

## Roles of Trypanothione *S*-Transferase and Tryparedoxin Peroxidase in Resistance to Antimonials<sup>∇</sup>

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The clinical value of antimonial drugs, the mainstay therapy for leishmaniasis, is now threatened by the emergence of acquired drug resistance, and a comprehensive understanding of the underlying mechanisms is required. Using the model organism *Leishmania tarentolae*, we have examined the role of trypanothione *S*-transferase (TST) in trivalent antimony [Sb(III)] resistance. TST has *S*-transferase activity with substrates such as chlorodinitrobenzene as well as peroxidase activity with alkyl and aryl hydroperoxides but not with hydrogen peroxide. Although *S*-transferase activity and TST protein levels were unchanged in Sb(III)-sensitive and -resistant lines, rates of metabolism of hydrogen peroxide, *t*-butyl hydroperoxide, and cumene hydroperoxide were significantly increased. Elevated peroxidase activities were shown to be both trypanothione and tryparedoxin dependent and were associated with the overexpression of classical tryparedoxin peroxidase (TryP) in the cytosol of *L. tarentolae*. The role of TryP in Sb(III) resistance was verified by overexpression of the recombinant *Leishmania major* protein in Sb(III)-sensitive promastigotes. An approximate twofold increase in the level of TryP activity in this transgenic cell line was accompanied by a significant decrease in sensitivity to Sb(III) (twofold;  $P < 0.001$ ). Overexpression of an enzymatically inactive TryP failed to result in Sb(III) resistance. This indicates that TryP-dependent resistance is not due to sequestration of Sb(III) and suggests that enhanced antioxidant defenses may well be a key feature of mechanisms of clinical resistance to antimonial drugs.

The protozoan parasite *Leishmania* elicits a broad range of human and animal infections ranging from life-threatening kala azar to disfiguring mucocutaneous and cutaneous forms of the disease. The World Health Organization reports over 12 million cases of all forms of leishmaniasis worldwide with 500,000 new infections each year, resulting in an annual death toll of 59,000 (39). Chemotherapy has proven to be the only effective way of controlling infections and is highly dependent upon antimony-containing drugs such as sodium stibogluconate (Pentostam). However, after more than half a century as the mainstay therapy for leishmaniasis, the clinical value of antimonials is now threatened due to the emergence of acquired drug resistance (5). In the past 15 years, failure of antimonials in regions of Bihar, India, where the disease is endemic has become an escalating problem, with primary unresponsiveness reportedly of over 50% (35). Identifying ways in which current antimony therapies can be adapted and improved to circumvent resistance is now a matter of urgency.

The unique thiol metabolism of *Leishmania* is thought to play a pivotal role in the mechanisms of action of antimonial drugs. In these parasites, the major low-molecular-mass thiol is trypanothione (T[SH]<sub>2</sub>) [*N*<sup>1</sup>,*N*<sup>8</sup>-bis(glutathionyl)spermidine],

in contrast to most other organisms, which utilize glutathione ( $\gamma$ -L-glutamyl-L-cysteinylglycine) (GSH) (7). Key functions of this essential metabolite include maintenance of thiol redox homeostasis, as well as defense against chemical (37) and oxidative stress (7). Antimonial drugs are administered as pentavalent antimony [Sb(V)], a prodrug requiring conversion to the trivalent form [Sb(III)], before becoming biologically active. However, the site of reduction (host macrophage, amastigote, or both) and mechanism of reduction (enzymatic or nonenzymatic) remain unclear (10, 31). Sb(III) interferes directly with thiol metabolism, decreasing thiol-buffering capacity in drug-sensitive *Leishmania donovani* by inducing rapid efflux of intracellular T[SH]<sub>2</sub> and GSH (41). Sb(III) also inhibits T[SH]<sub>2</sub> reductase in intact cells, resulting in the accumulation of the disulfide forms of both T[SH]<sub>2</sub> (T[S]<sub>2</sub>) and GSH. These two mechanisms act synergistically against *Leishmania* parasites, leading to a lethal imbalance in thiol homeostasis.

The ability to generate drug-resistant *Leishmania* lines in the laboratory, through stepwise exposure to Sb(III), has greatly facilitated the study of antimonial resistance mechanisms (2, 26). Indeed, several key features of in vitro drug resistance have since been identified in resistant clinical isolates (23, 25). A considerable body of evidence places thiol metabolism at the center of both clinical and laboratory-generated resistance mechanisms (2, 23). Elevated levels of T[SH]<sub>2</sub> and GSH, resulting from the overexpression of the rate-limiting enzymes involved in the synthesis of GSH ( $\gamma$ -glutamylcysteine synthase [ $\gamma$ GCS]) (11) and polyamines (ornithine decarboxylase [ODC]) (13), the two precursor metabolites of T[SH]<sub>2</sub>, have been observed in several laboratory-induced resistant lines of

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*Leishmania*. Interestingly, elevated thiols alone do not result in Sb(III) resistance; however, modulation of T[SH]<sub>2</sub> levels, through the use of inhibitors of thiol biosynthesis, reverts resistance (12) indicating that antimonial drug resistance is multifactorial. Overexpression of PgpA, an intracellular metal-thiol transporter from the ATP-binding cassette transporter family, has also been demonstrated to play a key role in resistance (11, 12). Cotransfection of genes encoding  $\gamma$ GCS and PgpA confers antimony resistance in a synergistic manner in partially revertant *Leishmania* parasites (12). Collectively, these studies have led researchers to hypothesize that Sb(III) is detoxified from resistant parasites via the formation of Sb(III)-thiol complexes which are then sequestered in an intracellular vacuolar compartment by PgpA.

