Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 10383–10387, September 1996
Medical Sciences

Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*

(arsenite/trypanothione/Pentostam/drug resistance/transport ATPase)

RITA MUKHOPADHYAY*, SAIBAL DEY*, NAXING XU†, DOUGLAS GAGE†, JAMES LIGHTBODY*, MARC OUELLETTE‡, AND BARRY P. ROSEN*§

*Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, MI 48201; †Department of Biochemistry, Michigan State University, East Lansing, MI 48824; and ‡Service d'Infectiologie Centre de Recherche du Centre Hospitalier de l'Université Laval and Département de Microbiologie, Faculté de Médecine, Université Laval, QC Canada, G1V 4G2

Communicated by H. Ronald Kaback, University of California, Los Angeles, CA, June 18, 1996 (received for review April 4, 1996)

ABSTRACT ***Leishmania* resistant to arsenicals and antimonials extrude arsenite. Previous results of arsenite uptake into plasma membrane-enriched vesicles suggested that the transported species is a thiol adduct of arsenite. In this paper, we demonstrate that promastigotes of arsenite-resistant *Leishmania tarentolae* have increased levels of intracellular thiols. High-pressure liquid chromatography of the total thiols showed that a single peak of material was elevated almost 40-fold. The major species in this peak was identified by matrix-assisted laser desorption/ionization mass spectrometry as N^1,N^8-bis-(glutathionyl)spermidine (trypanothione). The trypanothione adduct of arsenite was effectively transported by the As-thiol pump. No difference in pump activity was observed in wild type and mutants. A model for drug resistance is proposed in which Sb(V)/As(V)-containing compounds, including the antileishmanial drug Pentostam, are reduced intracellularly to Sb(III)/As(III), conjugated to trypanothione, and extruded by the As-thiol pump. The rate-limiting step in resistance is proposed to be formation of the metalloid-thiol pump substrates, so that increased synthesis of trypanothione produces resistance. Increased synthesis of the substrate rather than an increase in the number of pump molecules is a novel mechanism for drug resistance.**

The trypanosomatid protozoan parasite *Leishmania* is the causative agent of kala azar and other less severe forms of leishmaniasis (1). Between 10 and 15 million people worldwide have clinical symptoms, and 400,000 new cases are diagnosed each year (2). The treatment of choice for all forms of leishmaniasis depends on Sb(V)-containing drugs such as sodium stibogluconate (Pentostam) and *N*-methylglucamine (Glucantime). Unresponsiveness to antimonial drugs in mucocutaneous and visceral leishmaniasis has long been recognized and is now becoming a common problem, occurring in 5% of patients. Resistance rates as high as 70% have been described in some endemic areas (3).

To serve as models for resistance, strains of *Leishmania tarentolae* resistant to trivalent arsenicals and antimonials have been generated *in vitro*. The strains used in this study were the parental TarII wild type and two mutants, TarIIAs20.3 and TarIIAs50.1 (4). The mutants were independently selected in a stepwise selection *in vitro* for resistance to 20 and 50 μM sodium arsenite, respectively. They are crossresistant to potassium antimonial tartrate [Sb(III)] and Pentostam [Sb(V)] (5, 6). We have shown previously that high level arsenical resistance in promastigotes of the mutants is related to reduced intracellular accumulation of the drugs (5). We have recently identified an ATP-dependent As(III)-thiol pump in vesicles from leishmanial promastigotes enriched in plasma membranes, suggesting that *in vivo* it is not free arsenite but rather the thiol adducts that are extruded (7). The Pentostam–glutathione complex inhibited ^{73}As(III)-glutathione transport, pointing to a single transport system that is involved in extrusion of and resistance to both arsenite and Pentostam. Based on those results, we suggested that resistance entailed formation of a complex of As(III) or Sb(III) with a thiol that the results in this report show to be N^1,N^8-bis-(glutathionyl)-spermidine (trypanothione) [$T(SH)_2$], the major reduced thiol of trypanosomatidae (8). The ATP-coupled efflux pump would then extrude the As(III)/Sb(III)-$T(S)_2$ conjugate from the cell. Thus, both conjugation and extrusion would be required for resistance.

