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Review

Redox control in trypanosomatids, parasitic protozoa with trypanothione-based thiol metabolism

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ABSTRACT

Trypanosomes and leishmania, the causative agents of several tropical diseases, possess a unique redox metabolism which is based on trypanothione. The bis(glutathionyl)spermidine is the central thiol that delivers electrons for the synthesis of DNA precursors, the detoxification of hydroperoxides and other trypanothione-dependent pathways. Many of the reactions are mediated by tryparedoxin, a distant member of the thioredoxin protein family. Trypanothione is kept reduced by the parasite-specific flavoenzyme trypanothione reductase. Since glutathione reductases and thioredoxin reductases are missing, the reaction catalyzed by trypanothione reductase represents the only connection between the NADPH- and the thiol-based redox metabolisms. Thus, cellular thiol redox homeostasis is maintained by the biosynthesis and reduction of trypanothione. Nearly all proteins of the parasite-specific trypanothione metabolism have proved to be essential.

1. Introduction

Trypanosomatids are protozoan organisms of the order *Kinetoplastida* that parasitize a wide variety of invertebrate and vertebrate hosts. The most relevant specimens for human and animal health belong to two genera: *Trypanosoma* and *Leishmania*, which account for over half a million annual human deaths in (sub)tropical regions around the world. In sub-Saharan countries *T. brucei rhodesiense* and *T. b. gambiense* are the causative agents of African sleeping sickness and Nagana cattle disease is caused by *T. b. brucei*, *T. vivax and T. congolense*. In the New World, *T. cruzi* is responsible of Chagas' disease. Different *Leishmania* species occur world-wide and cause *inter alia* black fever, espundia, oriental sore and Kala-Azar. *Crithidia fasciculata* is an apathogenic trypanosomatid that serves as a useful model organism. Trypanosomatids represent one of the earliest branches of eukaryotic evolution with mitochondria and microbodies [1]. The parasites show a large number of biochemical, morphological and genetic peculiarities with the thiol redox metabolism being one of the unique pathways. The genome sequencing projects of *T. brucei* [2], *T. cruzi* [3] and *L. major* [4] have revealed that trypanosomatids lack genes for glutathione reductase (GR) and thioredoxin reductase (TrxR) as well as catalase and selenocysteine-containing glutathione peroxidases. While in most eukaryotic organisms the glutathione (GSH)/GR and thioredoxin (Trx)/TrxR systems maintain the intracellular thiol redox homeostasis, trypanosomatids possess a redox metabolism that is based on the low molecular mass dithiol trypanothione [bis(glutathionyl) spermidine; $T(SH)_2$] [5] and trypanothione reductase (TR) — which keeps it in the reduced form (Table 1) [for reviews see [23,24]]. $T(SH)_2$ and/or TR have also been described in the flagellated green algae *Euglena gracilis* [19] and the amitochondriate pathogenic amoebae *Entamoeba histolytica* [20] and *Naegleria fowleri* [22]. Interestingly, *E. gracilis* and *N. fowleri* were reported to contain both GR as well as TR [19,22]. The absence of the trypanothione system in mammals, the lack of a functional redundancy within the parasite thiol system together with the sensitivity of trypanosomes against oxidative stress render the components of this metabolism attractive drug target molecules [for recent reviews see 24 and 25]. The thorough analysis of the trypanothione metabolism and its control mechanisms will certainly reveal additional unprecedented features and putative new targets for a selective antiparasitic drug development.

2. Thiol redox homeostasis in trypanosomes

In any living organism, the cellular redox homeostasis is affected by an excess of reactive oxygen (ROS) and nitrogen species originating as by-product of aerobic growth or from the environment. Redox-active thiol groups in proteins and low molecular mass compounds play key roles as redox buffers that balance any disturbance of the intracellular redox state [26,27]. Depending on the level and extension of the oxidative stress, the cells may establish short- or long-term

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Table 1
Organisms with documented (trypanosomatids) and putative (non-trypanosomatids) trypanothione metabolism

Organism	Protein[a]			Evidence	Pathology	Reference
	GspS[b]	TryS[c]	TR[d]			
Trypanosoma brucei brucei	Absent	AY155570	X63188	Characterization, RNA-interference and conditional knock-out	Nagana cattle disease	[2, 6–8]
T. b. gambiense	Absent	Tbgamb.1821	Tbgamb.31271	Genome	African sleeping sickness	[9]
T. vivax	Absent	tviv499f03.q1k_27	tviv1287e08.q1k_3	Genome	Nagana cattle disease	[9]
T. congolense	Absent	congo999f08.p1k_9	M21122	Genome and characterization of TR	Nagana cattle disease	[9,10]
T. cruzi	EAN98995	AF311782	M38051	Genome and characterization	Chagas' disease	[3,11,12]
					Leishmaniasis forms:	
Leishmania major	LmjF25.2380 (pseudogene)	AJ311570	CT005244	Genome and characterization	Cutaneous and diffuse	[4,13]
L. donovani	n.r.	AJ430863	Z23135	Genome and characterization of TR	Visceral	[13,14]
L. infantum	AM502243	AM502245	AM502223	Genome	Visceral	[15]
L. amazonensis	n.r.	EF583872	EF583873	cDNA sequence	Diffuse cutaneous	Lin et al. (unpublished)
L. brasiliensis	n.r.	AM494964	AM494942	Genome	Mucocutaneous	[15]
Crithidia fasciculata	U66520	AY603101	CAA78264	Characterization	Insect pathogen	[6,16–18]
Euglena gracilis	n.r.	n.r.	Peptide sequences	Characterization	None	[19]
Entamoeba histolytica	n.r.	n.r.	AF503571	TR activity, $T(SH)_2$ detection and DNA sequence	Dysentery	[20,21]
Naegleria fowleri	n.r.	n.r.	n.r.	TR activity and detection of $T(SH)_2$	Meningoencephalitis	[22]

n. r., not reported;
[a] Accession numbers; [b]Glutathionylspermidine synthetase; [c]Trypanothione synthetase; [d]Trypanothione reductase.

adaptive responses. Kinetoplastids are equipped with a number of unique low molecular mass thiols and redox proteins. The redox homeostasis in these parasites appears to be efficiently regulated since they can successfully withstand the oxidative burst during host infection and perfectly adapt to the different metabolic and environmental conditions imposed by their digenetic life-cycle.

2.1. Low molecular mass thiols

Depending on the species, life stage and growth phase, trypanosomatids contain varying levels of four major low molecular mass thiols: GSH, mono-glutathionylspermidine (Gsp), $T(SH)_2$ and, in some species, ovothiol A (N^1-methyl-4-mercaptohistidine; OvSH; Fig. 1). As in other organisms, GSH is synthesized via two ATP-dependent steps. The first one is catalyzed by γ-glutamylcysteine synthetase (GSH1) [28], an enzyme shown to be essential for *T. brucei* [29], and the second reaction is catalyzed by glutathione synthetase (Fig. 2). Significant amounts of the tripeptide are present as $T(SH)_2$ and, to a lesser extend, as Gsp (Table 2, Section 2.2).

When exponentially growing *C. fasciculata* enter the stationary phase, the $T(SH)_2$ concentration decreases from 1.51 to 0.37 mM and Gsp increases from 0.9 to 2.28 mM [33]. Inoculation of the stationary phase cells into fresh medium is followed by a rapid recovery of $T(SH)_2$ with a concomitant increase in the level of free spermidine [33]. The

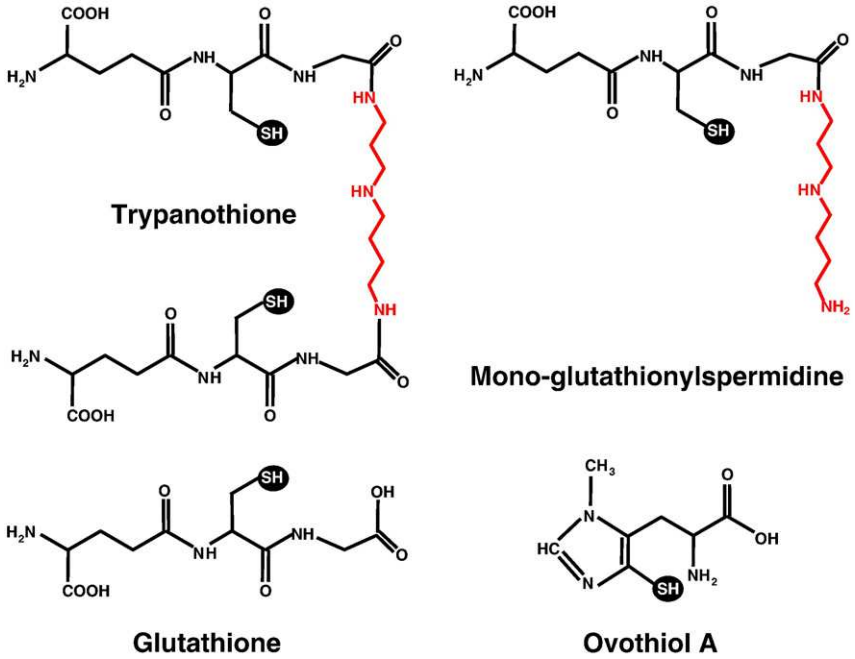

Fig. 1. Low molecular mass thiols occurring in trypanosomatids. The polyamine moiety in the trypanothione [bis(glutathionyl)spermidine] and mono-glutathionylspermidine molecules are depicted in red. Sulfhydryl groups are highlighted by a black background.

