Roles for mitochondria in pentamidine susceptibility and resistance in *Leishmania donovani*

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Abstract

Pentamidine resistant *Leishmania donovani* was raised in the laboratory by stepwise exposure to increasing drug pressure until a line capable of growth in 8 μM pentamidine (R8) had been selected. An IC50 value of 40 μM was determined for this line, some 50-fold higher than that recorded for the parental wild-type line. The pentamidine resistant promastigotes were cross-resistant to other toxic diamidine derivatives but not to antimonials or substrates of multi drug resistance pumps. Decreased mitochondrial transmembrane potential was observed in pentamidine resistant promastigotes. A substantial net decrease in accumulation of [3H]-pentamidine accompanied the resistance phenotype. Inhibitors of P-glycoprotein pumps, including prochlorperazine and trifluoperazine, did not reverse this decreased drug uptake, which distinguishes the *L. donovani* resistant line studied here from *L. mexicana* promastigotes previously studied for pentamidine resistance. Kinetic analysis identified a carrier with an apparent Km value of 6 μM for pentamidine. No significant difference between wild-type and resistant parasites could be detected with respect to this transporter in rapid uptake experiments. However, in longer-term uptake experiments and also using concentrations of pentamidine up to 1 mM, it was demonstrated that wild-type cells, but not resistant cells, could continue to accumulate pentamidine after apparent saturation via the measured transporter had been reached. Agents that diminish the mitochondrial membrane potential inhibited this secondary route. A fluorescent analogue of pentamidine, 2,3-bis-(4-amidophenyl)-3,4-dimethylfuran (DB99), accumulated in the kinetoplast of wild-type but not resistant parasites indicating that uptake of this cationic compound into mitochondria of wild-type cells was more pronounced than in the resistant line. These data together indicate that resistance to pentamidine in *L. donovani* is associated with alterations to the mitochondria of the parasites, which lead to reduced accumulation of drug.

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Keywords: *Leishmania donovani*; Pentamidine; Drug resistance; Mitochondria

1. Introduction

*Leishmania donovani*, a flagellated protozoan parasite, is the causative agent of visceral leishmaniasis. Sandflies trans-
tance to the drug in *Saccharomyces cerevisiae* [23]. The protein encoded by this gene localizes to the inner membrane of the mitochondrion and appears to play a role in secretion of proteins from the mitochondrion [24]. Disruption of mitochondrial function has been proposed as a likely toxic effect of pentamidine in *S. cerevisiae* [24]. The mitochondrion has also been implicated in the action of pentamidine against trypanosomatids. Electron microscopy revealed that treatment of *Leishmania* species with pentamidine leads to disintegration of the kinetoplast and mitochondrion [25,26], and a collapse in mitochondrial potential [27], is one of the first manifestations of treatment of these parasites with the drug.