Although resistant cells accumulate less arsenite than wild-type cells as a result of active extrusion (5), *in vitro* no difference was found in rate of $As(GS)_3$ accumulation in everted plasma membrane-enriched vesicles prepared from wild-type cells and from arsenite-resistant mutants (7). We therefore predicted that the limiting step in efflux *in vivo* would be formation of the As(III)–thiol complex, the substrate of the pump, rather than overexpression of the pump genes. While amplification of the genes for drug resistance pumps such as P-glycoprotein has been observed (9, 10), an efflux-related resistance resulting from increased synthesis of the substrate of the pump would be a novel mechanism. In this paper, the physiological thiol involved in arsenical extrusion was determined to be trypanothione. In addition, As(III)-resistant mutants overproduced trypanothione as much as 40-fold. These results are consistent with our hypothesis that formation of the substrate of the pump is rate-limiting for resistance in *Leishmania*.

MATERIALS AND METHODS

Cell Line and Culture. The cell lines used in this study are the wild-type *L. tarentolae* (TarII WT) (11) and the sodium arsenite-resistant mutants TarII As20.3 and TarII As50.1, selected stepwise for resistance to 20 and 50 μM sodium arsenite, respectively (4). Promastigotes were grown in SDM-79 medium (12) supplemented with 10% fetal bovine serum (GIBCO/BRL) at 29°C with gentle shaking. For preparation of plasma membrane vesicles, cells were grown up to 5–6 × 10^7 cells per ml, as determined by counting in a hemocytometer.

Preparation of Plasma Membrane Vesicles. Membranes vesicles were prepared from promastigotes of *L. tarentolae* as described previously (7). These preparations have been re-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: As(III), trivalent arsenic; As(V), pentavalent arsenic; Sb(III), trivalent antimony; Sb(V), pentavalent antimony; $T(SH)_2$, reduced trypanothione; $T(S)_2$, oxidized trypanothione; $As(GS)_3$, As(III)-glutathione conjugate; $AsT(S)_3$, As(III)-trypanothione conjugate.

§To whom reprint requests should be addressed.

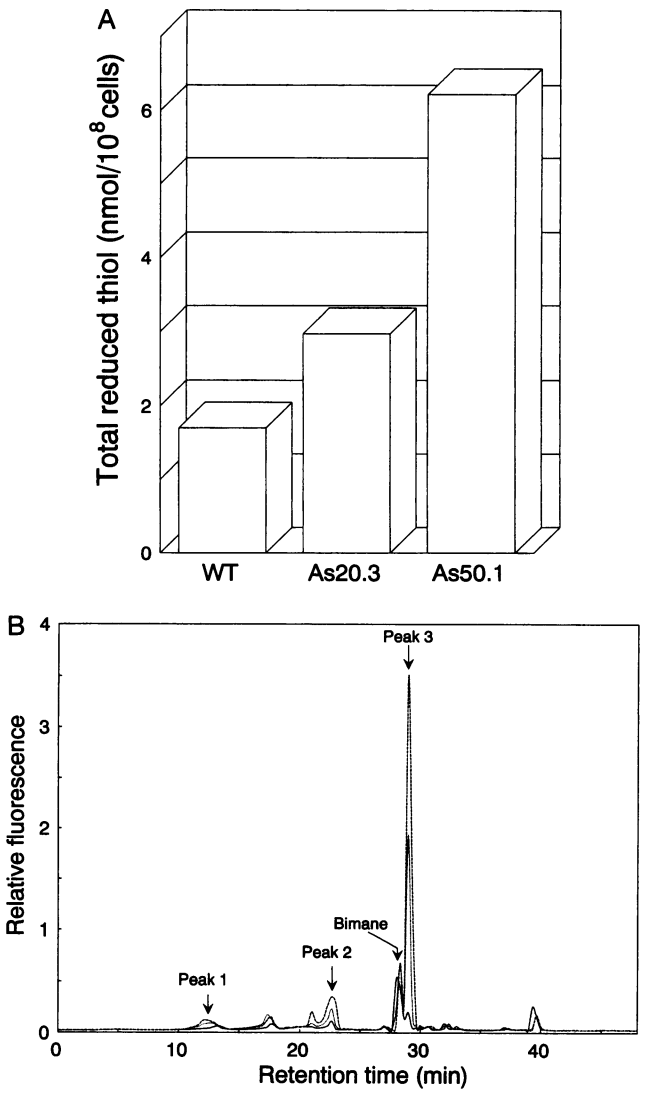

FIG. 1. Overproduction of intracellular thiols in arsenite-resistant *L. tarentolae*. (*A*) Reduced intracellular thiol content in wild-type and arsenite-resistant promastigotes were quantified with DTNB. Each experimental data represents the mean of three independent experiments with duplicate parallel cultures. (*B*) Separation of reduced intracellular thiols. Samples were analyzed by HPLC from TarII wild type (solid line), As20.3 (dotted line), and As50.1 (dashed line). Retention times of the standards are indicated.