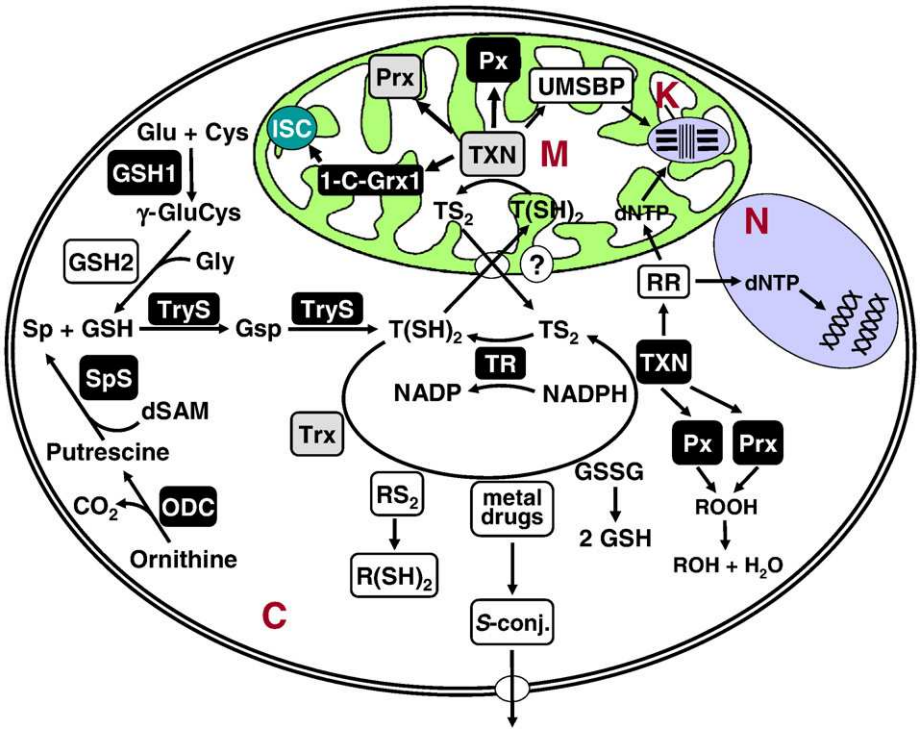

Fig. 2. The trypanothione-based thiol redox metabolism of African trypanosomes. C, cytosol; K, kinetoplast (mitochondrial DNA); M, mitochondrion; N, nucleus. Trypanothione [$T(SH)_2$] is synthesized from glutathione (GSH) and spermidine. GSH is formed from glutamate, cysteine and glycine in two steps catalyzed by γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2). Spermidine (Sp) is obtained from ornithine. In the first step, ornithine decarboxylase (ODC) generates putrescine which is then linked with an aminopropylic group derived from decarboxylated *S*-adenosyl-L-methionine (dSAM) in a reaction catalyzed by spermidine synthase (SpS). The step-wise conjugation of two molecules of glutathione and spermidine is catalyzed by trypanothione synthetase (TryS). In the cytosol, $T(SH)_2$ reduces disulfides in proteins (RS_2) such as thioredoxin (Trx), tryparedoxin (TXN) and glutathione disulfide (GSSG) as well as dehydroascorbate (not shown). Trypanothione disulfide (TS_2) generated in these reactions is reduced back to the active dithiol by the NADPH-dependent trypanothione reductase (TR). $T(SH)_2$ can be conjugated to metal-containing drugs (S-conj) that are sequestered inside the cell or extruded, probably by specific transporter. 2-Cys-peroxiredoxins (Prx) and glutathione-peroxidase-type enzymes (Px) act as tryparedoxin peroxidases which detoxify hydroperoxides (ROOH) by converting them into the respective alcohol (ROH). TXN delivers also electrons to ribonucleotide reductase (RR) which synthesizes the deoxyribonucleotides (dNTP) required for DNA synthesis. In the mitochondrion (depicted with a green background), an isoform of TXN occurs that can transfer reducing equivalents to peroxidases, the universal minicircle sequence binding protein (UMSBP) and a monothiol glutaredoxin (1-C-Grx1). UMSBP participates in the initiation of the replication of kinetoplast (K)-DNA. 1-C-Grx1 is probably involved in iron metabolism and/or in the biogenesis of iron sulfur clusters (ISC). Since TR and TryS are not detectable in this organelle, it remains to be elucidated if the parasite possesses a redox shuttle mechanism [$T(SH)_2/TS_2$ transporter] between cytosol and mitochondria. Proteins that were experimentally shown to be essential and dispensable for *T. brucei* are highlighted with black and light grey background, respectively.

authors suggested that this redistribution of conjugated and free spermidine represents an efficient and rapid mechanism to make available high levels of polyamine, required for cell growth.

The insect forms of *L. major*, *L. donovani* and *T. cruzi* contain 1 to 2 mM $T(SH)_2$ (Table 2). The concentration does not change significantly during the different logarithmic growth phases but decreases when the cells enter the stationary phase. After re-dilution of the non-dividing cells in fresh medium the $T(SH)_2$ levels recover within 24 h. In *L. donovani*, the decrease of $T(SH)_2$ in the stationary phase is not accompanied by a conversion into Gsp but reflects a decrease of the total thiols [44]. Taken together, the data show that in growing cells the $T(SH)_2$ concentration is significantly higher than in growth-arrested cells.

The cellular $T(SH)_2$ content depends on the availability of spermidine. Treatment of bloodstream *T. brucei* with a competitive inhibitor of ornithine decarboxylase (Fig. 2), namely difluoromethylornithine, depletes the cells of $T(SH)_2$ [36]. *T. cruzi* epimastigotes grown in polyamine-deficient medium contain 2 mM GSH but only marginal concentrations of Gsp and $T(SH)_2$ (Table 2) [37,45]. The OvSH concentration is even lower than that in cells grown in the presence of putrescine which rules out a compensatory up-regulation of the mercaptohistidine. Although a detailed analysis of the thiol content in different growth stages is not yet available for the mammalian stages of *T. cruzi*, it is obvious that the thiol concentration in the infective stages is generally lower than that in the corresponding insect stage (Table 2) [38].

Several physiological stimuli trigger an increase/decrease in the cellular concentration of thiols. i) Depletion of the mRNA of trypanothione synthetase (TryS) in African trypanosomes causes a decrease of the glutathionylspermidine conjugates and an increase in the GSH content (Table 3) [8,52]. The impaired synthesis of the conjugates probably entails the accumulation of substrate [8]. Prolonged suppression of TryS may also be associated with a slightly enhanced expression of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis [52]; ii) Down-regulation of tryparedoxin (TXN) by RNA interference in *T. brucei* leads to 2- to 4-fold elevated levels of the low molecular mass thiols [35]; iii) Resistance to melarsoprol and antimonials in *T. brucei* and *L. donovani*, respectively, is associated with higher intracellular levels of low molecular mass thiols [34,63,64]. Interestingly, the depletion of TR in *T. brucei* is not accompanied by an up-regulation of thiol biosynthesis [53] whereas down-regulation of $T(SH)_2$ synthesis leads to a 2-fold increase in TR activity [52]. Probably parasites with 10% of wild-type TR activity can still sustain the thiol redox balance in resting cells [53]. In contrast, interfering with the biosynthesis of $T(SH)_2$ has a more severe impact on the redox homeostasis that is partially compensated by an up-regulation of TR.