In addition to elevated intracellular thiols and elevated PgpA, a third resistance determinant has been identified. In work with the model organism *Leishmania tarentolae*, Hameur and colleagues demonstrated that, despite having lost amplification of  $\gamma$ GCS, ODC, and PgpA (12), a formerly Sb(III)-resistant revertant cell line retained significant resistance. These researchers hypothesized that an enzyme facilitating the formation of thiol-Sb(III) conjugates for subsequent sequestration by PgpA may well be responsible. Indeed, GSH *S*-transferase, which catalyzes the conjugation of xenobiotics to GSH in mammalian cells, is elevated in arsenite-resistant Chinese hamster ovary cells (22). Since GSH *S*-transferase is not detectable in *Leishmania* spp., it has been suggested that the recently identified T[SH]<sub>2</sub> *S*-transferase (TST) enzyme may facilitate this conjugation in Sb(III)-resistant *Leishmania* parasites (12). Unusually, TST activity in these cells is associated with the eukaryotic translation elongation factor 1B (38). In the current study we investigate the potential role of TST activity in antimony resistance.

#### MATERIALS AND METHODS

**Cell lines and culture conditions.** The cell lines used in this study were wild-type *L. tarentolae* (TarII), the Sb(III)-resistant TarII Sb1.1, and its partially resistant revertant TarII Sb1.1rev (12). Promastigotes were propagated in SDM-79 medium supplemented with 10% (vol/vol) fetal calf serum and grown at 24°C with shaking. TarII Sb1.1 cells were grown in the presence of 250  $\mu$ M potassium antimony tartrate to maintain resistance.

In order to examine the effects of antimony on growth, triplicate cultures containing Sb(III) (as potassium antimony tartrate) were seeded at  $5 \times 10^5$  promastigotes ml<sup>-1</sup>. Cell densities were determined microscopically after culture for 72 h, and 50% effective concentrations (EC<sub>50</sub>s) were determined using the EC<sub>50</sub> four-parameter equation provided with GraFit.

**Analysis of intracellular thiols.** Mid-log-phase promastigotes ( $5 \times 10^7$ ) were collected by centrifugation (1,600  $\times$  g, 10 min, 4°C) and derivatized with monobromobimane, as described previously (32). Acid-soluble thiols were separated by ion-paired, reverse-phase high-pressure liquid chromatography on a Beckman Ultrasphere C<sub>18</sub> column using a Beckman System Gold instrument fitted with a Gilson-121 fluorometer.

**Cell lysis.** Frozen *L. tarentolae* cell pellets were thawed on ice and resuspended in an equal volume of ice-cold lysis buffer (50 mM potassium phosphate [pH 7.0], 1 mM EDTA). The organisms were lysed under pressure (30 kpsi) using a one-shot cell disruptor (Constant Systems). After centrifugation (100,000  $\times$  g, 1 h, 4°C), the clarified extracts were dialyzed against the same buffer (4 times, 50 volumes) and assayed immediately for enzyme activity.

**Enzyme assays.** T[SH]<sub>2</sub> reductase was assayed spectrophotometrically at 340 nm in 40 mM Na<sup>+</sup> HEPES (pH 7.5), 1 mM EDTA, 150  $\mu$ M NADPH, and 100  $\mu$ M T[SH]<sub>2</sub> (6). TST was assayed in 100 mM Na<sup>+</sup> phosphate (pH 6.5) with 400  $\mu$ M 1-chloro-2,4-dinitrobenzene and 400  $\mu$ M T[SH]<sub>2</sub> as substrates. The rate of formation of the T[SH]<sub>2</sub> *S*-dinitrobenzene conjugate was followed at 340 nm, using the published extinction coefficient of 9.2 mM cm<sup>-1</sup> (36). T[SH]<sub>2</sub>-dependent peroxidase activities were measured as previously described (15). Briefly, the

rates of metabolism of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), *t*-butyl hydroperoxide (*t*BuOOH), and cumene hydroperoxide (CuOOH) were monitored in assays containing 50 mM K<sup>+</sup> phosphate (pH 7.0), 0.05 mM T[SH]<sub>2</sub>, 0.05 mM peroxide, 0.25 mM NADPH, and 0.3 U ml<sup>-1</sup> T[SH]<sub>2</sub> reductase. Where specified, assays were supplemented with 1  $\mu$ M recombinant *L. major* tryparedoxin. Peroxidase activity was measured by the consumption of NADPH at 340 nm. All enzymatic activities were proportional to the amount of protein assayed and heat labile.

**Western analysis of whole-cell lysates.** Polyclonal antisera against *Leishmania major* tryparedoxin, tryparedoxin peroxidase (type I), tryparedoxin peroxidase (type II), and T[SH]<sub>2</sub> synthetase and *Trypanosoma brucei* T[SH]<sub>2</sub> reductase were raised in adult male Wistar rats. An initial injection of 100  $\mu$ g of purified antigen, emulsified in complete Freund's adjuvant, was followed by two identical booster injections of antigen emulsified in Freund's incomplete adjuvant at 2-week intervals.