ported to contain primarily plasma membrane vesicles free from mitochondrial contamination, although a small amount of endoplasmic reticulum could not be excluded (13, 14). Membrane vesicles were rapidly frozen in liquid nitrogen in small aliquots and stored at −80°C until use. Protein content was determined by a micromodification of the procedure of Lowry *et al.* (15).

Chemical Synthesis of $^{73}AsO_2^-$, $^{73}As(GS)_3$, and $^{73}AsT(S)_2$. $^{73}AsO_2^-$ was prepared by chemical reduction of arsenate (16). The $^{73}As(GS)_3$ conjugate was synthesized according to the method of Delnomdedieu *et al.* (17) using a 4-fold molar excess of reduced glutathione. $^{73}As(TS)_2$ was prepared by a similar method using an equimolar concentration of dihydrotrypanothione.

Assay of $^{73}AsT(S)_2$ and $^{73}As(GS)_3$ Transport. ATP-dependent uptake of $^{73}AsT(S)_2$ and $As(GS)_3$ in everted plasma membrane-enriched vesicles was assayed with 10 mM ATP as an energy source as described previously (7).

Determination of Total Cellular Thiol Content. The level of total intracellular thiol was measured in deproteinized cell extracts (18). Cells were grown in 10 ml of arsenite-free SDM-79 medium supplemented with 10% fetal calf serum to mid exponential phase (6×10^7 cells per ml). The cells were harvested, washed with a buffer consisting of 0.14 M sodium phosphate, 0.14 M potassium phosphate, 0.14 M NaCl, and 3 mM KCl (pH 7.4), and suspended in 0.6 ml of 25% tricholoracetic acid. After 10 min on ice, denatured protein and cell debris were removed by centrifugation in a microfuge for 20 min at 4°C. The thiol content of the supernatant solution was determined with 0.6 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in 0.2 M sodium phosphate buffer (pH 8.0). The concentration of DTNB derivatives of thiols was estimated spectrophotometrically at 412 nm.

Separation of Thiols by High-Pressure Liquid Chromatography (HPLC). Samples for analysis by HPLC were prepared from cells derived from 10 ml of culture medium suspended in a buffer consisting of 0.1 ml of 50 mM Hepes (pH 8.0) containing 5 mM EDTA (19, 20). The cells were transferred to a dark tube and 0.1 ml of 2 mM monobromobimane in ethanol was added with mixing, and the suspension was incubated at 70°C for 3 min. The suspension was mixed with 0.2 ml of cold 25% trichloroacetic acid and incubated on ice for 20 min, after which denatured protein and cell debris were removed by centrifugation. Samples were analyzed by HPLC using an ion-paired reversed-phase Synchropack C18 column with a linear gradient of 0–90% methanol in 0.25% acetic acid (pH 3.5). Millimolar solutions of cysteine, glutathione, trypanothione, dithiothreitol (DTT), and lipoic acid derivatized with monobromobimane were used as standards. Thiols were identified from bimane fluorescence with excitation at 360 nm and emission at 450 nm using an on-line fluorescence detector.

Identification of HPLC Peaks by Mass Spectrometry. Matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy was carried out on a Voyager Elite time-of-flight instrument (PerSeptive Biosystems, Cambridge, MA) equipped with a nitrogen laser (337 nm) using α-cyano-4-hydroxycinnamic acid as the matrix. Samples were prepared by adding 1 μl of the sample solutions from the collected HPLC fractions (containing an estimated 5–50 pmol of the trypanothione bimane derivative). Spectra were obtained by averaging 50–100 laser shots.