The first indication that interfering with the thiol metabolism is detrimental for the parasites was the observation that buthionine sulfoximine (BSO), an irreversible inhibitor of GSH1 (Table 3, Fig. 2), exerts trypanocidal activity [46]. As expected, BSO blocks

Table 2
Low molecular mass thiols in different life and growth stages of *Kinetoplastida*

Species	Life stage	Strain/growth phase	Cellular concentration (mM)[a]				
			GSH	Gsp	$T(SH)_2$ (Percentage of total thiol)	OvSH	Reference
T. brucei	Bloodstream	927/mid-log and stationary	1.2 and 0.17	n.d.	0.33 and 0.1. (35 and 27)	n.d	[34]
		427/early-log	0.3	0.01	0.15 (49)	n.d	[35]
		427, purified from rats	0.23–0.26	0.05–0.04	0.34–0.07 (71–32)	<0.005	[36,37]
	Procyclic	427/late-log	0.22	0.07	0.34 (67)	0.04	[37]
T. cruzi	Amastigote	Y_0/n.s.	0.43	0.07	0.12 (30)	0.05	[38]
		MF/n.s.	0.80	0.26	0.36 (40)	n.d.	[39]
	Epimastigote	Silvio/late-log[b]	0.70	0.27	2.13 (76)	0.43	[37]
		Silvio/late-log[c]	2.07	<0.02	0.04 (4)	0.08	[37]
		MF/log	1.26	0.47	1.36 (61)	n.d.	[39]
		Tulahuen/early stationary	0.23	0.07	0.82 (85)	n.d.	[40]
	Trypomastigote	Silvio/n.s.	0.34	0.14	0.79 (55)	0.82	[37]
		LQ, Dm28c, Brener and MF/n.s.	0.68–1.18	0.29–0.68	0.47–1.94 (46–68)	n.d.	[39]
L. aethiopica	Promastigote	L100/late-log	0.24	0.09	0.66 (40)	1.61	[37]
L. donovani	Amastigote	BOB/mid-log (axenic)	2.27	n.d.	1.75 (61)	n.d.	[32]
		LV9, obtained from hamsters	0.25	0.04	0.46 (76)	0.04	[37]
	Promastigote	LV9/late-log	1.68	0.56	2.20 (23)	3.50	[37]
L. infantum	Promastigote	Clone MAP263[d]/early stationary	0.17	n.d.	1.06 (93)	n.d.	[41]
L. major	Amastigote	JISH118, obtained from mice	0.39	0.09	0.36 (25)	1.65	[37]
	Promastigote	JISH118/late-log	0.21	1.22	1.01 (25)	4.79	
L. mexicana	Promastigote	M379/late-log	0.31	0.14	1.22 (43)	2.80	[37]
L. tarentolae	Promastigote	TarII WT/mid-log and late-log	0.03 and 0.05	n.d.	0.03 and 0.05 (67)	n.d.	[42,43]
L. tropica	Promastigote	K27/late-log	0.59	0.14	0.52 (41)	0.77	[37]
C. fasciculata		HS6/early-, late-log and stationary	0.51, 0.44 and 0.34	0.90, 0.96 and 2.28	1.51, 1.05 and 0.37 (68, 61 and 22)	0.2[e]	[33,37]

n.d., not determined. n.s., not specified.
[a] The thiol concentrations were calculated using the following cell volumes: 58 fl [30] and 96 fl (M. Engstler, personal communication) for bloodstream and procyclic *T. brucei*, respectively; 30 and 12 fl/cell [31] for epimastigote and trypomastigote *T. cruzi*, respectively; as for amastigote *T. cruzi* data are missing and the form is microscopically indistinguishable from *Leishmania* amastigotes, we assumed a cell volume of 9.7 fl [32]; for *Leishmania* spp. promastigote and amastigote, the cell volumes reported for *L. donovani* promastigote (28.6 fl) and amastigote (9.7 fl), respectively [32] were used; 115 fl for *C. fasciculata* [33].
[b] Parasites cultured in the presence of 100 μM putrescine.
[c] Parasites cultured in polyamine deficient medium.
[d] Clone MHOM/MA67ITMAP263 [41].
[e] Value reported for late-log cultures, where OvSH and $T(SH)_2$ represents 9 and 59 %, respectively, of the total thiol concentration [37].

the biosynthesis of GSH as well as of the glutathionylspermidine derivatives [29,44,48].

Treatment with BSO prolongs the survival of or even cures mice infected with African trypanosomes [46] and potentiates the toxicity of nifurtimox and benznidazole, drugs that alter the thiol-redox metabolism of American trypanosomes [47, 48]. BSO has been reported to have only a minute effect on the growth of axenic *L. donovani* amastigotes although GSH and $T(SH)_2$ were depleted by more than 95% [44]. This effect may be related to the culture conditions because BSO strongly impairs multiplication of the parasites in macrophages [49] (see Table 3). As shown more recently, depletion of GSH1 in procyclic *T. brucei* lowers the cellular GSH and $T(SH)_2$ pools and finally results in parasite death [29].

The heteroaromatic OvSH (Fig. 1) occurs in millimolar concentrations in different *Leishmania* spp. and is also found in *C. fasciculata* and in *T. cruzi* but is probably absent from the mammalian form of *T. brucei* [37,65,66] (Table 2). Although 4-mercaptohistidines are general powerful radical scavengers and metal chelators [67,68] the contribution of OvSH to the hydroperoxide metabolism of trypanosomes is not clear. The second order rate constant for the non-enzymatic reaction of H_2O_2 with OvSH is half of that with $T(SH)_2$. On the other hand, in parasites that possess OvSH the concentration of the heteroaromatic thiol can be significantly higher than that of $T(SH)_2$. An OvSH-dependent peroxidase activity was not detectable in *Leishmania* extracts [37]. OvSH has been shown to decompose the *S*-nitroso groups of nitrosoglutathione and dinitrosotrypanothione and thus may act in synergy with trypanothione in the degradation of these compounds [69]. Considering the differential occurrence and abundance of GSH, Gsp and OvSH in the different life stages of trypanosomatids one can envisage specific functions for each thiol in the redox metabolism of these protozoa. It remains to be investigated if OvSH is involved in cellular redox control as it has been reported for the green alga *Dunaliella salina* [70] and/or if it can form mixed disulfides with protein thiols as it is the case with glutathione and mono-glutathionylspermidine [71].

Another redox active low molecular mass thiol present in trypanosomes is lipoic acid [5-(1,2-dithiolan-3-yl)pentanoic acid]. Synthesis of lipoic acid takes place in the mitochondrion where a type II fatty acid synthase generates the precursor molecule octanoate [72]. Lipoic acid has a redox potential of −288 mV. The dithiol can participate in thiol/disulfide exchange reactions, efficiently scavenges ROS and heavy metals and reduces metabolites such as dehydroascorbate or glutathione disulfide (GSSG). *In vitro*, the complexes formed between melarsen oxide (arsenical drug) and lipoate are much more stable than those formed with $T(SH)_2$. Arsenical-resistant *T. brucei* contain significantly less lipoic acid than the respective sensitive cell line. This led to the suggestion that lipoic acid is either involved in the uptake or is the final target of these drugs [73]. The marginal cellular concentration of free lipoate however raises doubts about its physiological role as a general reductant.