The mechanism of action of pentamidine remains a matter of debate but selective toxicity may relate to high levels of accumulation of the drug in the case of African trypanosomes [28,29]. Damper and Patton [28,30] first reported that high affinity transporters that are energy dependent and competitively inhibited by other diamidines mediate the uptake of pentamidine by *Trypanosoma brucei* bloodstream forms. The P2 amino-purine transporter of *Trypanosoma brucei* [31] appears to play a key role in the transport of this drug, although the situation is complex [29,32], with at least two additional transporters also capable of carrying the drug [33]. Loss of P2 nucleoside transporter can mediate resistance to diamidines including dimenazine aceturate (berenil) in African trypanosomes [34–36]. However, the presence of multiple pentamidine transporters in *T. brucei* ensures that P2-defective parasites are still sensitive to this drug [33]. In *Leishmania* sp. uptake of pentamidine has also been shown to be saturable, involving a carrier-mediated, energy dependent process [37,38]. Pentamidine was found to be a competitive inhibitor of arginine transport [39,40] and a non-competitive inhibitor of putrescine and spermidine transport in *L. infantum* [41], *L. donovani* and *L. mexicana* [42]. However, experiments using radiolabeled pentamidine in *L. mexicana*, failed to show reciprocal inhibition with arginine or polyamines. The physiological substrate for the transporter that carries pentamidine has yet to be identified in *Leishmania*. Exclusion of diamidines from the mitochondrion, in a manner associated with diminished mitochondrial membrane potential, has been proposed to contribute to the resistance phenotype in *L. mexicana*. A separate study showed that episomal expression of a P-glycoprotein could confer low-level resistance to pentamidine in *L. major* [43]. The subcellular localization of that transporter was not clear. It has recently been proposed [44] that inhibition of respiratory chain complexes can induce apoptosis in *L. donovani*. Moreover, inhibitors of complex II of the respiratory chain are synergetic with pentamidine’s leishmanicidal activity—again pointing to a possible role of the mitochondrion in the action of this drug. While biological phenomena characterized in one *Leishmania* species are frequently taken as pointers to similar activity in other species of the genus it is important to actually study each individual species. Crucial differences between species exist, and this is clearly manifest in pharmacological responses to drug. For example, *L. donovani* is markedly more sensitive to a variety of drugs than is *L. mexicana* [45]. In this study we set out to determine the biochemical basis of resistance to pentamidine in *L. donovani*, the most important pathogenic *Leishmania* species that is responsible for visceral leishmaniasis in India. Loss of mitochondrial membrane potential appears to be key to the development of drug resistance in this species, while drug efflux appears to play some role in *L. mexicana* but not in *L. donovani*.

2. Materials and methods

2.1. Materials

([Ring-3H] pentamidine (98 Ci/mmole, 5 mCi/ml) was custom synthesized by Amersham Pharmacia Biotech (Buckinghamshire, UK). Pentamidine isethionate, rhodamine 123 (Rh123), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), berenil, 4',6-diamidino-2-phenylindole (DAPI), verapamil, trifluoperazine (TFP), prochlorperazine (PCP), vinblastine, sodium arsenite were purchased from Sigma (St. Louis, MO). Pentostam was a gift from The Wellcome Foundation Ltd., UK, and glucantime from Rhône-Poulenc, Specia, France. Propamidine and stilbamidine were kindly donated by Dr. Harry De Koning (University of Glasgow), and DB99 was kindly donated by Dr. David Boykin (University of Georgia). ATP, sodium succinate, NADH, potassium ferricyanide, ethylene glycol-bis(β-aminoethyl) ether (EGTA), cytochrome c, sodium orthovanadate, percoll, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), N,N'-dicyclohexylcarbodiimide (DCCD), valinomycin, oligomycin, sodium azide, monensin and ouabain were also procured from Sigma.

2.2. Strains and culture conditions

*L. donovani* AG83 (MHOM/IN/1983/AG83) promastigotes were grown in vitro at 24 °C in modified M199 medium (Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco BRL, Life Technologies, Scotland, UK). The pentamidine sensitive wild-type strain of *L. donovani* was cloned on semisolid M199 medium containing 1% Bacto Agar (Difco) and 10% heat inactivated fetal bovine serum. Colonies were picked and transferred separately into the above-described liquid medium. The cloned wild-type cells were then cultured in the presence of 1 µM pentamidine. The culture was stabilized for six subcultures before increasing the drug level at one passage a week. The line growing in 1 µM drug was plated on M199-agar plates and a single clone (R1) was picked. This was subjected to stepwise increasing drug pressure with final concentrations of 2, 4, and 8 µM. Cells from cultures growing at each concentration were cloned on semi-solid medium in the presence of drug. The clones were designated as R2, R4 and R8, respectively.

2.3. Cross-resistance studies

The growth sensitivities of WT and R8 to different cytotoxic agents were determined by plating the cells in 96-well microtitre plates. Briefly, 1 x 10^5 parasites in 200 µl medium were incubated with different concentrations of the drugs. Drugs were
added at 20 times the final concentration in a volume of 10 μl in triplicate (with the concentration range depending on the drug). After 72 h of drug addition cell densities were determined by a Neubauer hemocytometer. The effective concentration of the drugs, which inhibited 50% of cell proliferation of wild-type and R8 after 72 h, was denoted as the IC50 value.