Reagents. $H_3{}^{73}AsO_4$ was purchased from Los Alamos National Laboratories. Oxidized trypanothione was obtained from Bachem, and was reduced to $T(SH)_2$ with a 2-fold molar excess of tris(2-carboxyethyl)phosphine immediately before

Table 1. Quantification of intracellular cysteine, glutathione, and trypanothione in wild-type and arsenic-resistant promastigotes of *L. tarentolae*

Strain	Cysteine, nmol per 10^8 cells	Fold wild type	Glutathione, nmol per 10^8 cells	Fold wild type	Trypanothione, nmol per 10^8 cells	Fold wild type
Wild type	0.13	1	0.09	1	0.1	1
As20.3	0.34	2.6	0.41	4.6	1.71	17.1
As50.1	0.67	5.2	0.91	10.1	3.69	36.9

Concentrations were determined from the data in Fig. 1*B* using values from known amounts of authentic standards.

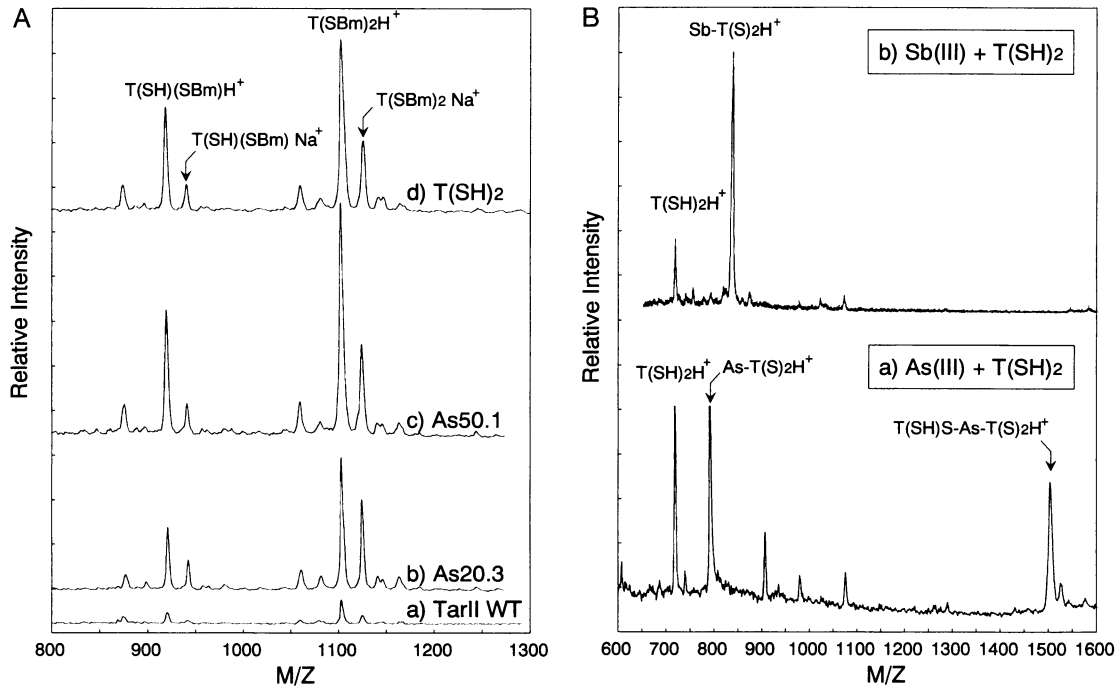

FIG. 2. MALDI mass spectroscopy of the increased thiol peak. (*A*) Mass spectroscopic analysis of HPLC peaks 3 from (*a*) TarII wild type, (*b*) As20.3, (*c*) As50.1, and (*d*) an authentic standard of bimane-derivatized trypanothione. (*B*) Mass spectroscopic analysis of (*a*) As(III) and (*b*) Sb(III) adducts of trypanothione.

use (21). All other chemicals were obtained from commercial sources.

RESULTS

Overproduction of Intracellular Thiols in Arsenite-Resistant *L. tarentolae*. Total intracellular reduced thiol was quantified in the parental TarII wild type and the two mutants, TarIIAs20.3 and TarIIAs50.1. Total reduced thiol levels were increased 1.7-fold in the mutant TarIIAs20.3 and 3.7-fold in TarIIAs50.1 compared with the parental strain (Fig. 1*A*). Individual thiols from each of the three strains were separated and quantified by HPLC after derivatization with monobromobimane. Three peaks contained most of the thiol species (Fig. 1*B*). Peak 1 eluted with a retention time corresponding to the cysteine-bimane adduct. The retention time of peak 2 corresponded to the bimane adduct of glutathione. The trypanothione-bimane and lipoic acid-bimane adducts had retention times corresponding to that of peak 3. However, as shown below, the major component in peak 3 is the bimane derivative of trypanothione, and no lipoic acid derivatives were observed. All three HPLC peaks were increased in the two mutants (Table 1). Peak 3 increased 17-fold in extracts from TarIIAs20.3 and 37-fold from TarIIAs50.1 compared with the parental TarII strain. The compound in peak 3 increased from slightly less than half of the total thiol in the wild type to more than 80% of the total thiol in the mutants.

