Leishmania major LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug Pentostam

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Abbreviations:  GSH, reduced glutathione; DTT, dithiolthreitol; PCR, polymerase
chain reaction; Grx, glutaredoxin; HPLC, high pressure liquid chromatography; ICP-MS, inductively coupled plasma mass spectroscopy.
Abstract

Arsenicals and antimonials are first-line drugs for the treatment of trypanosomal and leishmanial diseases. To create the active form of the drug, Sb(V) must be reduced to Sb(III). Since arsenic and antimony are related metalloids, and arsenical-resistant Leishmania strains are frequently cross-resistant to antimonials, we considered the possibility that Sb(V) is reduced by a leishmanial As(V) reductase. The sequence for the arsenate reductase of Saccharomyces cerevisiae, ScAcr2p, was used to clone the gene for a homologue, LmACR2, from Leishmania major. LmACR2 was able to complement the arsenate sensitive phenotype of an arsC deletion strain of Escherichia coli or a ScACR2 deletion strain of Saccharomyces cerevisiae. Transfection of Leishmania infantum with LmACR2 augmented Pentostam sensitivity in intracellular amastigotes. LmACR2 was purified and shown to reduce both As(V) and Sb(V). This is the first report of an enzyme that confers Pentostam sensitivity in intracellular amasitoges of Leishmania. We propose that LmACR2 is responsible for reduction of the pentavalent antimony in Pentostam to the active trivalent form of the drug in Leishmania.
Introduction

*Leishmania* species are distributed worldwide. During its life cycle, the parasite goes through two developmental stages. The promastigote form of the parasite resides in the intestinal tract of the insect vector. The amastigote form of the parasite resides in macrophages and other mononuclear phagocytes in the mammalian host. Between 10 and 15 million people have clinical symptoms of leishmaniasis, and 400,000 new cases are diagnosed each year. Increases in personal and business travel and intervention in regional conflicts such as the last two Iraqi wars have resulted in a significant rise in cases of *Leishmania* in the United States (1).

The pentavalent antimonial drugs Pentostam and Glucantime are the first-line treatment for leishmaniasis, and resistance to those drugs is a serious clinical problem (2,3). To create the active form of the drug, Sb(V) is first reduced to Sb(III) (4). Reduction occurs preferentially in the amastigotes, which are more sensitive to Pentostam than are promastigotes (5,6). Arsenic and antimony have similar properties and are transported by the same channels (7,8), carriers (9,10) and pumps (11,12). We propose that at least a portion of the Sb(V) in Pentostam is reduced by a leishmanial As(V) reductase. Arsenate reductases are ubiquitous in prokaryotes and archaea and are essential for conferring resistance to arsenate (13). In these organisms arsenate reductase enzymes apparently arose independently at least
twice. Recently a *L. donovani* enzyme related to glutathione S-transferases has been shown to catalyze GSH-dependent reduction of arsenate and antimonate *in vitro* (14). While this protein is a reasonable candidate for a Pentostam reducing enzyme, there are no data that directly demonstrate a relationship of this enzyme and drug sensitivity in *Leishmania* amastigotes.

In contrast, the ScAcr2p arsenate reductase from *Saccharomyces cerevisiae* reduces arsenate to arsenite both *in vivo* and *in vitro* (15-18). ScAcr2p is homologous to the Cdc25a cell cycle protein tyrosine phosphatase (19) and to rhodanese, a thiosulfate sulfurtransferase (20). ScAcr2p uses GSH and glutaredoxin as cofactors (17). Because of its relatedness to protein phosphatases, it is difficult to identify paralogous eukaryotic proteins solely by homology searches of DNA databases. We used the ScAcr2p sequence to identify a closely related *Leishmania major* sequence. This open reading frame, designated *LmACR2*, was cloned into both yeast and *E. coli* expression vectors. *LmACR2* functionally complements the arsenate sensitive phenotypes of the *ScACR2* deletion of *S. cerevisiae* and an deletion of the *Escherichia coli* *arsC* gene, which encodes a bacterial arsenate reductase. LmACR2 was purified and characterized. The *Leishmania* enzyme requires GSH and glutaredoxin for arsenate reductase activity, with a Km of 6 mM for As(V) and is inhibited by As(III), Sb(III) and phenylarsine oxide. LmACR2 also reduces Sb(V) to Sb(III) with a Km of 7 µM. To demonstrate a relationship between
LmACR2 and Pentostam activity, \textit{LmACR2} was transfected into \textit{L. infantum} promastigotes. Macrophage-infected amastigotes derived from these transfected cells exhibited increased sensitivity to Pentostam. This is the first demonstration of an enzyme that is involved in and probably required for action of the drug Pentostam in intracellular \textit{Leishmania} amastigotes.