2.4. Determination of mitochondrial transmembrane potential

Wild-type and resistant cells, treated or not treated with pentamidine were stained with 0.05 μM rhodamine 123 for 30 min to determine the relative mitochondrial membrane potential. Mitochondrial membrane potential was assessed by flow cytometry as previously described [46]. Data were gathered with a Becton Dickinson LSR flow cytometer. Rhodamine 123 fluorescence was collected in a photomultiplier tube designated as FL1. Data analysis was carried out with Win MDI software.

2.5. Isolation of mitochondria from promastigotes

Promastigotes of wild-type L. donovani and R8 were collected by centrifugation and washed twice in ice-cold phosphate buffered saline with 2 mM glucose, pH 7.4 (PBGS). Subsequent steps were performed at 4°C. Cells were resuspended at 1.2 x 10^9 ml^-1 in 1 mM Tris, pH 8.0, 1 mM EDTA, briefly homogenized in an all glass homogenizer and lysed by passage through a 26-gauge needle. Sixty percent sucrose was immediately added to the above lysates so that the final concentration of sucrose was 250 mM. The lysate was centrifuged for 10 min at 25,800 x g (Sorvall SS-34 rotor, 11,500 rpm). The pelleted material was resuspended at one-fifth of the original lysate volume in 250 mM sucrose, 20 mM Tris, pH 8.0, 2 mM MgCl2 and treated for 30 min with 9 μg/ml RNAse free DNase (Qiagen). This pellet was further diluted five-fold with 250 mM sucrose, 20 mM Tris, pH 8.0, 2 mM EDTA and centrifuged at 15,600 x g as described above. The pelleted material was resolved on a 20–35% Percoll gradient (45 min, Beckman SW rotor, 24,000 rpm), and a mitochondrial vesicle fraction was collected. Mitochondria were washed four times with an excess of 250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA to remove percoll. Mitochondria could be stored for up to a month in 50% glycerol, 250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA [47].

2.6. Biochemical assays of mitochondrial enzymes

All enzymatic assays from mitochondrial fractions except for the ATPase assay were assessed by a modified method originally described by Denicolae-Seoane et al. [48] The assays were performed using a final protein concentration of 0.04 mg/ml in 1 ml cuvettes using 50 mM phosphate buffer pH 7.5 with 5 mM azide to inhibit cytochrome oxidase and divert electrons to the artificial acceptor.

(I) Succinate dehydrogenase (SDH) activity was measured spectrophotometrically at 600 nm (ε = 20.5 mM^-1 cm^-1) using 3 mM succinate, 0.5 mM 2,6-dichloro-phenolindophenol, 0.1 mM phenazine methosulphate.

(II) NADH dehydrogenase (NDH) activity was determined spectrophotometrically at 420 nm by measuring the rate of potassium ferricyanide reduction in the presence of NADH (ε = 1 mM^-1 cm^-1).

(III) Succinate- and NADH–cytochrome c reductase (SCC and NCC) activities were measured spectrophotometrically at 550 nm (ε = 18.9 mM^-1 cm^-1) in the presence of 20 μM cytochrome c and either 5 mM succinate or 0.2 mM NADH.

(IV) Mitochondrial ATPase activity was measured by an end point inorganic phosphate (Pi) release colorimetry assay. The amount of inorganic phosphate (Pi) release over 10 min at 25°C was measured. Briefly, a standard 100 μl reaction mixture containing mitochondrial extract (0.04 mg) was incubated with ATPase buffer (150 mM Tris–HCl, pH 7.5, 1 mM MgCl2, 0.5 mM EGTA), 100 μM Na-orthovanadate (inhibitor of P-type ATPases), supplemented with 3 mM ATP [49]. The transfer of the mixture from ice to room temperature initiated the reaction. The reaction was terminated by addition of 1 ml stop solution (0.5% SDS, 2% sulphuric acid and 0.5% ammonium molybdate), followed by addition of 10 μl of freshly prepared coloring reagent (10% ascorbic acid) and absorbance was taken at 750 nm using UV–vis 160A Shimadzu spectrophotometer. ATPase activity was plotted using a standard curve for 0–100 mM of inorganic phosphate.