Analysis of HPLC Peaks by Mass Spectrometry. Analysis of the HPLC peak 3 from wild type and mutants by MALDI mass spectrometry showed the presence of trypanothione-bimane derivatives in each (Fig. 2*A*, curves a–c). Peaks at m/z of 1105.5 and 1127.6 correspond to the mass of proton and sodium ion adducts of T(S-bimane)$_2$, respectively. Peaks at m/z of 915.0 and 937.3 are the mass of protonated and sodium ion species, respectively, in which one bimane had been lost. It is unlikely that mono- and di-substituted bimane species coeluted on HPLC and probably arose from post-HPLC sample processing. Although quantification of the peaks must be considered tentative because different complexes can exhibit differential MALDI responses, the ratio of the intensities of the signals for the protonated molecule correspond to the ratio of the peak areas in the HPLC analysis of the three samples (greatest in TarIIAs50.1 and smallest in the parental). The structure of T(S-bimane)$_2$ was confirmed by post-source decay analysis

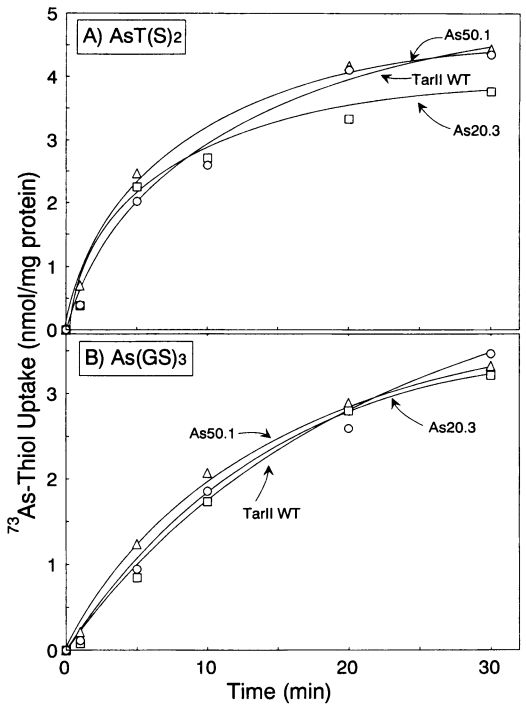

FIG. 3. ATP-dependent uptake of (*A*) ^{73}AsT(S)$_2$ and (*B*) ^{73}As(GS)$_3$ in everted plasma membrane-enriched vesicles of *L. tarentolae* promastigotes. Vesicles were prepared and transport assayed with 0.1 mM ^{73}AsT(S)$_2$ or ^{73}As(GS)$_3$ and 10 mM ATP as energy source. The values at each time were corrected for nonspecific binding by subtraction of the values obtained with 10 mM AMP. Vesicles were prepared from promastigotes of TarII wild type (●), As20.3 (■), and As50.1 (▲).