$T(SH)_2$ is the most powerful reducing agent when compared with Gsp, GSH and OvSH. Although the redox potentials are nearly identical [23], the dithiol character of $T(SH)_2$ favors formation of an intramolecular disulfide in comparison to the intermolecular disulfide formed upon oxidation of GSH. In addition, the p*K*-value of $T(SH)_2$ is 7.4 [74] and thus similar to the physiological pH which renders $T(SH)_2$ highly reactive in thiol disulfide exchange reactions [24]. The redox potential of the imidazolium form of OvSH ($E^{\circ\prime}$=−92 mV) [75] is 150 mV more positive than that of $T(SH)_2$ ($E^{\circ\prime}$=−242 mV) at physiological pH, and thus the cellular OvSH should be maintained in the reduced form by $T(SH)_2$. Also, the free glutathione in the parasite is kept reduced by the spontaneous reaction between GSSG and $T(SH)_2$. In addition, *T. cruzi* contains a protein (P52) that displays trypanothione-glutathione disulfide thioltransferase as well as dehydroascorbate reductase activities [76,77]. $T(SH)_2$ is also an excellent reductant of dehydroascorbate, the

Table 3
Phenotypic changes associated with the manipulation of proteins of the thiol-redox metabolism in trypanosomatids

Protein[a]	Species	Method	Phenotype	Reference
GSH1	*T. brucei*	dsRNAi[b] and enzyme inhibition (BSO)[c]	Depletion of GSH and $T(SH)_2$, cell death and loss of virulence	[29]
		BSO treatment of infected mice		[46]
	T. cruzi	Enzyme inhibition (BSO)	Increased susceptibility against nifurtimox and benznidazole	[47,48]
	L. donovani		None in axenic cultures	[44]
			Impaired multiplication within macrophages	[49]
ODC	*T. brucei*	Enzyme inhibition (DFMO)[d]	Thiol depletion and cell growth arrest	[36]
SpS	*L. donovani*	KO[e]	Spermidine auxotrophy, depletion of $T(SH)_2$ and cell death	[50]
	T. brucei	dsRNAi	Growth arrest	[51]
TryS	*T. brucei*	dsRNAi	Depletion of Gsp and $T(SH)_2$, growth arrest, loss of viability, enhanced sensitivity against hydroperoxides and drugs	[8,52]
TR	*T. brucei*	Conditional KO	Growth arrest, loss of viability and virulence	[53]
	L. donovani	Dominant negative mutants and KO	Failure to proliferate in macrophages	[54,55]
cTXN	*T. brucei*	dsRNAi	Growth arrest	[56]
			Impaired proliferation and increased sensitivity towards H_2O_2	[35]
mTXN	*T. brucei*	dsRNAi	None	[56]
		Overexpression	Rescue of the loss of kinetoplast replication caused by the overexpression of mPrx and cytochrome-b_5-reductase	[57]
Trx	*T. brucei*	dsRNAi and conditional KO	None	[58]
cPrx	*T. brucei*	dsRNAi	Impaired cell growth and enhanced H_2O_2 sensitivity	[56]
	L. amazonensis	Antisense RNAi	Increased sensitivity towards oxidants	[59]
		Overexpression	Enhanced resistance towards arsenite, hydroperoxide and NO	
	L. infantum	Overexpression	Increased resistance against H_2O_2	[60]
mPrx	*T. brucei*	dsRNAi	None	[56]
		Overexpression	Loss of kinetoplast replication	[57]
	L. amazonensis	Antisense RNAi	Increased sensitivity towards oxidants	
		Overexpression	Enhanced resistance against arsenite, *t*-butyl hydroperoxide and NO	[59]
	L. infantum	overexpression	Increased resistance against *t*-butyl hydroperoxide	[60]
		KO	None	[61]
Px	*T. brucei*	dsRNAi	Cell death and enhanced H_2O_2 sensitivity	[56,62]
GLX II	*T. brucei*	KO	None	Wendler et al., unpublished
1-C-Grx1	*T. brucei*	overexpression	Enhanced sensitivity to iron depletion and hydrogen peroxide	Comini et al. 2008, submitted

[a] GSH1, γ-glutamylcysteine synthetase; ODC, ornithine decarboxylase; SpS, Spermidine synthase; TryS, trypanothione synthetase; TR, trypanothione reductase; cTXN and mTXN, cytosolic and mitochondrial tryparedoxin, respectively; Trx, thioredoxin; cPrx and mPrx, cytosolic and mitochondrial 2-Cys-peroxiredoxin-type tryparedoxin peroxidase, respectively; Px, glutathione peroxidase-type tryparedoxin peroxidases; GLX II, glyoxalase II; 1-C-Grx1, mitochondrial monothiol glutaredoxin 1.
[b] Double-stranded RNA-interference.
[c] Buthionine sulfoximine.
[d] Difluoromethylornithine.
[e] Gene knock-out or replacement.

reaction being three orders of magnitude faster than that with GSH [78]. Hydrogen peroxide, peroxynitrite and radiation-induced radicals are all scavenged by $T(SH)_2$ with remarkable efficiency [40,79,80]. As outlined below, $T(SH)_2$ is the donor of reducing equivalents for the parasite's Trx [81], TXN [82, 83], monothiol glutaredoxin-1 (1-C-Grx1) [84] and ribonucleotide reductase (RR) [85]. It acts as cofactor of the trypanosomatid glyoxalase systems [86,87] and can spontaneously reduce protein sulfenic acids in the model protein glyceraldehyde-3-phosphate-dehydrogenase [84].

Apart from being an intermediate in the biosynthesis of trypanothione, Gsp has been suggested to function as a store of spermidine and GSH [23] but a specific role for Gsp has not yet been evaluated *in vivo*. Its disulfide form is reduced by TR with an efficiency comparable to that for trypanothione disulfide (TS_2) [88]. In addition, glutathionylspermidine forms mixed disulfides with specific protein thiols [71] and can replace trypanothione in the TXN-mediated detoxification of hydroperoxides (Fig. 3) [89].

2.2. Synthesis and reduction of trypanothione

The bifunctional glutathionylspermidine/trypanothione synthetase (TryS) catalyzes the ATP-dependent stepwise conjugation of two GSH and one spermidine molecules to yield $T(SH)_2$. In the first reaction, Gsp is formed that is subsequently combined with another GSH molecule to give $T(SH)_2$. In the absence of ATP, the synthetase exerts an amidase activity and hydrolyses its products back to the substrates GSH and spermidine [[18] and references quoted therein]. In addition to TryS, *C. fasciculata* possesses a specific glutathionylspermidine synthetase (GspS) and the genome of *T. cruzi* encodes also a putative GspS that has not yet been characterized [12,16–18] (Table 1). In contrast, *T. brucei* and *L. major* have only TryS for the *in vivo* synthesis of $T(SH)_2$ and either lack or encode a pseudo GspS-gene [8,13,52].

TS_2 and Gsp-disulfide are recycled back to the thiol state by the flavoenzyme TR at the expense of NADPH (Fig. 2). TR belongs to the family of FAD-cystine-oxidoreductases and shares many physical and chemical properties with GR, lipoamide dehydrogenase, eukaryotic TrxRs and the bacterial mercuric ion reductase [90]. All these enzymes are homodimers with subunit molecular masses of about 50 kDa. The closest relative of TR is GR. The major difference between the two enzymes is their exclusive specificity towards the respective disulfide substrates. When compared with GR, TR has a much wider and negatively charged disulfide substrate binding site that accommodates the positively charged glutathionylspermidine conjugates. Subcellular fractionation of bloodstream and procyclic *T. brucei* followed by determination of enzymatic activity [91] or Western blot analysis [62] revealed that TR is a cytosolic enzyme. In *T. cruzi*, immunolocalization and electron microscopy using a peptide antibody suggested an additional mitochondrial localization [92].

Since trypanosomatids lack GR and TrxR, TR is the only enzyme that connects the NADPH- and the thiol-based redox systems in these parasites. TR is an essential enzyme. Viable *L. donovani* devoid of TR activity could not be obtained and gene replacement was only

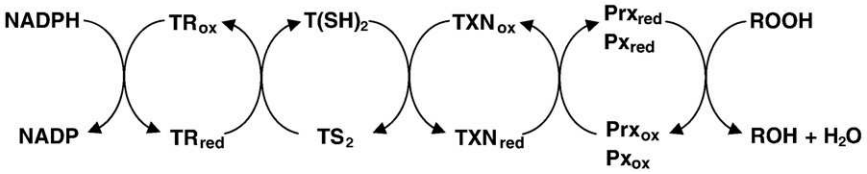

Fig. 3. The trypanothione-mediated hydroperoxide metabolism of trypanosomatids. Detoxification of hydroperoxides (ROOH) is accomplished by 2-Cys-peroxiredoxin (Prx) and glutathione-peroxidase-type (Px) enzymes which both derive their reducing equivalents from a cascade composed of tryparedoxin (TXN), trypanothione [T(SH)$_2$] and trypanothione reductase (TR) with NADPH as the primary electron source. The subscripts red and ox refer to the redox state, dithiol and disulfide, respectively, of the proteins.

possible upon episomal co-expression of the TR-coding sequence [55]. Leishmania with partial TR knock-out and 15% remaining TR activity did not show growth defects under axenic culture conditions but exhibited a significantly impaired ability to survive inside activated macrophages [54,55]. In conditional knock-out cell lines of bloodstream *T. brucei*, the TR activity could be lowered to less than 10% of wild type activity [53] which caused a remarkable growth arrest although the total thiol levels remained constant. Probably the *de novo* synthesis of T(SH)$_2$ together with the residual TR activity is sufficient for maintaining the thiol levels in a resting cell. The growth arrest may be due to the diminished turnover of TS$_2$ and thus an increased TS$_2$/T(SH)$_2$ ratio (which would not be detectable when measuring total thiol concentrations) that could impair the reduction of TXN and thus of RR [85]. This would suggest that the RR-catalyzed reaction is more sensitive to alterations in the metabolic flux of trypanothione than other T(SH)$_2$/TXN-mediated reactions such as the removal of hydroperoxides. However, expectedly the TR-deficient parasites become highly sensitive towards exogenous hydrogen peroxide [53].