\textbf{Experimental procedures}

\textbf{Reagents.} All reagents were from Sigma-Aldrich. As(V), Sb(V), As(III) and Sb(III) were purchased in the form of sodium arsenate, potassium hexahydroxy antimonate, sodium arsenite and potassium antimonyl tartrate, respectively. DNA manipulation reagents were obtained from Qiagen and Invitrogen.

\textbf{Cloning of LmACR2.} The \textit{Leishmania} Genome Network GeneDB was inspected for a \textit{L. major} homologue of \textit{ScACR2}. The closest to \textit{ScACR2} (Systematic Name: LmjF32.2740) is 384 bp in length and localized in chromosome 32. The full-length gene, designated \textit{LmACR2}, was cloned by polymerase chain reaction (PCR) using 5'-CCATGGCGAACTACACGTACATAAAGCCGG-3' as forward primer and 5'-AAGCTTCACGTACATGAGGTCTGGC-3' as reverse primer. \textit{NcoI} and \textit{HindIII} sites were created at the 5' and 3' end of the gene, respectively, for cloning into pBAD-Myc-HisA (Invitrogen). For cloning into the yeast expression vector pYES2.0 (Invitrogen), \textit{LmACR2} was cloned using 5'
ATGACGAACTACACGTACATAAAGCCGG-3’ as forward primer and 5’-
TCACACGTACATGAGGTCTGGCCGCACGTC-3’ as reverse primer. For cloning
into *Leishmania* expression vector pSPαHYGα, *LmA CR2* was cloned using 5-
TCTAGAATGACGAACTACACGTACATAAAGCCGG-3 as forward primer and
AAGCTTTTCACACGTACATGAGGTCTGGC as reverse primer. Each PCR fragment
was first cloned into pGEM-T (Promega) cloning vector and sequenced (CEQ 2000,
Beckman) to confirm the integrity of the gene. PCR reactions were performed in a
Peltier Thermal Cycler (PTC 200) from MJ Research. The fragments were then
cloned into respective expression vectors, giving rise to LmA CR2-pBADA for
expression in *E. coli*, LmA CR2-pYES for expression in yeast and pSP-LmA CR2-
HYG for expression in *Leishmania*.

**Metalloid sensitivity assays.** Cells of *E. coli* were grown in a low phosphate medium
(21) at 37°C supplemented with 50 to 125 µg/ml ampicillin, as appropriate. The
arsente resistance phenotype of cells expressing *ACR2* was determined in *E. coli*
strains W3110 (wild type) or WC3110 (ΔarsC), as described previously (17). Overnight cultures were diluted 100-fold in low phosphate medium containing various
centration of sodium arsenate and 0.2% arabinose. Growth was estimated from
the absorption at 600 nm after 15 h of growth at 37°C.

In *Saccharomyces cerevisiae* W303-1B and RM1 (15) bearing plasmids
pYES2.0, LmACR2-pYES, or pACR2-3 (ScACR2-pYES) were grown overnight at 30° C in a basal salts medium (15) containing either 2% glucose or galactose supplemented with 0.2 mg/ml each of histidine and/or uracil, as appropriate. Spots of 3 µl of serial dilutions of cultures were then applied onto 1% agar plates with minimal media containing 2 mM sodium arsenate. The plates were incubated at 30° C for four to five days.