Mid-log-phase *L. tarentolae* promastigotes ( $1 \times 10^7$  ml<sup>-1</sup>) were pelleted by centrifugation (1,600  $\times$  g, 10 min, 4°C), washed twice in phosphate-buffered saline (PBS), and resuspended directly in 2 $\times$  Laemmli buffer containing 200 mM dithiothreitol. Whole-cell extracts ( $1 \times 10^7$  parasites per lane) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose. After blocking with 5% skim milk in PBS at room temperature for 1 h, blots were incubated with polyclonal antiserum at room temperature for 1 h, washed in PBS containing 0.1% (vol/vol) Tween 20, and then incubated with a secondary rabbit anti-rat (immunoglobulin G [IgG]) antibody (1/10,000 dilution). Immunoblots were developed using the ECL Plus enhanced chemiluminescence system from Amersham Biosciences. Identical immunoblots were prepared and probed with the following primary antisera: tryparedoxin (1/700 dilution), T[SH]<sub>2</sub> synthetase (1/500 dilution), T[SH]<sub>2</sub> reductase (1/500 dilution), tryparedoxin peroxidase type I (1/5000 dilution), tryparedoxin peroxidase type II (1/1000 dilution), and TST (1/300 dilution) (38). The relative intensities of protein bands in each Western blot were determined by densitometry using Labworks software (UVP).

**Subcellular fractionation.** Subcellular fractions of *L. tarentolae* were prepared as previously described (27). Each fraction (30  $\mu$ g) was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. After blocking, the blot was probed with *L. major* tryparedoxin peroxidase (type I) polyclonal antiserum and detected by chemiluminescence, as described above. Identical immunoblots were probed using the following primary antibodies: anti-Hsp60 monoclonal antibody (1/5,000 dilution; Stressgen); anti-*L. major* T[SH]<sub>2</sub> synthetase antiserum (1/1,000 dilution) (27), and anti-*T. brucei* BiP antiserum (1/5,000 dilution) (1). Rabbit anti-rat (IgG) antibody (Dako; 1/10,000 dilution) and anti-mouse (IgG-horseradish peroxidase) antibody (Sigma; 1/10,000 dilution) were used as secondary antibodies where appropriate, and immunoblots were once again developed by enhanced chemiluminescence.

**Cloning and expression of *L. major* tryparedoxin peroxidase in *L. tarentolae*.** The *L. major* tryparedoxin peroxidase gene (LmjF15.1120) was amplified by PCR from *L. major* Friedlin genomic DNA using the sense primer 5'-GGATCCATGTCCTGCGGTAACGCCAAGATCAAC-3' and the antisense primer 5'-GGATCCTTACTGCTTGCTGAAGTATCCCTCGAC-3', both with additional BamHI sites (underlined). The PCR product was then cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and sequenced. The pCR-Blunt II-TOPO-LmTryP construct was then digested with BamHI and the fragment cloned into the pIRISAT expression vector, resulting in a pIRISAT-LmTryP construct. Mid-log-phase *L. tarentolae* promastigotes (wild type, TarII) were transfected with either pIRISAT-LmTryP or with the empty vector by electroporation using a high-voltage protocol (28). Following transfection, cells were allowed to grow for 16 to 24 h in SDM-79 medium with 10% fetal calf serum and then plated on semisolid medium containing 1% Noble agar (Difco) and 100  $\mu$ g ml<sup>-1</sup> nourseothricin (Jena Bioscience, Germany). Individual colonies were picked and grown in liquid medium. Clones were maintained in selective medium and then removed from drug selection for one passage prior to experiments.

**Cloning and expression of an inactive *L. major* tryparedoxin peroxidase gene mutant in *L. tarentolae*.** Site-directed mutagenesis was carried out following the QuikChange protocol (Stratagene) and using the KOD HotStart DNA polymerase (Novagen). Using the pIRISAT-LmTryP construct as a template, an R128D mutant of *L. major* TryP was generated by PCR with the sense primer 5' GCCAGGGCGTGCTACGACGGTCTCTTCATCATCG and antisense primer 5' CGATGATGAAGAGACCGTCGTAGGCCACGCCCTGGC, with mutated bases underlined. The modified construct, pIRISAT-LmTryP(R128D), was transfected into mid-log-phase *L. tarentolae* promastigotes (wild type, TarII) as described above.