2.3. Tryparedoxins and other small dithiol redox proteins

The predominant low molecular mass dithiol proteins in trypanosomatids are TXNs. The parasite proteins have a WCPPCR active site motif and form a distinct molecular clade within the superfamily of thioredoxin-type proteins [83,93]. Despite the low sequence similarity, which is restricted to the active site region and a few other vicinal amino acid residues, TXNs and Trxs have the same core structure. An insertion of 24 to 36 residues between α-2 and ß-5 is responsible for the substantially larger size of TXNs (16 kDa) in comparison to Trxs (12 kDa). Functionally, TXNs are oxidoreductases that share many features with Grxs and/or Trxs. In analogy to Grxs, TXNs are reduced by a low molecular mass thiol, namely T(SH)$_2$ [82,94] and can reduce glutathione-protein mixed disulfides as shown for *T. brucei* 1-C-Grx1 [71]. Like Trxs and Grxs, TXN transfers electrons to RR [85]. Similar to Trxs, TXN is the electron donor for different peroxidases [82,95]. The more N-terminal cysteine of the active site CPPC motif is solvent exposed and its nucleophilicity is warranted by a fast proton shuttling that involves the second cysteine and a network of uncharged internal residues [96]. The thiolate anion reacts with specific protein disulfides leading to mixed disulfides between TXN and the respective target molecule [97,98]. Attack of this mixed disulfide by the vicinal Cys of TXN releases the reduced target protein and oxidized TXN. Reduced TXN is then regenerated by the spontaneous reaction with T(SH)$_2$ (Figs. 2 and 3).

Although trypanosomatids lack GRs and TrxRs [2–4], the genomes of *T. brucei*, *T. cruzi* and *L. major* encode genes for Trxs as well as glutaredoxins (Grxs). The Trx gene is transcribed in both bloodstream and procyclic *T. brucei* [99], but the protein was not detectable by Western blot analysis even not in cells that expressed an ectopic copy of the gene at a 30-fold higher level than the wild-type transcript [58]. Only the enrichment of the protein from 10^{10} procyclic *T. brucei* on an antibody column indicated the presence of the protein in the subsequent Western blot analysis (unpublished data). In epimastigote *T. cruzi* Trx was also only detectable after enrichment by immunoprecipitation. Immunohistochemistry showed a diffuse cytoplasmic staining with extension to the flagellum [100]. Recombinant *T. brucei* Trx has a redox potential of −267 mV which is comparable to that of other Trxs ($E^{\circ\prime}$ = −240 to −270 mV). Both active site cysteines display a p*K*-value of about 7.5 that matches the parasite's intracellular pH [81]. Despite its unusually basic p*I* value of 8.5 (in contrast to p*I*-values of around 4 of most Trxs) and the replacement of a highly conserved Asp by a Trp, the *T. brucei* protein behaves structurally [101] and functionally like a classical Trx. *In vitro*, *T. brucei* Trx is an electron donor for RR [81,99], 2-Cys-peroxiredoxin (Prx) [99] and glutathione peroxidase-type enzymes (Pxs) [95] as well as for 1-C-Grx1 [84]. T(SH)$_2$ and dihydrolipoamide, but not Gsp, proved to be efficient reductants of *T. brucei* Trx [81]. Given a total cellular lipoate concentration of about 3 μM [73], T(SH)$_2$ is the most likely physiological reductant of Trx. However, in comparison to TXN, which is reduced by T(SH)$_2$ with a rate of 5×10^4 M^{-1} s^{-1} [94], the respective reaction of Trx is much slower, the calculated rate being 14 M^{-1} s^{-1} [81]. *T. brucei* Trx possesses a third cysteine residue that is susceptible to specific glutathionylation (Section 7) [71]. Although Trxs are usually key players in numerous cellular processes [27], their role in trypanosomatids remains to be elucidated. The cellular concentration of Trx in the parasite is very low especially when compared with TXN. In addition, deletion of both *trx* alleles in bloodstream *T. brucei* did not result in a growth phenotype at least under cell culture conditions [58].

In terms of their structure and catalytic properties, Grxs have been classified into three categories [102]: i) classical Grxs with the CPYC active site motif and thioredoxin/glutaredoxin fold; ii) glutathione-*S*-transferase-related proteins which possess Grx activities but have a structural organization similar to glutathione-*S*-transferases; iii) 1-C-Grxs that contain a single cysteine in their active site (usually CGFS). The genes encoding the two putative dithiol Grxs (Acc. N° XP_828228 and CAJ16191) have been cloned from *T. brucei* (Ceylan and Krauth-Siegel, unpublished). The kinetoplastid genomes [2–4] also contain genes for putative glutathione-*S*-transferase related glutaredoxins [*T. brucei* (XP_845973), *L. major* (Acc. N° Q4QFI5 and Q4Q940) and *T. cruzi* (Acc. N° XP_806420, XP_810222 and XP_807380)], but none of the proteins has yet been characterized. Three 1-C-Grxs from *T. brucei* are currently characterized [[84]; Comini et al. 2008, submitted] (Section 6).

3. The central role of T(SH)$_2$ and TXN in the thiol metabolism of trypanosomes

The T(SH)$_2$/TXN system delivers electrons for the reduction of ribonucleotides, the detoxification of hydroperoxides and other cellular processes that are regulated by thiol/disulfide exchanges. Mitochondrial and cytosolic isoforms of TXN have been identified in different trypanosomatids [56,57,103,104]. TXNs are abundant proteins. In *C. fasciculata*, the cytosolic TXN accounts for 5% of the total soluble protein [82]. Also in bloodstream *T. brucei* TXN achieves a concentration of ≥100 μM [35,105]. *In vitro*, TXN is readily reduced by human TrxR [105]. However, the lack of TrxR in all trypanosomatid organisms studied so far strongly suggests that

$T(SH)_2$ is the physiological reductant of TXN. K_m values between 30 and 150 μM for $T(SH)_2$ have been reported for different TXNs [83,93,94]. The cellular $T(SH)_2$ concentrations range from 0.2 to 1.5 mM (Table 2). This, together with their nearly identical redox potentials [E_0' −249 and −242 mV for TXN and $T(SH)_2$, respectively [23,105]], indicates that the $T(SH)_2$/TXN system can readily respond to changes in the cellular redox milieu. Also the thiol p*K* values of TXN (7.2) [105] and $T(SH)_2$ (7.4) [74] are very similar and coincide with the intracellular pH of the parasites. Since the second order rate constants for thiol/disulfide exchange reactions exhibit an optimum when the thiol p*K* is equal to the pH of the surrounding solution [106], this probably contributes to the reactivity of these thiols. The activity of TXN may be regulated by the redox status of the parasite. A $T(SH)_2/TS_2$ ratio of 1:10, reflecting severe oxidative stress conditions, caused a significant inhibition of the $T(SH)_2$/TXN-mediated RR activity [85]. In the bloodstream forms of African trypanosomes, depletion of $T(SH)_2$ to low micromolar concentrations by RNA interference towards TryS impairs the growth and antioxidant defense [8]. Also in procyclic *T. brucei*, a growth arrest was observed together with an increased sensitivity against trypanocidal compounds but no alterations of the thiol redox potential [52]. Thus the growth defect is probably not due to changes in the cellular thiol redox balance but may result from the impairment of TXN-mediated reactions by the limited availability of $T(SH)_2$.