*L. infantum* strain strain MHOM/M/67/ITMAP-63, which expresses the firefly luciferase gene (22), was used for susceptibility testing in macrophages. Cells were maintained in SDM79 media (23) supplemented with 10 µM of 6-biopterin at 25° C as promastigotes. Plasmids pSP±HYG± and pSP-LmACR2-HYG were transfected by electroporation of promastigotes. The promastigote parasites were used to infect the human leukemia monocyte cell line THP-1, as described previously (22). THP-1 cells were differentiated with phorbol myristate acetate and infected with *L. infantum* promastigotes at a ratio of 2:1 for 2 hr. Non-internalized parasites were washed away, and infected macrophages were treated with varying concentrations of Pentostam. After 5 days of culture, wells containing adherent differentiated THP-1 cells were washed, and luciferase activity was determined essentially as described using a microtiter plate luminometer (24). Values are expressed as relative light units.
Purification of LmACR2 and glutaredoxins. *E. coli* Grx2 and yeast Grx1p were purified as described previously (17,25). LmACR2 was purified from *E. coli*. Cells of *E. coli* strain TOP10 bearing LmACR2-pBADA were grown in 4 liters of LB medium containing 50 µg/ml ampicillin with shaking at 37 °C. At an A\textsubscript{600 nm} of 0.5, L(+)-arabinose was added to a final concentration of 0.02% as inducer, and the culture was grown for an additional 3 h at 37 °C. The cells were washed once with Buffer A (10 mM Tris-HCl, 0.1 M KCl, pH 7.5), suspended in Buffer B (50 mM MOPS, pH 7.5, containing 20 mM imidazole, 0.5 M NaCl, 10 mM β-mercaptoethanol and 20% glycerol) at a ratio of 5 ml of buffer per g of wet cells, and lysed by a single passage through a French pressure cell at 20,000 p.s.i. Diisopropylfluorophosphate (2.5 µl/g of wet cells) was added to the lysate immediately after lysis. The lysate was centrifuged at 100,000 x g for 60 min at 4 °C, and the supernatant solution was loaded at a flow rate of 0.5 ml/min onto a Ni\textsuperscript{2+}-NTA column pre-equilibrated with Buffer B. The column was then washed with 250 ml of Buffer A followed by elution with 125 ml of Buffer B with the concentration of imidazole increased to 0.2 M. LmACR2 was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Fractions containing purified LmACR2 were pooled and
concentrated. The concentrated protein from the Ni\textsuperscript{2+}-NTA column was applied to a 1.5 cm diameter column filled to 75 cm with Sephacryl S-100 (Amersham Pharmacia Biotech) pre-equilibrated with Buffer C (50 mM MOPS, pH 6.5, containing 0.5 M NaCl, 10 mM β-mercaptoethanol, 20% glycerol, and 0.5 mM EDTA), eluted with buffer C, pooled and concentrated. All purified proteins were stored at -70 °C until use. Protein concentrations were determined from the absorbance at 280 nm using the following extinction coefficients calculated by the method of Gill and von Hippel (26): Grx2, 21,860 M\textsuperscript{-1} cm\textsuperscript{-1}; Grx1p, 5,360 M\textsuperscript{-1} cm\textsuperscript{-1}; LmA CR2, 16,410 M\textsuperscript{-1} cm\textsuperscript{-1}.

**Assay of arsenate reductase activity.** Arsenate reductase activity was assayed at 37 °C using a coupled assay (17). The assay buffer contained 50 mM MOPS, 50 mM MES, pH 6.5, 0.1 mg/ml bovine serum albumin, 0.25 mM NADPH, 5 nM yeast glutathione reductase (Calbiochem), 1 mM GSH, and 50-100 µM LmA CR2. Reduction of 2-hydroxyethyldisulfide was used to ensure that the rate of the coupling system was not rate limiting in the presence of inhibitors. Sodium arsenate, glutaredoxins and inhibitors were added as indicated. Reductase activity was monitored at 340 nm and expressed as nmol NADPH oxidized per mg of LmA CR2.
using a molar extinction coefficient of 6,200 for NADPH. The data were analyzed using SigmaPlot 2000. Each assay was repeated at least three times with two separate batches of purified protein.