TABLE 1. Intracellular thiol levels in Sb(III)-sensitive *L. tarentolae* TarII (wild type), Sb(III)-resistant TarII Sb1.1, and partially resistant revertant TarII Sb1.1rev

Cell line	EC <sub>50</sub> (μg ml <sup>-1</sup> )	Thiol content (nmol 10 <sup>8</sup> cells <sup>-1</sup> ) <sup>a</sup>			
		GSH	GspdSH	T[SH] <sub>2</sub>	Total SH
TarII (wild type)	7.35 ± 0.14	0.75 ± 0.04	0.325 ± 0.08	1.6 ± 0.03	4.28
TarII Sb1.1rev (revertant)	56.5 ± 1.9 <sup>c</sup>	0.8 ± 0.05 <sup>c</sup>	0.37 ± 0.02 <sup>c</sup>	1.70 ± 0.07 <sup>c</sup>	4.56 <sup>c</sup>
TarII Sb1.1 (resistant)	377 ± 38.4 <sup>d</sup>	8.76 ± 0.11 <sup>d</sup>	1.4 ± 0.01 <sup>d</sup>	10.05 ± 0.1 <sup>d</sup>	30.26 <sup>d</sup>
TarII Sb1.1 – Sb <sup>b</sup>	Not applicable	11.1 ± 0.2 <sup>d</sup>	1.8 ± 0.09 <sup>d</sup>	12.1 ± 0.15 <sup>d</sup>	37.1 <sup>d</sup>

<sup>a</sup> Thiol levels represent the means ± standard deviations from triplicate determinations.

<sup>b</sup> Cells grown in the absence of Sb(III) for two passages prior to analysis.

<sup>c</sup> Not significantly different from wild-type values ( $P > 0.05$ ).

<sup>d</sup> Significantly different from wild-type value ( $P < 0.0001$ ).

## RESULTS

**Intracellular thiol levels in Sb(III)-sensitive and -resistant *L. tarentolae*.** A comprehensive understanding of the mechanisms of resistance to antimonials is crucial, not only in the ongoing fight to save these mainstay therapies but also for the future design of more effective anti-*Leishmania* drugs. With this in mind, the previously characterized *L. tarentolae* TarII (wild type), TarII Sb1.1 [Sb(III)-resistant] and TarII Sb1.1rev [Sb(III)-resistant revertant] cell lines were used to examine the putative role of TST in Sb(III) resistance (12). Initially, the relative Sb(III) sensitivities of all three cell lines were determined in potassium antimony tartrate. In keeping with previously published data (12), the Sb(III)-resistant cell line was approximately 50-fold less sensitive to potassium antimony tartrate than the parental wild-type cells, while the revertant cell line was 8-fold less sensitive (Table 1).

Intracellular thiol levels in the parental wild-type, revertant, and resistant cell lines were determined (Table 1). In contrast to the data of Haimour and Ouellette (14), we could detect no significant difference in the thiol levels of revertant versus wild-type cells. However, Sb(III)-resistant cells maintained significantly higher levels of GSH, GspdSH, and T[SH]<sub>2</sub> ( $P < 0.0001$ ). Indeed, the levels of total acid-soluble low-molecular-mass thiol groups were approximately 7.5-fold higher in TarII Sb1.1 than in revertant and wild-type cells, suggesting that elevated intracellular thiol levels are associated with high levels of Sb(III) resistance but are not responsible for the residual resistance of the Sb1.1rev cell line.

Since Sb(III) is known to induce the rapid efflux of intracellular GSH and T[SH]<sub>2</sub> in *Leishmania donovani* (41), thiols in Sb(III)-resistant promastigotes were reanalyzed following growth for two passages in the absence of Sb(III). Interestingly, these cells maintained even higher levels of intracellular thiols (37.1 nmol 10<sup>8</sup> cells<sup>-1</sup> total SH) than the original resistant cell line (30.3 nmol 10<sup>8</sup> cells<sup>-1</sup> total SH), presumably due to continued thiol synthesis in the absence of thiol-Sb(III) conjugate efflux.

**TST activity in clarified *L. tarentolae* lysates.** Clarified extracts of all three *L. tarentolae* cell lines were prepared and assayed directly for TST activity (Table 2). Using 1-chloro-2,4-dinitrobenzene as a substrate, no difference in the TST activities of extracts of wild-type, revertant, and resistant promastigotes could be detected, suggesting that TST overexpression is not associated with Sb(III) resistance in these parasites. T[SH]<sub>2</sub> reductase (TryR) activity was similar in all three ex-

tracts, suggesting that an elevated TryR level is not responsible for residual resistance either.

In our previous studies we demonstrated that *L. major* TST displays T[SH]<sub>2</sub>-dependent peroxidase activity against a variety of organic hydroperoxides. To further verify that TST is not an Sb(III) resistance determinant in *L. tarentolae*, the rates of metabolism of H<sub>2</sub>O<sub>2</sub>, CuOOH, and tBuOOH were measured in dialyzed extracts. Unexpectedly, the rates of metabolism of H<sub>2</sub>O<sub>2</sub> and tBuOOH were considerably higher in the resistant and revertant lysates than in the wild type (Table 2). Specifically, H<sub>2</sub>O<sub>2</sub> metabolism was enhanced 4-fold and 8.5-fold in Sb1.1rev and Sb1.1 extracts, respectively, in keeping with the 8-fold- and 50-fold-increased levels of Sb(III) resistance found in whole cells. CuOOH was less efficiently reduced. Since *L. major* TST is known to preferentially metabolize hydrophobic hydroperoxides and is unable to utilize hydrogen peroxide as a substrate (38), it is unlikely that this enzyme is responsible for the peroxidase activities detected in these extracts.

