes take place (reviewed in ref. 69). In *T. cruzi* and *T. brucei* glycosomes, the presence of nsGPX-A enzymes was reported (24, 109), which likely constitute an important line of defense against oxidative damage. As for *Leishmania* spp., the presence of nsGPX-As in glycosomes has never been addressed. Although not yet experimentally demonstrated, *Leishmania* spp. possibly possesses a 2-Cys peroxiredoxin in glycosomes (PRX3 in Table 1).

Kinetoplastida organisms possess a single mitochondrion, wherein the electron transport chain leading to molecular oxygen reduction, constitutes one of the most relevant sources of oxidants. In homology to that described for the cytosol, two classes of TXN-dependent peroxidases (the 2-Cys PRXs and the nsGPX-As) are probably in charge of peroxide metabolism in the mitochondrion (Table 1). This situation may, nevertheless, differ with respect to the parasite species, because no nsGPX-A was found in the mitochondrial fractions of *T. cruzi* (109). Apart from general antioxidant defense, other functions have been postulated for 2-Cys peroxiredoxins operating in the mitochondrion of trypanosomatids. First, the enzyme may be indirectly implicated in regulation of mitochondrial DNA replication by competing with UMSBP for reducing equivalents derived from TXN (72). Second, in analogy to mitochondrial peroxiredoxins of higher eukaryotes (21), it could be involved in parasite protection from H_2O_2-induced programmed cell death, as reported for *L. donovani* (45). Another function proposed for the mitochondrial peroxiredoxin is to protect the mitochondrial genome from direct or indirect peroxide-mediated damage (108). The same role has been suggested for the mitochondrial nsGPX-A3 of *T. brucei*, for which thymine hydroperoxide was found to be a good substrate (93).

The importance of the mitochondrial 2-Cys peroxiredoxin for trypanosomatids has been investigated in two organisms. In the case of *L. infantum*, enzyme depletion was shown to significantly impair parasite infectivity in an animal model (H. Castro and Ana M. Tomás, unpublished). The situation may be somewhat different in *T. brucei*, wherein downregulation of the homologous enzyme does not seem to affect growth of the mammalian stage of the parasite (112). This could partially be explained by the fact that, unlike *Leishmania* spp. and *T. cruzi*, the mammalian stage of *T. brucei* lacks oxidative phosphorylation (87). Still, low levels of H_2O_2 are constantly produced (35) and depletion of the mitochondrial peroxiredoxin is possibly complemented by other mechanisms, such as the mitochondrial nsGPX-A redox pathway. For *T. brucei*, the mitochondrial nsGPX-A was alleged to play an important role within the mitochondrion (93), although no definitive evidence for this exists.

Concluding Remarks

In recent years, the combined effort of worldwide researchers has led to important findings concerning the pathways for peroxide elimination in trypanosomatids. Contradicting early convictions, it is presently recognized that these organisms possess an elaborate peroxide metabolism. This consists of a series of redox cascades, wherein reducing equivalents derived from trypanothione are transferred to enzymes belonging to three major families, (a) 2-cysteine peroxiredoxins, (b) nonselenium glutathione peroxidases, and (c) ascorbate peroxidases. These peroxidases possess different physiological reductants and substrate specificities (Fig. 2). In addition, they are strategically localized in specific parasite compartments, where they play precise and complementary roles. Despite all the advances in this area of research, the peroxidase repertoire of trypanosomatids may not yet be complete. Conclusion of the trypanosomatids genome projects will certainly add important findings to this, in the near future. Also, noteworthy is the possibility of exploring some of the unique characteristics of the peroxide metabolism of trypanosomatids to develop new chemotherapeutic agents for the treatment of these medically important parasites.

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Abbreviations

APX, ascorbate peroxidase; ER, endoplasmic reticulum; GPX, glutathione peroxidase; GSH, glutathione; GSPS, glutathionylspermidine synthetase; K_M, Michaelis–Menten constant; nsGPX, nonselenium glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase; PRX, peroxiredoxin; SeCys, selenocysteine; TR, trypanothione reductase; TRYS, trypanothione synthetase; TS_2, trypanothione disulfide; $T(SH)_2$, dihydrotrypanothione; TXN, tryparedoxin; UMSBP, Universal Minicircle Sequence Binding Protein

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