**Assay of antimonate reductase activity.** Purified LmACR2 (20-40 μM) was incubated with indicated concentrations of sodium hexahydroxy antimonate at 37 °C for the indicated times with the assay buffer used for arsenate reduction, as described above (pH 7.5). The reaction was terminated with addition of an equal volume of 70% HNO₃ and rapidly diluted with HPLC grade water. A ion exchange column PRP-X100 (Hamilton) was used for HPLC separation of Sb(V) and Sb(III) with 20 mM EDTA, pH 4.7 as the mobile phase at a flow rate of 1.2 ml/min (27). Sb(III) most likely elutes after Sb(V) because Sb(V) forms a negatively charged chelate with EDTA. The amount of antimony in each fraction was calculated by ICP-MS using commercial Sb(V) and Sb(III), which were nearly completely free of the other oxidization state. The values were corrected for the amount of Sb(III) produced in the absence of LmACR2, which was usually only a few percent of the Sb(III) produced with enzyme. No more than 10% of the total Sb(V) was consumed during the enzymatic reaction. During the time course of these assays and under the conditions used Sb(III) remained in the reduced form.
Results

Identification and cloning of LmACR2. The Leishmania Genome Network GeneDB (www.genedb.org/) contains one *L. major* sequence (systemic name LmjF32.2740) for a ScAcr2p homologue that is located in chromosome 32. PCR was performed with 5’ forward and 3’ reverse gene-specific primers, as described under *Experimental procedures*, to produce a single fragment that was cloned and sequenced, giving rise to a 384 bp gene that we designate *LmACR2* (Accession # AY567836). LmACR2 and ScAcr2p are similar in size and mass. LmACR2 has 127 residues with a mass of 14,493 Da, compared to ScAcr2p, which has 130 residues and a mass of 14,883 Da. ScAcr2p has an active site cysteine within the sequence FHC(X)₅R (16), and LmACR2 has the same FHC(X)₅R motif. The two proteins exhibit 28% sequence identity and 44% similarity (Fig.1).

Expression of *LmACR2* functionally complements the arsenate sensitive phenotype of an arsenate reductase deletion of *E. coli*. LmACR2 was cloned behiind the arabinose promoter in plasmid pBAD-Myc-HisA with a C-terminal six-histidine tag. ACR2 genes from either *S. cerevisiae* (*ScACR2*) or *L. major* (*LmACR2*) with six histidine codons at the 3’ end of the genes restored arsenate resistance in the *E. coli arsC* deletion strain WC3110 (17) (Fig. 2). *LmACR2* complemented better than *ScACR2*, perhaps because of improved solubility. There was no complementation with...
either gene in the absence of arabinose. \textit{LmACR2} functionally complemented the As(V) sensitivity of an \textit{acr2''} strain of \textit{S. cerevisiae} (data not shown). Neither \textit{E. coli} nor \textit{S. cerevisiae} exhibited sensitivity to Sb(V) (data not shown).

**Purification of \textit{LmACR2}**. When expressed in \textit{E. coli}, \textit{LmACR2} is a soluble cytosolic protein that was purified by NiNTA chromatography. The molecular mass of purified \textit{LmACR2} was determined by gel filtration chromatography using a Sephacryl S-100 column (Fig. 3). From the nucleotide sequence of the \textit{LmACR2} gene with the C-terminal myc-epitope and six histidine tag, the gene product has a predicted mass 17,454 Da. From gel filtration, a mass of approximately 17 kDa was determined, consistent with a monomer. In contrast, yeast ScAcr2p elutes as a homodimer (17).

\textit{LmACR2} is an arsenate reductase. The ability of \textit{LmACR2} to reduce arsenate to arsenite was examined using a coupled assay developed for ArsC and ScAcr2p (17,28). Reduction of arsenate was catalyzed by \textit{LmACR2} in the presence of Grx2, the most effective \textit{E. coli} glutaredoxin for ArsC (25) (Fig. 4). Both yeast ScGrx1p and the \textit{E. coli} Grx2 could serve as electron donor for \textit{LmACR2} activity (Fig. 5). With Grx2 the \textit{Km} for arsenate was 6 mM, compared to 10 mM with ScGrx1p. The \textit{V_{max}} was approximately 10 nmol/mg/min with both glutaredoxins. \textit{LmACR2} exhibited hyperbolic kinetics (Fig. 5), in contrast to the yeast ScAcr2p, which shows sigmoidal kinetics and an apparent Hill coefficient of 2.7 (17). The effect of trivalent metalloids
on arsenate reductase activity was examined. Inorganic As(III) inhibited with an apparent \( K_i \) of 0.25 mM (Fig. 6A). The organic arsenical phenylarsine oxide and inorganic Sb(III) were better inhibitors, with apparent \( K_i \) values of approximately 20 \( \mu \)M (Fig. 6B and C). Sodium phosphate, nitrate and sulfate did not inhibit LmACR2 arsenate reductase activity in vitro (data not shown).