The apparent lack of TST involvement in Sb(III) resistance is in marked contrast to the resistance mechanisms employed by mammalian cells, in which GSH *S*-transferases accelerate the detoxification of the related metalloid As(III) via conjugation to GSH (18). Although the spontaneous formation of Sb(III)-thiol (GSH and T[SH]<sub>2</sub>) complexes has been demonstrated (34), some researchers have suggested that an enzyme such as TST may be required to accelerate this reaction in vivo (12). In light of the elevated levels of intracellular thiols and

TABLE 2. Specific activities of T[SH]<sub>2</sub> reductase, TST, and T[SH]<sub>2</sub>-dependent peroxidase in *L. tarentolae* soluble extracts

Enzymatic activity	Substrate	Sp act (nmol min <sup>-1</sup> mg <sup>-1</sup> ) <sup>a</sup>		
		Wild type	Revertant strain	Resistant strain
T[SH] <sub>2</sub> reductase	T[S] <sub>2</sub>	112 ± 9	120 ± 9 <sup>b</sup>	114 ± 7.9 <sup>b</sup>
TST	CDNB	1.2 ± 0.5	1.6 ± 0.8 <sup>b</sup>	1.0 ± 0.4 <sup>b</sup>
T[SH] <sub>2</sub> -dependent peroxidase	H <sub>2</sub> O <sub>2</sub>	3.5 ± 0.4	14.4 ± 0.9 <sup>c</sup>	25.7 ± 1.0 <sup>c</sup>
	CuOOH	1.0 ± 0.1	3.1 ± 0.1 <sup>c</sup>	8.9 ± 0.4 <sup>c</sup>
	tBuOOH	1.9 ± 0.2	8.8 ± 0.2 <sup>c</sup>	22.4 ± 0.4 <sup>c</sup>

<sup>a</sup> All enzymatic activities were assayed as described in Materials and Methods and were corrected for nonenzymatic background rates. Values are means ± standard deviations.

<sup>b</sup>  $P > 0.05$  compared to wild-type value.

<sup>c</sup>  $P < 0.0001$  compared to wild-type value.

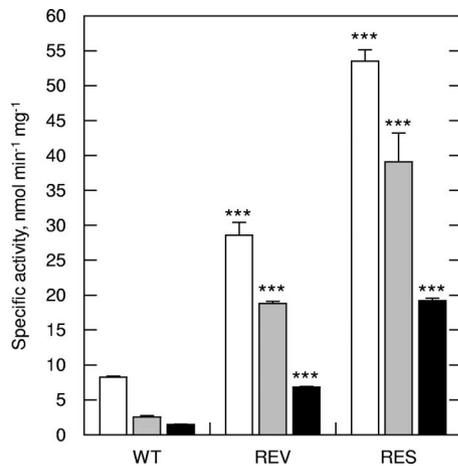


FIG. 1. Trypanodexin-dependent peroxidase activity in *L. tarentolae* clarified lysates. Metabolism of hydrogen peroxide (white bars), tBuOOH (gray bars), and CuOOH (black bars) in wild-type (WT), revertant (REV), and resistant (RES) extracts were measured in the presence of 1  $\mu$ M recombinant *L. major* trypanodexin. Data represent the means  $\pm$  standard deviations from triplicate determinations. \*\*\*,  $P < 0.0001$ .

overexpression of the metal-thiol transporter PgpA in resistant *Leishmania* parasites, the ability to more rapidly form Sb(III)-thiol conjugates for detoxification would be predicted to enhance Sb(III) resistance. However, the absence of accompanying TST overexpression in these cell lines implies that TST is not responsible for the resistance phenotype.

**Trypanodexin-dependent peroxidase activity in clarified extracts.** In *Leishmania* and other trypanosomatids, enzymes of the trypanodexin peroxidase family are thought to be principally responsible for detoxification of peroxides (9). This family consists of two classes of 2-Cys peroxidases, the decameric type I trypanodexin peroxidase (TryP) (8, 20) and the monomeric type II trypanodexin peroxidase (TDPX), a cysteine homologue of the classical GSH peroxidases (16, 29, 30). Both enzymes obtain their reducing equivalents from T[SH]<sub>2</sub> via the dithiol protein trypanodexin (TryX). To establish whether a TryX-dependent peroxidase was involved in Sb(III) resistance, clarified extracts were assayed for peroxidase activity following the addition of exogenous recombinant *L. major* TryX (Fig. 1). In the presence of TryX, peroxidase activities in all three extracts, and against all substrates, were at least doubled (Table 2). However, metabolism of H<sub>2</sub>O<sub>2</sub>, tBuOOH, and CuOOH remained significantly higher in revertant and resistant cell extracts compared to wild type ( $P < 0.0001$ ). These observations suggest that enhanced peroxide metabolism in Sb(III)-resistant cells is TryX dependent and that TryX is rate limiting under these assay conditions. TryPs from several *Leishmania* spp. have shown susceptibility to inactivation following exposure to high concentrations of hydrophobic peroxides (3). Therefore, it was interesting to note that preincubation with 1 mM CuOOH inactivated the elevated peroxidase activities in revertant and resistant extracts (data not shown).