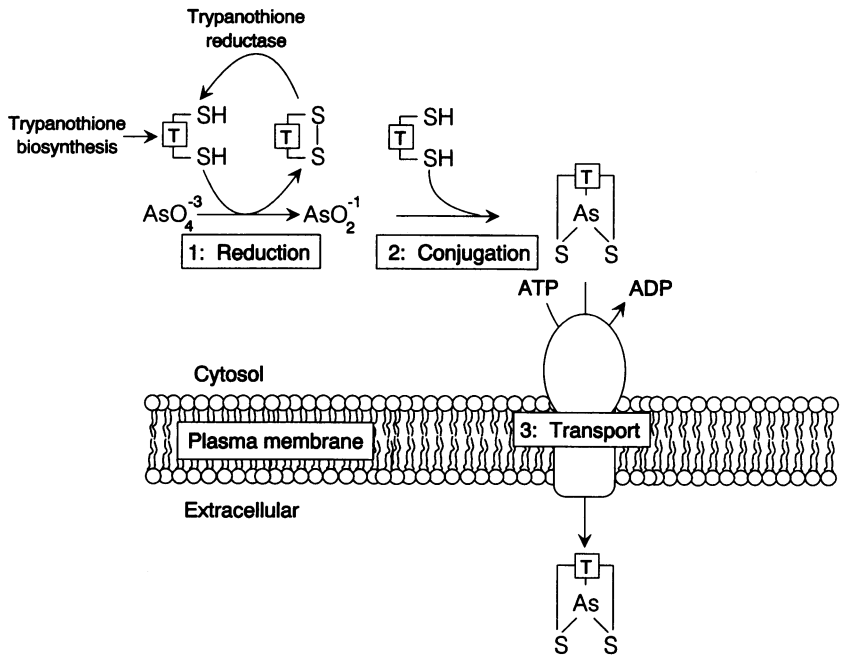

FIG. 4. Model for arsenical/antimonial resistance in *Leishmania*. The first step is reduction of Sb(V)/As(V) to Sb(III)/As(III) by trypanothione. Although it is not known whether this process is enzymatic, As(V) reductases from bacteria have been characterized (29, 30). The second step is conjugation of the reduced semimetal to trypanothione. Slow spontaneous formation of $As(GS)_3$ from arsenate and glutathione has been observed (17). As a diglutathione, trypanothione would be expected to react similarly. On the other hand, arsenite resistance in mammalian cells has been correlated with increased levels of glutathione *S*-transferase π (24), so it would not be unreasonable to postulate the existence of a trypanothione-conjugating enzyme. It is also possible that a conjugating enzyme exists for a thiol other than trypanothione; thus, even though trypanothione comprises over 70% of the total free thiol in resistant cells (Table 1), it might not necessarily be the As(III) conjugant. The final step in resistance is active extrusion by the ATP-coupled As-thiol pump (7), lowering the intracellular concentration of metalloid to subtoxic levels. The rate-limiting step in resistance is proposed to be formation of the As-trypanothione conjugate. Selection for mutations that increases trypanothione biosynthesis thus generates resistance.

(22) of the peak representing the intact molecule at m/z 1105.5. The fragmentation pattern of the trypanothione derivatives from the biological samples was identical to that of an authentic standard of bimane-derivatized trypanothione (data not shown). No signal corresponding to bimane derivatives of lipoic acid were observed in any samples. These results unambiguously identify the thiol increased in arsenite-resistant mutants of *L. tarentolae* as trypanothione.

Analysis of As(III)- and SB(III)-Trypanothione Complexes by Mass Spectrometry. Authentic As(III)- and Sb(III)-trypanothione complexes were analyzed by MALDI mass spectrometry. The reaction products between reduced trypanothione and arsenite or antimonite displayed a prominent peaks at m/z of 798.6 and 844.5, respectively, representing the 1:1 $AsT(S)_2$ and $SbT(S)_2$ complexes, respectively (Fig. 2*B*). A smaller peak in the As–trypanothione mixture was observed at m/z of 1521.5 that could be assigned to $T(S)_2AsT(SSH)$. Because higher m/z complexes may exhibit differential MALDI responses, quantitative assessments of the relative amounts of the two species cannot be conclusive. However, no peaks of higher m/z were observed with the Sb-trypanothione mixture. These results indicate that $AsT(S)_2$ and $SbT(S)_2$ are the predominant species present in solution.

ATP-Dependent Transport of ^{73}As–Glutathione/Trypanothione Complexes in Plasma Membrane-Enriched Vesicles. ATP-dependent accumulation of $^{73}AsT(S)_2$ and $^{73}As(GS)_3$ by plasma membrane-enriched vesicles of wild type and resistant cells was measured (Fig. 3). The transport activities of the wild type and mutants with either substrate were not significantly different from each other (Fig. 3). With $^{73}AsT(S)_2$ as substrate, the kinetics of uptake of vesicles prepared from the three strains were found to be similar (data not shown). These results indicate that resistance is due neither to a change in the number of pump molecules nor in their affinity for substrate. Moreover, transport of both $^{73}AsT(S)_2$ and $73As(GS)_3$ was similar, indicating that the pump uses the trypanothione adduct of arsenite as efficiently as the glutathione adduct.