In *T. brucei*, down-regulation of TXN expression affects cell growth [35,56] and is accompanied by elevated levels of low molecular mass thiols, in particular of $T(SH)_2$, whose concentration raises up to 4-fold when compared to the non-induced cells [35]. Interestingly, the concentration of $T(SH)_2$ increases already 24 h after induction of TXN depletion when still no growth phenotype is observed. This suggests that the cells can up-regulate the synthesis of the low molecular mass thiols as an adaptive mechanism and underlines again the critical role of the $T(SH)_2$/TXN system in the maintenance of the cellular redox balance.

TXN delivers reducing equivalents to different peroxidases (see Section 5). Depletion of cytosolic TXN to low micromolar concentrations impairs the antioxidative defense of bloodstream *T. brucei* against exogenous hydrogen peroxide [35]. Thus, although the $T(SH)_2$ levels are elevated, the spontaneous reaction of $T(SH)_2$ with the hydroperoxide is not sufficient to compensate for the TXN-mediated peroxidase-catalyzed reaction. The $T(SH)_2$/TXN system serves also as an electron source for RR, a pivotal enzyme for the biosynthesis of the DNA precursors [85]. So far, proteins that interact with TXN have been identified empirically and/or by homology with other organisms [82,85]. Recently we started an approach to identify novel covalent interaction partners of the cytosolic *T. brucei* TXN. An extract of procyclic *T. brucei* was incubated with His_6-TXN-C43S and applied onto a Ni-NTA column. Proteins covalently linked to TXN were eluted with 2-mercaptoethanol, subjected to SDS-PAGE and identified by MALDI-TOF-analysis of their tryptic peptides. A preliminary screening identified a number of known proteins, e.g. the universal minicircle binding protein (UMSBP) and the Pxs (Sections 4 and 5.2) as well as several novel protein partners (Comini, Melchers and Krauth-Siegel, unpublished).

4. Redox regulation of (kinetoplast) DNA replication

Deoxyribonucleotides are essential substrates for the replication and repair of DNA. Reduction of the precursor nucleoside diphosphates to the respective deoxyribonucleotides is catalyzed by RRs [107]. Trypanosomes possess a typical eukaryotic class I RR which is a heterotetramer composed of two subunits R1 and R2 each [108]. During catalysis the R1 subunit is oxidized leading to an intramolecular disulfide which is then reduced by the Grx or Trx systems [109]. *In vitro* studies revealed that $T(SH)_2$ can spontaneously reduce *T. brucei* RR and thus represents the first example of a natural low molecular mass thiol that directly reduces this enzyme [85]. However, *T. brucei* RR displays much higher affinities for TXN and Trx than for $T(SH)_2$, the apparent K_m values being 4 μM, 29 μM and 2 mM, respectively [81,85]. Thus, at submillimolar concentrations of $T(SH)_2$ the RR catalyzed reaction is strongly accelerated by the dithiol proteins. Taking into account the low cellular concentration of Trx [58,100] and its much slower reduction by $T(SH)_2$ when compared to TXN [81] (see Section 2.3), the $T(SH)_2$/TXN system is most likely the physiological reductant of RR.

Trypanosomatids have a single mitochondrion. The organelle contains a unique DNA network known as kinetoplastid DNA (kDNA) which consists of a large arrangement of interlocked DNA rings. Replication of the kDNA minicircles and maxicircles is a complex process that involves multiple proteins. UMSBP is involved in the initiation of the replication process of kDNA minicircles and the segregation of the replicated kDNA network [110]. UMSBP contains five CCHC-type zinc knuckle motifs. The C-terminal zinc fingers mediate the interaction between the protein and single stranded DNA, and the N-terminal motif is responsible for UMSBP oligomerization *via* formation of intermolecular disulfides with a concomitant loss of its DNA-binding activity [111].

In vitro, the $T(SH)_2$/*C. fasciculata* TXN system can re-activate oxidized UMSBP allowing its binding to the minicircle origin sequence which suggested that *in vivo* kDNA replication is subject to redox regulation *via* TXN [111]. More recently, Motyka et al. [57] reported that overexpression of a mitochondrial cytochrome b_5 reductase-like protein (CBRL) in *T. brucei* causes growth arrest and kDNA loss due to UMSBP oxidation/inactivation. Overexpression of the mitochondrial Prx, a known redox partner of TXN (see Section 5.1), yielded an identical phenotype. The phenotype caused by CBRL overexpression was partly reversed by the simultaneous overexpression of the mitochondrial, but not the cytosolic, isoform of TXN. This led to the assumption that overexpression of mitochondrial redox active proteins diverts the electron flow from TXN towards these proteins and, thus, increases the level of oligomerized (oxidized) UMSBP with concomitant impairment of the initiation of kDNA replication [57]. This conclusion, however, raises some doubts taking into account that neither depletion of mitochondrial TXN by RNA interference in *T. brucei* [56] nor overexpression of mitochondrial Prx *in T. cruzi* [104] cause a growth arrest, a phenotype expected if reduced mitochondrial TXN became limiting to sustain kDNA replication. In addition, overexpression of the mitochondrial form of Px, a protein that also requires TXN as reducing partner (see Section 5.2), does not impair the growth of *T. brucei* or induce a loss of kDNA (Diechtierow and Krauth-Siegel, unpublished). The proposed depletion of reduced TXN by overexpression of CBRL and mitochondrial Prx should imply that the two proteins are present at very high concentrations in mainly oxidized form. On the other hand, UMSBP is one of the proteins identified to covalently bind to the C43S mutant of TXN (see Section 3). Evidently, the detailed mechanism of the redox regulation of kinetoplast DNA replication merits further investigations.

5. Trypanothione-dependent detoxification systems in trypanosomatids

$T(SH)_2$ is the central molecule for the detoxification systems of trypanosomatids. The parasites lack catalases and selenocysteine-containing glutathione peroxidases, enzymes responsible for the removal of hydrogen peroxide and other hydroperoxides in mammalian cells. Hydroperoxide detoxification in trypanosomatids relies on a sophisticated cascade of reactions in which trypanothione, TR and TXN play central roles as the carriers of reducing equivalents from NADPH onto two types of peroxidases (Fig. 3). Both the Pxs and the cytosolic Prx are essential for *T. brucei* [56,62] (Table 3) although the enzymes have very similar *in vitro* activities and overlapping cellular localizations. This may suggest that the peroxidases fulfill additional

functions independent of the direct reduction of hydroperoxides. In addition, *T. cruzi* and *Leishmania*, but not *T. brucei*, possess an ascorbate-dependent peroxidase which also obtains its reducing equivalents from $T(SH)_2$ [for a recent review see 112].

The elongation factor 1B complexes from *C. fasciculata* and *L. major* have been reported to have trypanothione *S*-transferase activities [113,114] but the physiological significance of this reaction remains to be elucidated. Also the glyoxalase system detoxifying ketoaldehydes is trypanothione-dependent in these parasites [86,87,115,116]. Finally, the conjugation of metal-containing drugs with $T(SH)_2$ and the active extrusion of the complexes protects the parasites against their cytotoxic effects [64,117].

5.1. 2-Cys-peroxiredoxins

All trypanosomatid organisms studied so far possess Prxs whereas 1-Cys-peroxiredoxins could not be detected in the genomes of *T. brucei*, *T. cruzi* and *L. major* [2–4]. The parasites encode multiple (nearly) identical copies for cytosolic Prxs and on another chromosome a single gene for a mitochondrial analogue. The cytosolic and mitochondrial localization of the proteins was verified in *T. cruzi* [104], *T. brucei* [118] and *L. infantum* [60] by immunoelectron microscopy and/or immunofluorescence assays.

The minimum concentration of Prx in *C. fasciculata* was estimated to amount to 6% of the total soluble protein [82]. In *T. cruzi*, the cytosolic and mitochondrial Prxs occur in all life stages with cellular concentrations of about 2 μM [119]. These values probably reflect minimum concentrations. At least in the case of the mitochondrial protein the local concentration should be one order of magnitude higher given that the volume of the organelle corresponds to about 9% of the total cell volume [120].