**Western analysis of whole cell lysates.** Immunoblots of *L. tarentolae* promastigote whole-cell lysates were probed with a series of polyclonal antisera against all the enzymes of the trypanodexin peroxidase pathway (Fig. 2). In keeping with our

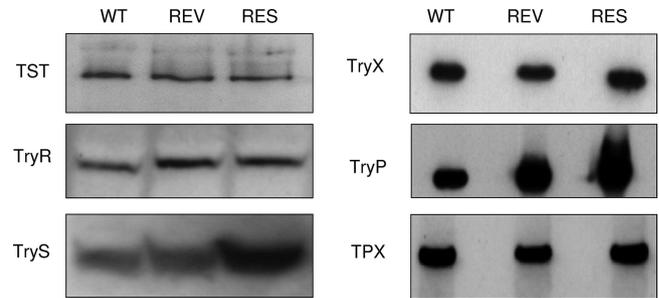


FIG. 2. Immunoblot analysis of *L. tarentolae* whole-cell lysates. Blots of cell extracts from *L. tarentolae* wild-type (WT), revertant (REV), and Sb(III)-resistant (RES) cell lines ( $1 \times 10^7$  parasites in each lane) were probed with antisera to *L. major* TST, *T. brucei* T[SH]<sub>2</sub> reductase (TryR), *L. major* T[SH]<sub>2</sub> synthetase (TryS), *L. major* trypanodexin (TryX), *L. major* trypanodexin peroxidase type I (TryP), and *L. major* trypanodexin peroxidase type II (TDPX).

enzymatic analysis of whole-cell lysates, TST and TryR protein levels were similar in wild-type, revertant, and resistant cell lines. Surprisingly, levels of TryS protein were increased 2.5-fold in the resistant cell line but not in the revertant. Sb(III) up to 1 mM had no inhibitory effect on TryS activity, indicating that TryS, like ODC (spermidine synthesis) and  $\gamma$ GCS (GSH synthesis) is a critical, rate-limiting step in T[SH]<sub>2</sub> biosynthesis in highly resistant lines. Although there was no apparent association between Sb(III) tolerance and the levels of TryX or the recently characterized TDPX (19, 30), levels of TryP were elevated 2.8-fold and 8.9-fold (as determined by densitometry) in the revertant and resistant lysates, respectively. Overexpression of TryP in the revertant and resistant parasites correlates well with the fourfold and eightfold increases in peroxidase activity (Table 2 and Fig. 1).

**Western analysis of Sb(III)-sensitive and -resistant *L. tarentolae* subcellular fractions.** TryP localizes to both the mitochondria and cytosol of *Leishmania* parasites (4). To determine whether overexpression of TryP in a specific cellular location was associated with Sb(III) resistance, crude subcellular fractions of both wild-type and resistant *L. tarentolae* were prepared and immunoblots of each fraction were probed with an *L. major* TryP-specific polyclonal antiserum known to cross-react with both cytosolic and mitochondrial enzymes (Fig. 3). The purity of each fraction was demonstrated using antibodies against marker proteins in each subcellular location. The endoplasmic reticulum-localized BiP protein (immunoglobulin heavy chain-binding protein) was used as a marker for the microsomal fraction, while Hsp60 and TryS were used as marker proteins for the large granular and cytosolic fractions, respectively (27). As expected, TryP was found in both the cytosolic and large granular fractions of both cell lines. The levels of mitochondrial TryP were marginally higher in resistant than in wild-type *L. tarentolae* (1.8-fold); however, cytosolic TryP levels were considerably higher in the Sb(III)-resistant line (6.5-fold). Specific overexpression of cytosolic TryP was also observed in revertant parasites (data not shown). These data suggest that overexpression of a TryP localized to the cytosol of *L. tarentolae* is associated with Sb(III) resistance. Interestingly, Southern analysis of genomic DNA isolated from wild-type, revertant, and resistant cells confirmed that in-

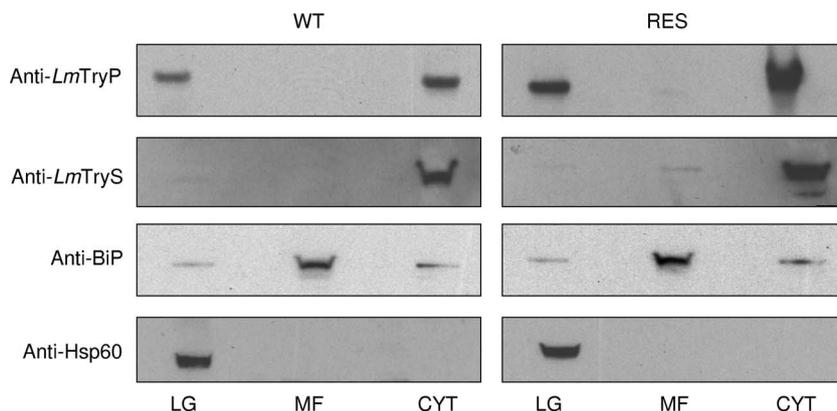


FIG. 3. Cellular location of elevated tryparedoxin peroxidase. Subcellular fractions of *L. tarentolae* promastigotes containing the large-granule (LG), microsomal (MF), and cytosolic (C) fractions were prepared as described in Materials and Methods. Western blots of these equally loaded fractions (30 µg of protein in each lane) were probed with antiserum to *L. major* tryparedoxin peroxidase (type I). In addition, blots were stripped and reprobed with antiserum to marker proteins for each subcellular fraction to demonstrate the purity of each fraction (LG, anti-Hsp60; C, anti-*L. major* T[SH]<sub>2</sub> synthetase; MF, anti BIP).

creased TryP expression was due to gene amplification (data not shown).