2.7. Transport studies

Parasites were harvested during the mid logarithmic phase of growth by centrifugation at 2100 x g, for 10 min at 4°C. Cells were then washed twice with phosphate buffered saline supplemented with 1% d-glucose (PBGS) at pH 7.4. Cells were resuspended in this buffer at densities appropriate for the procedure to be used, as indicated in the text and figure legends. Parasite suspensions (100 μl, containing 2 x 10^7 cells) were warmed to 25°C and mixed with 100 μl of assay buffer containing labeled molecule plus or minus other test compounds at the concentration indicated in the figure legends. Transport was terminated after times indicated in the figure legends by the rapid separation of parasites from the buffer components by centrifugation through a 9:1 mixture of dibutylphthalate (specific gravity, 0.2) and mineral oil (specific gravity, 0.875–0.885). The sample tubes were immediately flash frozen in liquid nitrogen, and the tubes were cut to separate the pellet from the transport medium. The pellet was dissolved in 2% sodium dodecyl sulfate (200 μl) and 3 ml of scintillation fluid (Ecoscint A, National Diagnostics). These were left overnight to remove the effects of chemiluminescence, and then incorporated radioactivity was counted in a Scintillation counter.

2.8. Inhibition studies

The effect of various metabolic inhibitors, ionophores and uncouplers on pentamidine accumulation in wild-type and resis-
tant promastigotes was studied by resuspending cells in PBSG containing inhibitors at concentrations indicated in the figure legends for 15 min at 25°C. After this treatment, 5 μM [3H]pentamidine was added to the mixture and uptake was measured for 2 min. Accumulated label was counted as above.

2.9. Fluorescence microscopy

Cells from a mid-log phase culture were pelleted by centrifugation, washed (in PBSG) and incubated with 10 μM 2,5-bis-(4-amidophenyl)-3,4-dimethylfuran, DB99, for 5 min and then transferred to a microscope slide and viewed by fluorescence microscopy (excitation at 330 nm, emission at 400 nm) with a Zeiss Axioscope and images were captured using the Improvision OpenLab Software.

3. Results

3.1. Characterization of pentamidine resistant parasites

Four cloned lines of L. donovani promastigotes resistant to pentamidine were selected in vitro by increasing drug pressure. The cloned lines, designated R1, R2, R4 and R8, displayed 4-, 22-, 30- and 50-fold resistance to pentamidine, respectively. Doubling time for the exponentially grown wild-type L. donovani was 14 h and 22 h for the R8 resistant promastigotes grown in the presence of 8 μM pentamidine. The phenotype was stable in drug free medium for up to 2 months, although the IC50 of R8 had declined to 18 μM after a period of 6 months without drug pressure. The IC50 of pentamidine for amastigotes derived from R8 was greater than 50 μM in comparison to 1 μM for sensitive amastigotes (data not shown).

R8 expressed cross-resistance to the diamidines, propamidine (9-fold), stilbamidine (12-fold), berenil (4-fold), and fluorescent analogs including DAPI (19-fold) and DB99 (11-fold) (Table 1). These diamidines competitively inhibit pentamidine uptake, indicating a common transport system (data not shown). The R8 line did not show any cross-resistance to antimoniais (pentostam, glucantime), arsenite and to multidrug resistance pump substrates including vinblastine, adriamycin or the Ca2+ antagonist and P-glycoprotein inhibitor verapamil (data not shown) [50,51].