**LmACR2isanantimonatereductase.** Since the drugs for treatment of leishmaniasis such as Pentostam are pentavalent antimonials that must be activated by reduction to Sb(III), the ability of LmACR2 to reduce Sb(V) was examined. Pentostam is the noncovalent chelate of gluconate and antimonate. We used an ion exchange HPLC separation of trivalent and pentavalent antimonials in which the amount of each species is quantified by ICP-MS (27). Purified LmACR2 reduced Sb(V) to Sb(III) in a time-dependent manner (Fig. 7A). Antimonate reductase activity required both GSH and Grx2 (data not shown). The kinetics of Sb(V) reduction gave a \( K_m \) of 7.2 \( \mu \)M for Sb(V) and a \( V_{\text{max}} \) of 33 nmol/min/mg (Fig. 7B). By comparison, the \( K_m \) for Sb(V) is 1000-fold lower than for As(V), and the \( V_{\text{max}} \) is three-fold higher. These results clearly demonstrate that LmACR2 is a metalloid reductase with much higher affinity for Sb(V) than As(V).

**Expression of LmACR2confersPentostam sensitivity in Leishmania.** If LmACR2 is responsible for the physiological effect of Pentostam, then the reduction of Sb(V) to...
Sb(III) by LmACR2 should be related to sensitivity of the parasite to Pentostam. The effect of expression of LmACR2 in intracellular amastigotes was examined (Fig. 8).

LmACR2 in plasmid pSP-ACR2HYG was transfected into L. infantum promastigotes, which were used to infect macrophages. These parasites express luciferase to facilitate measurement of drug susceptibility (22). Susceptibility to Pentostam was determined in these intracellular (macrophage infected) amastigotes (Fig. 8). Because this is a wild type strain diploid for LmACR2, reductase activity is expressed endogenously. Even so, there is an unambiguous increase in sensitivity to Pentostam in intracellular amastigotes expressing LmACR2 in trans compared to vector-transfected cells. We expect that the effect of LmACR2 expression would be much more dramatic in an ACR2 knockout. This is the first demonstration of an enzyme that is involved in and probably required for action of the drug Pentostam in intracellular amastigotes of Leishmania.

Discussion

Treatment of leishmaniasis is limited by the availability of effective drugs (29). Pentostam and Glucantime are the most widely used drugs. Inorganic pentavalent antimony is rather insoluble, which limits its utility as a drug. Pentostam and Glucantime are noncovalent chelates of Sb(V) with improved solubility and uptake properties than free Sb(V) and lower toxicity than Sb(III). In spite of their greater
solubility, they must still be administered parenterally, which is a serious limitation in their use for the treatment of leishmaniasis. In the human host, the parasite exists in the intracellular amastigote form inside of macrophages.

It is generally accepted that the Sb(V) in these drugs must be reduced to Sb(III) to create the active form of the drug, but does reduction occur in the macrophage, in the amastigote or both? On the one hand, axenic amastigotes of L. infantum produced in vitro have been reported to be more resistant to Pentostam than intracellular amastigotes grown in macrophages, leading to the suggestion that macrophages reduce Pentostam to Sb(III), which is then taken up by the intracellular amastigotes (4). As(III) is taken into most cells by aquaglyceroporins (7,8), and several Leishmania species have an aquaglyceroporin, AQP1, that we have shown catalyzes Sb(III) uptake (30). Yet axenic amastigotes of L. donovani have been reported to reduce Sb(V), and a Pentostam resistant mutant had lower reduction (6).