An association between TryP and heavy metal resistance has previously been established. In recent studies, Lin and colleagues described the concomitant overexpression of cytosolic and mitochondrial TryP in laboratory-generated, arsenite-resistant *Leishmania amazonensis* (17, 21). Arsenite-resistant *Leishmania* parasites are often found to be cross-resistant to Sb(III), and the mechanisms of resistance to both metalloids are believed to share many common features (12, 14).

**Overexpression of recombinant tryparedoxin peroxidase.**

To confirm the role of TryP in Sb(III) resistance, the cytosolic enzyme from *L. major* was overexpressed in wild-type (TarII), Sb(III)-sensitive *L. tarentolae*. Western analysis of parasites transfected with *L. major* TryP revealed an approximately 2.2-fold increase in TryP at the protein level compared with wild-type parasites and those transfected with the pIR1SAT vector alone (Table 3). The overexpression of TryP was further confirmed when metabolism of H<sub>2</sub>O<sub>2</sub> in crude lysates of these pIR1SAT-*Lm*TryP cloned transfectants (8.3 nmol min<sup>-1</sup> mg<sup>-1</sup>) was found to be approximately twofold higher than that in the vector-only control (4.3 nmol min<sup>-1</sup> mg<sup>-1</sup>). Most importantly, the TryP-overexpressing *L. tarentolae* cell line was

found to be significantly more resistant to Sb(III) (EC<sub>50</sub>, 22.7 ± 0.8 µg ml<sup>-1</sup>; *P* < 0.001) than either the wild-type cell line (EC<sub>50</sub>, 7.3 ± 0.4 µg ml<sup>-1</sup>) or promastigotes containing the empty vector (EC<sub>50</sub>, 9.24 ± 0.4 µg ml<sup>-1</sup>) (Table 3).

**Overexpression of an inactive tryparedoxin peroxidase.** In previous studies, Flöhe and colleagues have demonstrated that a highly conserved arginine residue at position 128 of both the *Crithidia fasciculata* (24) and *L. donovani* (8) TryPs is essential for peroxidase activity. In both organisms, replacement of R128 with an acidic residue resulted in a correctly folded TryP devoid of activity. To determine whether peroxidase activity itself is central to the Sb(III) resistance conferred by *L. major* TryP overexpression, a pIRSAT-*Lm*TryP(R128D) construct was generated and transfected into wild-type *L. tarentolae*. Western analysis of parasites expressing *L. major* TryP(R128D) revealed an approxi-

TABLE 3. Comparison of Sb(III) sensitivity and tryparedoxin peroxidase levels in wild-type, revertant, and transgenic *L. tarentolae* cell lines

Cell Line	Vector	Relative protein level <sup>a</sup>	Mean ± SD	
			Peroxidase activity (nmol min <sup>-1</sup> mg <sup>-1</sup> )	EC <sub>50</sub> (µg ml <sup>-1</sup> )
Wild type	None	1	3.5 ± 0.4	7.3 ± 0.4
	pIR1SAT	1	4.3 ± 0.2	9.3 ± 0.4
	pIR1SAT- <i>Lm</i> TryP	2.2	8.3 ± 0.6 <sup>b</sup>	22.7 ± 0.8 <sup>b</sup>
	pIR1SAT- <i>Lm</i> TryP (R128D)	3.3	4.4 ± 0.1	8.3 ± 0.5
	Revertant	None	2.8	14.4 ± 0.9 <sup>b</sup>

<sup>a</sup> As determined by densitometry of Western blots and compared to wild-type level.

<sup>b</sup> *P* < 0.0001 compared to wild-type value.

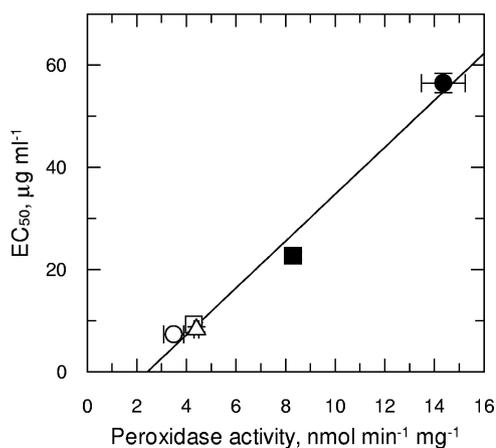


FIG. 4. Analysis of EC<sub>50</sub> values for Sb(III) against TryP activity. Peroxidase activities (H<sub>2</sub>O<sub>2</sub>) from the cell lysates of wild-type (open circles), REV (closed circles), vector-only control (open squares), TryP-overexpressing (closed squares), and inactive TryP(R128D)-overexpressing (open triangles) cloned cell lines were plotted against the EC<sub>50</sub>s for Sb(III). A linear regression was fitted to these data with a correlation coefficient of 0.99. Data are the mean of triplicate measurements ± standard deviations.