3.2. Mitochondrial transmembrane potential

The ability of wild-type parasites, and the R8 clone to accumulate the hydrophobic cation, rhodamine 123, which is widely used as an indicator of mitochondrial membrane potential [52] was also studied. Wild-type cells treated with pentamidine were also used for this study. After 30 min of staining, Rh123 showed decreased fluorescence in treated parasites and also in R8, indicating a decrease in the membrane potential (Fig. 1) compared to untreated wild-type cells.

3.3. Pentamidine uptake by wild-type and resistant parasites

Uptake of 1 μM pentamidine was measured over 3 h at 25°C in pentamidine resistant L. donovani and compared with that in wild-type cells. The resistant promastigotes accumulated significantly less drug in comparison to the sensitive strain. After 3 h of incubation, the intracellular pentamidine concentration was 0.77 μM in R8 whereas in the sensitive promastigotes the

| Table 1 Cross-resistance profile of wild-type and pentamidine resistant L. donovani promastigotes |
|----------------------|----------------------|----------------------|
| Drug                | Mean IC50 ± S.D. (μM) | R8                   |
| Pentamidine         | 0.81 ± 0.01           | 40 ± 6.36            |
| Propamidine         | 10.4 ± 1.5            | 95 ± 12.8            |
| Stilbamidine        | 8.5 ± 0.4             | 104 ± 7.5            |
| Berenil             | 24.0 ± 6.3            | 99 ± 1.4             |
| DAPI                | 1.03 ± 0.03           | 20 ± 2.5             |
| DB99                | 1.25 ± 0.06           | 14 ± 1.6             |

Effects of different drugs on in vitro growth of pentamidine sensitive and resistant promastigotes after a 72 h incubation period at 25°C. Cross-resistance was not evident for compounds like pentostam, glucantime, arsenite, verapamil, vinblastine and adriamycin. IC50 (μM) values are the mean values of three different experiments.

![Fig. 1](image.png) Rhodamine 123 staining of pentamidine resistant and sensitive promastigotes of L. donovani. Promastigotes of wild-type and resistant parasites were incubated with rhodamine 123 as described. Cell suspensions were subjected to flow cytometry and the fluorescence distribution was plotted as frequency histograms. The figure shows a set of histograms, representative of two duplicate determinations, for both the populations: (a) wild-type untreated, (b) wild-type treated with 2 μM pentamidine for 72 h and (c) R8. Differences in the degree of probe accumulation are seen.
in intracellular pentamidine concentration reached 3.8 μM, representing a 5.4-fold enhanced accumulation of the drug within these cells (Fig. 2).

Apparent kinetic constants for pentamidine uptake into resistant and wild-type promastigotes were determined with a range of substrate concentrations between 1 μM and 40 μM for 2 min. The apparent $K_m$ value and the $V_{max}$ value did not differ significantly in the wild-type and resistant lines (Table 2). The $K_m$ value indicates the presence of a relatively high affinity transporter for pentamidine. In addition to this saturable high affinity transporter, pentamidine uptake in wild-type *L. donovani* promastigotes also appears to occur through a secondary, non-saturable route. The presence of this second route was indicated by a failure of up to 1 mM cold pentamidine to completely inhibit uptake of 1 μM [3H] pentamidine (Fig. 3). In another experiment, increasing concentration of labeled pentamidine to 1 mM also showed the presence of a substantial non-saturable component in wild-type but not in resistant cells (Fig. 4).

### 3.4. Second route of entry of pentamidine into the sensitive strain

Both the wild-type and the resistant promastigotes have a high affinity transporter with similar $K_m$ and $V_{max}$ values. When pentamidine accumulation was measured in both the cell lines in the presence of a high range of extra-cellular pentamidine concentration (up to 1 mM), the accumulated pentamidine in the wild-type did not reach saturation (Fig. 4). This could indicate the presence of a secondary route with very low affinity into cells for pentamidine. This secondary route was not as pronounced in the R8 cell line. Treatment of wild-type cells with CCCP for 10 min led to loss of measurable uptake via the secondary route.