DISCUSSION

Drug resistance mediated by active efflux is widespread from prokaryotes to eukaryotes. Resistance to the metalloids arsenic and antimony in *Escherichia coli* is conferred by a plasmid-encoded ATP coupled pump (10). Multidrug resistance phenotype in mammalian cells is often correlated with the overexpression of P-glycoprotein (Pgp) or multidrug resistance-associated protein (9, 23). In eukaryotes, arsenical resistance has also been shown to be transport-mediated (9, 10, 24–26). We have previously shown that oxyanion-resistant *Leishmania* cell lines actively extrude As(III) by a process that is independent of PgpA (5). In addition, we recently characterized an ATP-dependent As(III)-glutathione transport system in plasma membrane-enriched vesicles of *L. tarentolae* (7). In contrast, the amplification of pump genes frequently observed in drug-resistant mammalian cell lines (9, 10, 23), this ATP-driven pump is not overexpressed in the As(III)-resistant mutants.

On the other hand, the promastigotes of the As(III)-resistant mutants of *L. tarentolae* have higher total reduced thiol levels compared with the parental strain (Fig. 1). Cysteine, glutathione, and trypanothione are all increased in the mutants, but the large majority of the increase could be accounted for increased trypanothione levels (Table 1). In contrast, in melarsen-resistant *Trypanosoma brucei brucei* trypanothione levels are not increased; activity of the uptake system for the organic arsenical is reduced instead (27, 28). Trypanothione is known to play a major role in the oxidant defense system of the kinetoplastida group and is a major thiol

species in this group of parasitic protozoa. Trypanothione forms a complex with As(III) and Sb(III) (Fig. 2), and this species was transported by the plasma membrane-enriched vesicles as efficiently as $AS(GS)_3$ (Fig. 3). Since more than 70% of the total thiol in promastigotes of TarII As50.1 is trypanothione, $AsT(S)_2$ would comprise the bulk of the substrate of the pump, indicating that it is most likely the natural substrate of the pump *in vivo*. The data suggest that the increase in resistance results from increased biosynthesis of trypanothione. Since the levels of glutathione and cysteine are also increased, this would point to an early step in the biosynthetic pathway.

We propose a model for Pentostam resistance with a minimum of three steps: (*i*) reduction of Sb(V) to Sb(III), (*ii*) conjugation to trypanothione, and (*iii*) extrusion of $SbT(S)_3$ by the ATP-coupled pump (Fig. 4). We cannot rule out the possibility that conjugation of As(III) or Sb(III) alone is sufficient to produce resistance; disruption of the thus far unidentified gene for the pump would be necessary to test that. However, several other glutathione-linked pumps have been shown to be related to drug and metal resistances, including the human multidrug resistance-associate protein, which is an ATP-dependent export pump for Leukotriene C_4 (23, 31, 32) and structurally related conjugates and the yeast YCF1 protein, a vacuolar cadmium-glutathione conjugate pump in yeast (33). The model points to several targets for new chemotherapeutic agents that could reverse drug resistance. Intervention to lower trypanothione levels might be effective. A more selective target may prove to be the efflux pump if it is present in the parasite and not the host. For that reason, it is of importance to identify the pump protein and the gene(s) that encode it.

This work was supported in part by U. S. Public Health Service Grants AI19793 (B.P.R.) and NCRR00480 (D.G.), which supports in part the Michigan State University–National Institutes of Health Mass Spectrometry Facility, and by a grant from the Medical Research Council of Canada (M.O.). M.O. is a chercheur boursier junior of the Fonds de Recherche en Santé du Québec and a recipient of a New Investigator award from the Burroughs Wellcome Fund.