Contrasting with the results of Piñeyro et al. [119], the proteome analysis of *T. cruzi* indicated elevated levels for the mitochondrial Prx together with several other antioxidant enzymes such as TXN and TryS in the infectious metacyclic trypomastigotes when compared with the epimastigote insect form [121]. These changes would be consistent with a pre-adaptation of metacyclic parasites to the potential respiratory burst of phagocytic cells in the mammalian host and are supported by the fact that metacyclic parasites show a comparably low sensitivity to peroxynitrite-mediated nitrotyrosine formation [122].

For nearly all parasite Prxs, hydrogen peroxide is a preferred substrate but the enzymes can also reduce linoleic acid hydroperoxide [82]. Another substrate of the *T. brucei* and *T. cruzi* Prxs is peroxynitrite ($ONOO^-$) which is reduced with second order rate constants of about $8\times10^5\ M^{-1}\ s^{-1}$ [122,123]. The kinetic parameters, catalytic mechanism and structural properties of trypanosomatid Prxs have recently been reviewed [112,124].

In the infective form of *T. brucei*, the cytosolic but not the mitochondrial Prx was shown to be crucial for protection against H_2O_2-mediated oxidative stress [56]. Overexpression of the cytosolic or the mitochondrial Prx in epimastigote *T. cruzi* confers resistance towards hydrogen peroxide and tert-butyl hydroperoxide [104]. *T. cruzi* epimastigotes overexpressing these peroxidases are also less susceptible towards reactive oxygen and nitrogen species generated either *in vitro* or released by co-cultured activated macrophages [122]. In addition, these cells are more efficient in the overall infection (but not specifically in invasion) and the parasite Prxs were discussed as virulence factors. Probably, wild type and Prx-overexpressing parasites invade host cells with the same efficiency but higher concentrations of the peroxidases result in an improved survival rate once the pathogen is inside the host cell [119]. In *L. infantum*, the cytoplasmic and mitochondrial Prxs appear to have complementary functions in antioxidant defense when overexpressed [60]. The mitochondrial isoform confers higher resistance against tert-butyl hydroperoxide in comparison to overexpression of the cytosolic Prx, and the opposite effect was observed with H_2O_2 as the source of oxidative stress. Probably H_2O_2 does not reach cytotoxic concentrations in the mitochondrion of parasites overexpressing mitochondrial Prx because it is readily detoxified in the cytoplasm by the authentic cytosolic Prx. In *L. major*, Prx has also been studied as potential antigen for vaccine development [125].

5.2. Glutathione peroxidase-type tryparedoxin peroxidases

Pxs are the second class of hydroperoxide-detoxifying enzymes in trypanosomatids. The proteins differ from the selenoenzymes found in mammals and some other organisms [126] in the replacement of the active site selenocysteine by a cysteine residue. The trypanosomal enzymes are monomers and thus are closer related to the monomeric phospholipid hydroperoxide glutathione peroxidase than to the other tetrameric mammalian enzymes. They exert very low activity with GSH but – as the Prxs of the parasites – use the $T(SH)_2$/TXN system (Fig. 3) [95,127]. The rate constants for hydroperoxide reduction of about $10^5\ M^{-1}\ s^{-1}$ [95] are in the same order of magnitude than those of the Prxs but significantly lower than the values of the mammalian selenoenzymes ($10^7\ M^{-1}\ s^{-1}$) [128,129]. In the *T. brucei* Px, Cys47, Gln82, and Trp137 correspond to the SeCys, Gln, and Trp catalytic triad of the mammalian selenoenzymes. Site directed mutagenesis of Cys47 and Gln82 resulted in inactive enzyme species and a glycine mutant of Trp137 had only 13% of wild type activity which suggested a putative role in catalysis [98]. However, the recently solved NMR structure of the enzyme revealed that neither Gln82 nor Trp137 are in the vicinity of the active site Cys47 (Melchers et al., manuscript in preparation). Cys95, conserved in related fungi and plant proteins but not in the selenoenzymes, proved to be essential as well. Treatment of the C76S/C95S but not of the C47S/C76S double mutant of *T. brucei* Px with H_2O_2 induced formation of a sulfinic acid and covalent homodimers in accordance with Cys47 being the peroxidative thiol. In the wild type protein, these oxidations are prevented by formation of an intramolecular disulfide bridge between Cys47 and Cys95. Regeneration of the reduced enzyme involves a transient mixed disulfide between Cys95 of Px and Cys40 of TXN [98]. Such an intramolecular disulfide bond also occurs in atypical Prxs as well as in Cys-homologous glutathione peroxidases of yeast, plants and insects [130–133]. The catalytic mechanism of the *T. brucei* Px resembles that of atypical Prxs but is clearly distinct from that of the selenoenzymes. In the endoplasmic reticulum of *T. cruzi*, a second Px (PxII) has been described that displays specificity for fatty acid and phospholipid hydroperoxides [134]. The authors speculate that the enzyme may have a specific role in the protection of newly synthesized lipids. African trypanosomes encode three nearly identical genes for Pxs [95]. The proteins occur in the cytosol and mitochondrion of the parasite [62]. Recombinant *T. brucei* Px hydrolyses H_2O_2 and the hydroperoxides of thymine and linoleic acid with comparable efficiency [62]. Silencing of Pxs expression by RNA-interference demonstrated the indispensability of the enzymes for African trypanosomes [56,62] (Table 3). Interestingly, bloodstream cells depleted of the Pxs mRNA displayed a similar susceptibility against H_2O_2 as wild type parasites [56] but overexpression of a myc-tagged version of the mitochondrial Px form in procyclic parasites resulted in the myc-tagged protein in the mitochondrion as well as in the cytosol and led to an increased tolerance against H_2O_2 (Diechtierow and Krauth-Siegel, unpublished).

The inability of the Prxs to substitute for the Px-type enzymes may be due to distinct substrate specificities or compartment-specific functions. In addition, this suggests that the enzymes have specific pivotal cellular functions. Future work will reveal if they can act as hydroperoxide sensors that transduce a redox signal to downstream protein thiols as it has been shown for yeast peroxiredoxin-1 and the glutathione peroxidase-type Gpx 3 [130,135,136] as well as for a related plant peroxidase [137].

5.3. Ascorbate peroxidases

In *T. cruzi* [138] and *L. major* [139], but not in *T. brucei*, plant-like ascorbate-dependent heme-peroxidases have been described. The *T. cruzi* enzyme occurs in the endoplasmic reticulum [138]. The parasite enzymes reduce H_2O_2 at the expense of ascorbate [138], and the dehydroascorbate generated is then reduced by the $T(SH)_2$/TR system (Fig. 3; see Section 2.1). Overexpression of the ascorbate-dependent peroxidase in *T. cruzi* confers a 2-fold increase in resistance towards H_2O_2. Based on the different substrate preferences but identical location with PxII (see Section 5.2), the authors proposed a complementary function of both enzymes in the oxidant environment of the endoplasmic reticulum [138].

5.4. Trypanothione-S-transferases

The eukaryotic elongation factor 1B complexes (eEF1B) from *L. major* [113] as well as from *C. fasciculata* [114] have trypanothione-*S*-transferase activity probably associated with its gamma subunit. Moreover, the *L. major* complex displays low peroxidase activity towards a variety of hydrophobic hydroperoxides but not with hydrogen peroxide [113]. The enzyme may be localized to the surface of the endoplasmic reticulum and has been suggested to play a putative role in the protection of the parasite from lipid peroxidation. Interestingly, the beta subunit of mammalian eEF1B is susceptible to glutathionylation [140] and the gamma subunit contains a redox active cysteine pair [141], but it is not yet known if the regulation of these proteins is involved in the established sensitivity of translation elongation towards reversible inhibition by GSSG.

5.5. Glyoxalase system

Ketoaldehydes are highly reactive and toxic compounds. The predominant physiological ketoaldehyde is methylglyoxal which is mainly generated from triosephosphates during glycolysis [142]. In nearly all organisms, methylglyoxal is detoxified by the GSH-dependent consecutive reactions of glyoxalase (GLX) I and II. In trypanosomatids, the glyoxalase system is $T(SH)_2$ dependent. *T. brucei* possesses a GLX II that specifically hydrolyzes trypanothione-based thioesters [86]. The genome sequencing revealed that African trypanosomes lack a GLX I gene [2], and GLX II seems not to be essential since deletion of both alleles in procyclic *T. brucei* did not cause a growth phenotype (Wendler and Krauth-Siegel, manuscript in preparation). Recently we could show that *T. brucei* GLX II hydrolyses thioesters that were obtained in GLX I-independent reactions. Nevertheless, the physiological role of the enzyme remains unknown. Although *T. cruzi* [115], *L. major* [87,143] and *L. donovani* [116,144] have a complete $T(SH)_2$-based glyoxalase system, the enzymes are also not compartmentalized in glycosomes, the organelles where glycolysis occurs in these parasites [145].