### Table 2

<table>
<thead>
<tr>
<th>Cells</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/(10^7 cells min))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.8455 ± 0.52</td>
<td>0.5848 ± 0.08</td>
</tr>
<tr>
<td>Resistant</td>
<td>7.0057 ± 0.87</td>
<td>0.5716 ± 0.0775</td>
</tr>
</tbody>
</table>

The apparent kinetic parameters were determined using Michaelis–Menten equation to a non-linear fitted curve using Enzyme Kinetics Pro (Version 2.36) software. The results show mean values ± S.D. of two independent experiments performed in triplicates.
the extent of inhibition was less pronounced than for pentamidine resistant L. donovani promastigotes.

3.5. Relative accumulation of pentamidine in wild-type and pentamidine resistant L. donovani promastigotes in the absence and presence of different metabolic inhibitors

Cells were pretreated with inhibitors at 25°C for 5 min and then incubated with 5 μM pentamidine for 1 min in PBSG. All values are given as a percentage of wild-type and resistant control (without inhibitor pretreatment) and are means ± S.D. of three independent experiments.

Uptake into CCCP treated wild-type cells was very similar to uptake into the resistant line (Fig. 4). It is possible that the secondary route is an independent low affinity carrier. Alternatively, the unsaturatable component could relate to a limitation in the kinetic experiments and at higher concentrations uptake into the mitochondrion of wild-type, but not resistant cells, could alter transmembrane equilibria and influence measurable uptake.

3.5. Relative accumulation of pentamidine in wild-type and pentamidine resistant L. donovani promastigotes in the absence and presence of different metabolic inhibitors

Pentamidine uptake was also investigated in the presence and absence of different inhibitors to investigate the nature of the force-driving uptake (Table 3). Inhibitors of mitochondrial metabolism including KCN and sodium azide, led to significant reductions in net pentamidine uptake in wild-type parasites. Ouabain, an inhibitor of plasma membrane Na⁺/K⁺ ATPase, and monensin, a Na⁺ ionophore, did not decrease pentamidine uptake in the promastigotes. These data indicate that pentamidine uptake is independent of Na⁺ ions. The K⁺ ionophore valinomycin and the protonophore carbonylcyanide (CCCP) reduced pentamidine uptake. The non-specific H⁺ATPase inhibitor, N,N'-dicyclohexylcarbodiimide, which reduced the plasma membrane pH gradient and the membrane potential in Leishmania sp. [53,54] and Oligomycin A, a mitochondrial ATPase inhibitor had inhibitory effects on pentamidine uptake. These results suggest that energy is required for the entry of drug into the promastigotes and that an actively metabolizing mitochondrion plays an important role in the net accumulation of pentamidine in L. donovani.

In L. mexicana promastigotes, the energy dissipating activity of mitochondrial inhibitors stimulated drug uptake, indicating that energy was expended in excluding the drug from the cell [55]. However, pentamidine uptake in resistant L. donovani also decreased in the presence of mitochondrial inhibitors, although the extent of inhibition was less pronounced than for wild-type cells. The protonophore CCCP did not alter the uptake of the drug significantly in comparison to control. Resistant parasites already have a lowered mitochondrial membrane potential which may contribute to the exclusion of drug from the mitochondrion.

In L. mexicana, PCP and TFP which are known to inhibit P-glycoprotein mediated efflux of compounds from Leishmania [56] increased the rate at which pentamidine could accumulate into the resistant cells [55]. This was taken to indicate that drug efflux was operative in these cells. Pentamidine accumulation over a 3-h period was also investigated in wild-type and R8 L. donovani in the presence of 10 μM PCP and 10 μM TFP (wild-type cells) and 20 μM PCP and 20 μM TFP (resistant cells). In the presence of these inhibitors the resistant cells accumulated drug to similar levels as in control cells (without drug) (Fig. 5), indicating that P-glycoprotein pumps do not contribute to the net accumulation of pentamidine in L. donovani to the same extent as they do in L. mexicana [55].