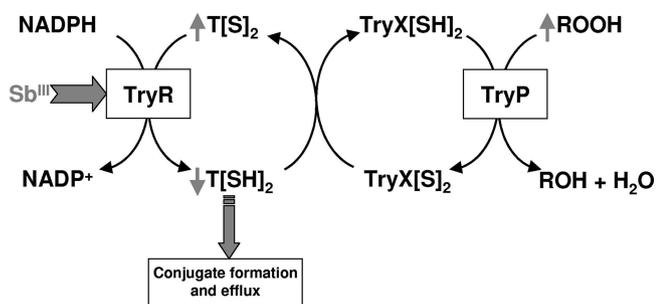


FIG. 5. Model for the mode of action of Sb(III) on *Leishmania*. Sb(III) is known to attack the thiol metabolism of *Leishmania* parasites in two distinct ways (41). First, Sb(III) inhibits TryR in intact cells, resulting in accumulation of the disulfide forms of T[S]<sub>2</sub>. Second, Sb(III) decreases thiol buffering capacity by inducing rapid efflux of intracellular T[SH]<sub>2</sub>. These two mechanisms combine to profoundly compromise the thiol redox potential in drug-sensitive parasites and lead to the accumulation of ROS (23).

mately 3.3-fold increase in TryP at the protein level, in keeping with the levels of overexpression seen in the pIR1SAT-*L. major* TryP transfectants (Table 3). However, in this instance *L. major* TryP(R128D) overexpression was not accompanied by a concomitant increase in the metabolism of H<sub>2</sub>O<sub>2</sub> (4.45 nmol min<sup>-1</sup> mg<sup>-1</sup>) or by an increase in Sb(III) tolerance (EC<sub>50</sub>, 8.3 ± 0.5 μg ml<sup>-1</sup>).

TryP activity (H<sub>2</sub>O<sub>2</sub>) in these cloned, transgenic lines and also in the parental wild-type and revertant lines was found to directly correlate with Sb(III) resistance, with a correlation coefficient of 0.99 (Fig. 4). The direct correlation between peroxidase activity and resistance is of particular interest, since it suggests that TryP overexpression may be the principal resistance determinant in the revertant cell line. Not surprisingly, TryP activity did not directly correlate with Sb(III) resistance in the highly resistant Sb1.1 line, since resistance in this cell line is known to be multifactorial (12). These findings confirm that an enhanced ability to metabolize specific peroxide substrates plays a direct and quantifiable role in the mechanisms of Sb(III) resistance in these *Leishmania* parasites.

## DISCUSSION

The relevance of elevated intracellular thiol levels, due to overexpression of γGCS and ODC, and an increased efflux of thiol-Sb(III) conjugates, due to the overexpression of PgpA, in high-level resistance is well defined (12, 25). However, the involvement of TryP in resistance has not been reported previously. The data presented here establish for the first time that overexpression of an active TryP directly contributes to the multifactorial Sb(III) resistance mechanisms in *Leishmania tarentolae*. Our observations with Sb(III)-resistant *L. tarentolae* are broadly supported by the recent studies of Hsu and colleagues, who reported the overexpression of several components of both the cytosolic and mitochondrial trypanothione pathways in laboratory-generated, arsenite-resistant *Leishmania amazonensis* (17, 21). TryPs form the backbone of parasite antioxidant defenses. In our previous studies we have demonstrated that Sb(III) has pleiotropic effects on Sb T[SH]<sub>2</sub> metabolism which severely compromise thiol redox homeostasis within Sb(III)-sensitive parasites, leading to accumulation of

reactive oxygen species (ROS) (Fig. 5) (23, 41). Thus, inhibition of TryR, depletion of intracellular T[SH]<sub>2</sub>, and inhibition of TryP, alone or in combination, could account for the increased levels of ROS.

Overexpression of TryP could confer resistance to Sb(III) by two mechanisms: by sequestration of Sb(III) to this abundant protein (1 to 4% of the total protein in *L. major* [19]) or by increased enzyme activity to reduce levels of ROS induced by exposure to Sb(III). The former mechanism can be excluded, since overexpression of a mutant TryP devoid of peroxidase activity but maintaining a full complement of cysteine residues failed to provide protection from Sb(III). Thus, peroxidase activity of TryP is essential to confer resistance in these cell lines. Preliminary studies indicate that Sb(III) binds to TryP sulfhydryl groups as well as the other components of the trypanothione peroxidase system (TryR, T[SH]<sub>2</sub>, and TryX), but we have yet to establish whether TryP is a specific target for antimonials (S. Wyllie, J. König, and A. H. Fairlamb, unpublished data). Although TryP has not yet been shown to be essential in *Leishmania* parasites, RNA interference studies have demonstrated that it is essential for the viability of bloodstream *T. brucei* (40). Further studies should clarify these possibilities.

The use of *Leishmania tarentolae* is open to the criticism that it is not a relevant model for clinically observed antimony resistance. However, elevated thiol levels and amplification of γGCS and PgpA have been identified in clinical isolates (25, 33). In preliminary studies with some of these Sb-resistant isolates, we have noted elevated levels of trypanothione peroxidase (S. Wyllie, G. Mandal, M. Chatterjee, and A. H. Fairlamb, unpublished data). Collectively, these data suggest that enhanced antioxidant defenses, through overexpression of TryP, may well be a key feature of mechanisms of clinical resistance to antimonial drugs.

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