1. Neva, F. A. & Brown, H. W. (1994) *Basic and Clinical Parasitology* (Appleton and Lange, Norwalk, CT), 6th Ed., pp. 57–106.
2. Ashford, R. W., Desjeux, P. & deRaadt, P. (1992) *Parasitol. Today* **8,** 104–105.
3. Jackson, J. E., Tally, J. D., Ellis, W. Y., Mebrahtu, Y. B., Lawyer, P. G., Were, J. B., Reed, S. G., Panisko, D. M. & Limmer, B. L. (1990) *Am. J. Trop. Med. Hyg.* **43,** 464–480.
4. Ouellette, M., Hettema, E., Wust, D., Fase-Fowler, F. & Borst, P. (1991) *EMBO J.* **10,** 1009–1016.
5. Dey, S., Papadopoulou, B., Roy, G., Grondin, K., Dou, D., Rosen, B. P. & Ouellette, M. (1994) *Mol. Biol. Parasitol.* **67,** 49–57.
6. Papadopoulou, B., Roy, G., Dey, S., Rosen, B. P. & Ouellette, M. (1994) *J. Biol. Chem.* **296,** 11980–11986.
7. Dey, S., Ouellette, M., Lightbody, J., Papadopoulou, B. & Rosen, B. P.(1996) *Proc. Natl. Acad. Sci. USA* **93,** 2192–2197.
8. Fairlamb, A. H. & Cerami, A. (1992) *Annu. Rev. Microbiol.* **46,** 695–729.
9. Gottesman, M. M. & Pastan, I. (1993) *Annu. Rev. Biochem.* **62,** 385–427.
10. Dey, S. & Rosen, B. P. (1995) in *Drug Transport in Antimicrobial and Anticancer Chemotherapy*, ed. Georgopapadakou, N. H. (Dekker, New York), pp. 103–132.
11. Urbina, J. A., Vivas, J., Ramos, H., Larralde, G., Auilar, Z. & Avilan, L. (1988) *Mol. Biochem. Parasitol.* **30,** 185–196.
12. White, T. C., Fase-Fowler, F., van Luenen, H., Calafat, J. & Borst, P. (1988) *J. Biol. Chem.* **263,** 16977–16983.
13. Benaim, G. & Romero, P. J. (1990) *Biochim. Biophys. Acta* **1027,** 79–84.
14. Cohen, B. E., Ramos, H., Gamargo, M. & Urbina, J. (1986) *Biochim. Biophys. Acta* **860,** 57–65.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
16. Reay, P. F. & Asher, C. J. (1977) *Anal. Biochem.* **78,** 557–560.
17. Delnomdedieu, M., Basti, M. M., Otvos, J. D. & Thomas, D. J. (1994) *Chem. Biol. Interact.* **90,** 139–155.
18. Moron, M. S., Depierre, J. W. & Mannervik, B. (1979) *Biochim. Biophys. Acta* **582,** 67–78.
19. Fahey, R. C. & Newton, G. L. (1987) *Methods Enzymol.* **143,** 85–96.
20. Fairlamb, A. H., Henderson, G. B., Bacchi, C. J. & Cerami, A. (1987) *Mol. Biochem. Parasitol.* **24,** 185–191.
21. Burns, J. A., Butler, J. C., Moran, J. & Whitesides, G. M. (1991) *J. Org. Chem.* **56,** 2648–2650.
22. Kaufmann, K., Kirsch, D. & Spengler, B. (1994) *Int. J. Mass Spectrom. Ion Processes* **131,** 355–385.
23. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deeley, R. G. & Keppler, D. (1994) *J. Biol. Chem.* **269,** 27807–27810.
24. Wang, H. F. & Lee, T. C. (1993) *Biochem. Biophys. Res. Commun.* **192,** 1093–1099.
25. Singh, A. K, Liu, H. Y. & Lee. S. T. (1994) *Mol. Biochem. Parasitol.* **66,** 161–164.
26. Wang, Z., Dey, S., Rosen, B. P. & Rossman, T. G. (1996) *Toxicol. Appl. Pharmacol.* **137,** 112–119.
27. Fairlamb, A. H., Carter, N. S., Cunningham, M. & Smith, K. (1992) *Mol. Biochem. Parasitol.* **53,** 213–222.
28. Carter, N. S. & Fairlamb, A. H. (1993) *Nature (London)* **361,** 173–176.
29. Ji, G. & Silver, S. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9474–9478.
30. Gladysheva, T. B., Oden, K. L. & Rosen, B. P. (1994) *Biochemistry* **33,** 7287–7293.
31. Jedlitschky, G., Leier, I., Buchholz, U., Center, M. & Keppler, D. (1994) *Cancer Res.* **54,** 4833–4836.
32. Müller, M., Meijer, C., Zaman, G. J. R., Borst, P., Scheper, R. J., Mulder, N. H., deVries, E. G. E. & Jansen, P. L. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 13033–13037.
33. Li, Z, Szczypka, M., Lu, Y., Thiele, D. J. & Rea, P. A. (1996) *J. Biol. Chem.* **271,** 6509–6517.