5.6. Mode of action of and resistance against metal-containing drugs

The trypanothione system is involved in the mode of action of and the resistance against metal-containing drugs. Trivalent antimonials – the active form of the pentavalent antimonial drugs – induce a rapid efflux of trypanothione and glutathione and thus decrease the thiol buffering capacity of *Leishmania*. The compounds inhibit TR which results in the accumulation of TS_2 and GSSG [32]. Cultured parasites selected for resistance to arsenite- or antimony-containing drugs frequently show amplification of the PGPA (Gp-glycoprotein-like protein A) gene. PGPA is a member of the multidrug resistance protein (MRP) family, ABC (ATP-binding cassette) transporters that transport different types of conjugated compounds [117]. In *Leishmania*, PGPA is located in intracellular membranes close to the flagellar pocket. PGPA-containing vesicles catalyze the ATP-dependent extrusion of conjugates between arsenicals and GSH whereby the physiological substrates are most probably the $T(SH)_2$ conjugates [117]. Recently it was shown that in field isolates of *L. donovani* amplification of the MRPA gene together with an increase in the cellular thiol levels can confer resistance against antimony-containing drugs [64,146]. In *T. brucei*, overexpression of MRPA is sufficient for resistance against arsenicals *in vitro* [34] but not *in vivo* and an overexpression of MRPA was not observed in four melarsoprol resistant isolates [147].

6. Trypanosomal monothiol glutaredoxins

The genomes of *Kinetoplastida* encode three putative 1-C-Grxs [84] located as single copy genes on different chromosomes. 1-C-Grx1 and 2 consist of a single monothiol glutaredoxin domain while in 1-C-Grx3 this domain is linked to an N-terminal thioredoxin domain. Two structural features distinguish the trypanosomatid 1-C-Grxs from orthologous proteins: i) the active site motif is rather variable (C,G>A>R,F>Y,T>S) in comparison to the highly conserved CGFS motif of most orthologues [148]; ii) the thioredoxin domain of the trypanosomatid 1-C-Grx3 contains a CXXC dithiol motif whereas related eukaryotic proteins have a single conserved cysteine residue in their N-terminal thioredoxin-like domain. *T. brucei* 1-C-Grx1 exists as a non-covalent dimer (2×17 kDa) whereas 1-C-Grx2 (14 kDa) and 1-C-Grx3 (25 kDa) are monomeric proteins (Comini et al. 2008, submitted) as are the orthologous proteins from other organisms [149].

All three 1-C-Grxs occur in bloodstream and procyclic *T. brucei* [[84]; Comini et al. 2008, submitted]. In general, their levels increase in stationary-phase and starving parasites, with 1-C-Grx1 and 3 – in contrast to 1-C-Grx2 – being abundant proteins. The expression pattern of the trypanosomal proteins resembles that of the single 1-C-Grx of *E. coli* (Grx4) [150] but differs from the yeast homologues which show a general down-regulation of 1-C-Grxs transcript during growth [151–153]. 1-C-Grx1 is a mitochondrial protein [84] and its concentration in the rudimentary organelle of bloodstream *T. brucei* is probably >200 μM (Comini et al. 2008, submitted).

A thorough biochemical characterization revealed that the parasite 1-C-Grx1 lacks any activity of dithiol Grxs [84]. A specific substrate or interacting protein has not yet been identified for any 1-C-Grx. *T. brucei* 1-C-Grx1 is specifically thiolated by glutathione or glutathionylspermidine at a non-active site cysteine residue, which induces the formation of an intramolecular disulfide [71]. TXN acts as reductant cleaving both the mixed and the intramolecular disulfide forms [84]. 1-C-Grxs from different organisms have been shown to be involved in the iron sulfur cluster assembly and/or cellular iron homeostasis, but the specific function remains elusive [149]. Complementation studies in a *Saccharomyces cerevisiae grx5*-KO strain targeted the trypanosomal 1-C-Grxs to the yeast mitochondria but only 1-C-Grx1 was, to some extent, able to ameliorate the mutant phenotype [84]. The poor or completely lacking ability of the parasite proteins to substitute for yeast Grx5 – in contrast to a variety of prokaryotic and eukaryotic 1-C-Grxs [154] – was unexpected and indicates a significant structural or functional divergence of the Kinetoplastid proteins.

Both life stages of *T. brucei* were refractory to *1-c-grx1* gene silencing (by double strand RNA-interference) and deletion (classical gene replacement). In addition, the *1-c-grx1* alleles could only be deleted in procyclic cells if an ectocopic copy of the gene was expressed (Comini et al. 2008, submitted). Taken together, the data obtained so far point to an essential role for 1-C-Grx1 and the lack of functional redundancy among 1-C-Grxs in this parasite. *T. brucei* 1-C-Grx1 is certainly involved in the iron metabolism of the parasite (Comini et al. 2008, submitted) and the elucidation of its specific role(s) in the mitochondrion of trypanosomes is currently under study. The strikingly high concentration of 1-C-Grx1 in the

rudimentary and metabolically repressed organelle of bloodstream parasites raises the possibility of additional/alternative functions.

7. Glutathionylation of trypanosomal thiol redox proteins

Organisms adapt to changes in their environment by adjustments in gene expression, and the most important control point is that of transcription initiation. This is true for all organisms except trypanosomatids [155]. In these protozoa, polycistronic precursor RNAs are produced as primary transcripts that are co-transcriptionally processed into monocistronic RNAs. Although a few proteins/subunits distantly related to known transcription factors have been described [for a recent review see [156]], the major regulation of gene expression is clearly at post-transcriptional levels.

The proteome analysis of procyclic *T. brucei* revealed that most proteins appear in multiple spots and the majority of the isoforms seems to be due to post-translational modifications [157]. In the proteome of *T. cruzi*, a search for peptides with modifications detected a wide variety of acetylated, methylated and phosphorylated proteins [121]. Post-translational modification/regulation mechanisms seem to be extensive in trypanosomatids.

As a first step to reveal whether trypanosomes can employ *S*-thiolation for regulatory or protection purposes, we studied different recombinant parasite thiol redox proteins for their ability to form mixed disulfides with glutathione or glutathionylspermidine. TR and TXN proved to be not sensitive to thiolation. In contrast, *T. brucei* 1-C-Grx1 becomes specifically thiolated at Cys181. A mixed disulfide at this residue then triggers formation of an intramolecular disulfide bridge with the active site Cys104 [71]. This contrasts with 1-C-Grxs from other sources that have been suggested to be glutathionylated at the active site cysteine [150,158]. The other 1-C-Grxs of *T. brucei* are also susceptible to glutathionylation of non-active site cysteines (Melchers and Krauth-Siegel, unpublished data). In addition, both types of tryparedoxin peroxidases (Sections 5.1. and 5.2) are sensitive to glutathionylation [[71]; Melchers and Krauth-Siegel, unpublished data]. In *T. brucei* Trx, a non-active site cysteine (Cys68) becomes glutathionylated. Work is now in progress to reveal if these proteins are thiolated *in vivo*, specifically when the parasites are exposed to exogenous or endogenous stresses.

8. Outlook

Trypanosomes and *Leishmania* have a unique thiol redox metabolism that is based on trypanothione. The TR/T$(SH)_2$/TXN system is essential and is responsible for maintaining the cytosolic redox homeostasis. Various components of the trypanothione metabolism have been characterized but there are still many open questions. For instance, what is the role of free glutathione in organisms lacking a glutathione reductase? *In vitro* several thiol redox proteins have been shown to be susceptible to specific thiolation. The next step will be to investigate if the parasites employ protein glutathionylation for regulatory and/or protection purposes. The surprising lack of functional redundancy between the parasite hydroperoxide-metabolizing enzymes points to specific physiological functions. Future work should reveal if the peroxidases can act as redox sensors in/activating specific target proteins. The putative involvement of the trypanothione metabolism in differentiation as well as adaptation of the parasites to the different life stages, hosts, and site of infections offers other interesting and valuable fields.

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