These considerations suggest that Sb(V) is reduced to Sb(III) in both the human host and the parasite (Fig. 9). While axenic amastigotes reduce Pentostam (6), no protein has been associated with this activity. In this study we identified a L. major homologue of known arsenate resistance enzymes and showed that it is an Sb(V) reductase. Its properties are similar to those of the yeast enzyme but exhibit some differences. For one thing, the $V_{\text{max}}$ of purified LmACR2 is considerably less
than that of ScAcr2p, which is in the range of 300 nmol/mg/min. The low specific activity of purified LmACR2 may be due in part to the fact that trypanosomes and Leishmania do not utilize the wide-spread system of GSH and GSH reductase. In trypanosomatidae, these are replaced by \( N^1,N^8 \)-bis(glutathionyl)spermidine (trypanothione), and trypanothione reductase (31-34). Replacing glutaredoxin is tryparedoxin (34,35). Thus, it is reasonable to assume that a leishmanial homologue of an arsenate reductase would function more efficiently with trypanothione, trypanothione reductase and tryparedoxin, which were not available for this study. A second apparent difference between the parasite and yeast enzymes is that LmACR2 is a monomer while ScAcr2p is a homodimer. LmACR2 also exhibits hyperbolic kinetics (Fig. 5), in contrast to the yeast ScAcr2p, which shows a sigmoidal relationship between arsenate reduction and arsenate concentration, with an apparent Hill coefficient of 2.7 (17). The apparent cooperativity and dimeric state of the yeast enzyme may be related.

Finally, the activity of LmACR2 is likely to be physiologically significant. First, the \( LmACR2 \) gene can functionally complement the arsenate sensitive phenotypes of arsenate reductase deletions of \( E. \ coli \) and \( S. \ cerevisiae \). Second, and more importantly, expression of \( LmACR2 \) in \( L. \ infantum \) amastigotes sensitized intracellular amastigotes to Pentostam, which could only occur if the amastigotes takes up Sb(V).
Third, in preliminary experiments we have found the transfection of a field isolate from India with *LmACR2* made the intracellular amastigotes sensitive to 300 µg/ml of pentostam, whereas the same field isolate without the transfected gene was completely resistant to that level of drug (data not shown). These results are consistent with involvement of *LmACR2* in conversion of the drug to its active form. Expression of *LmACR2* may be a predictor of success of Sb(V) treatment. Conversely, mutations in *LmACR2* may be an unrecognized cause of drug failure.

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**References**


3. Sundar, S., More, D. K., Singh, M. K., Singh, V. P., Sharma, S., Makharia, A.,


Figure legends

Fig. 1. Alignment of *Saccharomyces cerevisiae* (ScAcr2p) (accession number Q06597) and *Leishmania major* (LmACR2) (accession number AAS73185) arsenate reductases. The active site motif HC(X)_5R is underlined. Sequences were aligned with Clustal W.

Fig 2. **LmACR2 confers arsenate resistance in *E. coli*.** Arsenate resistance in *E. coli* was assayed as described in Experimental procedures: (†), W3110 (wild type); (o), WC3110 pBAD/Myc-HisA; ( ), WC3110 pBAD-LmACR2 + 0.2% arabinose; (†), WC3110 pBAD-LmACR2 (uninduced); (¼), WC3110 pBAD-ScACR2 + 0.2% arabinose; (½), WC3110 pBAD-ScACR2 (uninduced).

Fig. 3. **Purification and aggregation state of LmACR2.** LmACR2 was purified as described under Experimental procedures. The mass of LmACR2 was determined from its elution position (arrow) on Sephacryl S-100 chromatography. The elution position of the standard proteins are indicated for albumin (66 kDa), ovalbumin (46 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (14.3 kDa).

Inset: Samples of each step of purification were analyzed by sodium dodecyl sulfate
polyacrylamide gel electrophoresis on a 15% polyacrylamide gel. **Lane 1:** 50 µg of cells of *E. coli* strain WC3110 pBAD-ScACR2 induced with 0.2% arabinose; **lane 2,** 50 µg of inclusion bodies; **lane 3,** 100 µg of cytosol; **lane 4-6,** 25, 62 and 125 µg of pooled LmA CR2-containing fractions from NiNTA chromatography.

**Fig. 4. Arsenate reductase activity of purified LmA CR2.** The arsenate reductase activity of LmA CR2 was assayed with 1 mM GSH and 20 mM sodium arsenate. Arsenate reductase activity was estimated from the oxidation of NADPH measured at 340 nm, as described under *Experimental procedures.* (○), 100 µM LmA CR2; (½), 100 µM purified *E. coli* Grx2; (¡), 100 µM LmA CR2 + Grx2.

**Fig. 5. Glutaredoxin supports arsenate reductase activity of purified LmA CR2.**

The arsenate reductase activity of LmA CR2 was assayed with 1 mM GSH and either 5 nM *E. coli* Grx2 (¡) or 5 nM *S. cerevisiae* Grx1p (○). The rate of arsenate reductase activity was estimated from the oxidation of NADPH measured at 340 nm at each concentration of sodium arsenate, as described under *Experimental procedures.* The error bars represent the standard deviations from two independent assays.

**Fig. 6. Inhibition of arsenate reductase activity by trivalent metalloids.**

Arsenate reductase at 20 mM (Ī) and 40 mM (Ø) of sodium arsenate was assayed in the presence of indicated concentrations of inhibitors. The inhibitors were sodium arsenite (A), phenyl arsine oxide (B) and potassium antimonyl tartrate (C). Solid lines
represent best fits of the data using Sigma Plot 2000. $K_i$ values were estimated from the concentration of inhibitor at the intersection of the lines.

Fig. 7. LmACR2 reduces Sb(V) to Sb(III). The Sb(V) reductase activity of LmACR2 was assayed with 5 nM purified *E. coli* Grx2 and 1 mM GSH at 37° C. Samples were digested with HNO$_3$. Sb(V) and Sb(III) were separated by HPLC, and the amount of Sb(III) produced was quantified by ICP-MS., as described under Experimental procedures. A. Reduction of 1 mM Sb(V) was assayed for the indicated times with 40 µM LmACR2. B. Initial rates of Sb(V) reduction were estimated from the amount of Sb(III) produced after 5 min with 20 µM LmACR2. The line is the best fit to the Michaelis-Menten equation using SigmaPlot 2000.

Fig. 8. Expression of LmACR2 in *Leishmania infantum* augments Pentostam sensitivity. *L. infantum* promastigotes expressing the luciferase gene (22) were transfected with a hygromycin phosphotransferase vector either with no insert (o) or with LmACR2 (½). Macrophages were infected with transfected parasites, and nonengulfed parasites were removed by a wash. Intracellular parasites were incubated for five days with the indicated concentrations of Pentostam. The number of parasites was calculated from luciferase activity. The error bars represent the average of duplicates calculated with SigmaPlot 2000. Similar results were obtained
with two additional sets of duplicate assays.

**Fig. 9. Model of Pentostam action in macrophage-associated amastigotes of *Leishmania***. Sb(V) is taken up by macrophages, and a portion is reduced to Sb(III), which is then transported into the amastigote by AQP1. The other portion of the Sb(V) is taken into the amastigote and reduced to Sb(III) by LmACR2 (and perhaps other enzymes such as TDR1). The relative contributions of the two pathways to drug action would depend on the relative rates and expression of their respective components in both the human host and parasite. This could be different in different strains of *Leishmania*, as well as in different infected individuals, leading to variability in drug response.
Fig. 3

The graph plots the relationship between the log of molecular weight (log MW) and the average partition coefficient ($K_{av}$). The x-axis represents log MW, ranging from 4.1 to 4.9, and the y-axis represents $K_{av}$, ranging from 0.1 to 0.6. The data points for Ribonuclease A (13.7 kDa), Chymotrypsinogen A (25 kDa), Ovalbumin (43 kDa), and Albumin (67 kDa) are plotted on the graph. The graph shows a linear relationship between $K_{av}$ and log MW.

Inset: A gel showing four lanes labeled Whole cells, Inclusion bodies, Cytoplasm, LmACR2 (25 µg), LmACR2 (82 µg), and LmACR2 (125 µg). The gel displays bands at 16 kDa.
Fig. 6

**A**

[Sodium arsenite] mM

**B**

$1/V$

[Phenylarsine oxide] μM

**C**

[Potassium antimonyl tartrate] μM
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