In L. mexicana it was possible to load pentamidine to equilibrium levels across the plasma membrane by exposing the cells to drug for 15 min on ice [55]. In L. donovani, however, we were unable to stimulate significant uptake of drug on ice, making simple efflux experiments difficult to conduct in this species.

3.6. Intracellular compartmentalization of diamidines

2,5-Bis(4-amidophenyl)-3,4-dimethylfluran (DB99) is a fluorescent analogue of pentamidine. The compound is toxic to L. donovani promastigotes with an IC50 close to that of pentamidine, and cells resistant to pentamidine are cross-resistant to DB99. Moreover, the compound inhibits pentamidine uptake into L. donovani, indicating that the two compounds share a common route of internalization. Distribution of 10 μM DB99 was analyzed by adding it to the cells and viewing with fluorescence microscopy. Within 2 min of staining bright blue fluorescence was found to be associated with the kinetoplast DNA of wild-type but not with the R8 resistant line (Fig. 6). Thus, diamidines selectively accumulate in the mitochondria of WT but not of resistant parasites.
Fig. 6. Fluorescent images of DB99 stained promastigotes of wild-type and pentamidine resistant *L. donovani*. Exponentially growing promastigotes were pelleted, washed and were incubated with 10 μM DB99 at 25°C. Bright fluorescence was associated with the kinetoplast of sensitive cells but not with resistant parasites within 5 min of staining. (A) and (C) represent phase contrast and fluorescence images of sensitive promastigotes. (B) and (D) represent that of resistant promastigotes.

3.7. Enzyme activity of mitochondrial dehydrogenases and ATPases in vitro in wild-type and pentamidine resistant promastigotes

Mitochondrial extracts were prepared as mentioned in Section 2, and different enzymatic activities were measured in the wild and pentamidine resistant strains. As shown in Table 4 in the resistant line, the activity of NADH dehydrogenase, also called complex I, the first complex in the electron transfer chain of mitochondria, NADH-cytochrome c reductase (complex I + complex III), succinate dehydrogenase (complex II) and mitochondrial ATPase also called ATP synthase, catalyzes phosphorylation of ADP to form ATP, were less in comparison to the wild-type strain. Thus, the activity of most of the enzymes responsible for maintaining the mitochondrial potential decreased in a coordinated fashion in the resistant parasites.

Table 4
Spectrophotometric analysis of in vitro mitochondrial dehydrogenase activities of a mitochondrial fraction from untreated wild-type (WT) and pentamidine resistant (R8) *Leishmania donovani* promastigotes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity nmol/(min mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>211 ± 10.4</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>29 ± 3.2</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase</td>
<td>35 ± 5.1</td>
</tr>
<tr>
<td>Succinate-cytochrome c reductase</td>
<td>49.05 ± 19</td>
</tr>
<tr>
<td>Mitochondrial ATPase</td>
<td>158.3 ± 34.6</td>
</tr>
</tbody>
</table>

The results are mean values ± S.D. of triplicates of two independent experiments.

No difference in the activity of succinate-cytochrome c reductase (complex II + complex III) was observed in wild-type compared with the resistant strain.

4. Discussion

Pentamidine, a cationic aromatic diamidine, is a drug, which has been widely used as second line of defense against visceral leishmaniasis. Previous studies have given insight into mechanisms of resistance to pentamidine in *L. mexicana* [55], but less is known of *L. donovani*. It is tempting to draw conclusions from one *Leishmania* species across the range of species. However, it is important to learn about what actually happens in each individual species as clear biological differences can be ascertained across the spectrum of *Leishmania* species. We therefore focused on *L. donovani*, the causative agent of Kala-azar in India.

The pentamidine resistance developed by the clone of *L. donovani* in vitro, studied here, is specific to pentamidine and other diamidines and does not involve the multidrug-resistance phenotype. Diamidine transport has been studied in *T. brucei* where the situation regarding pentamidine uptake into *T. brucei* is complex [25] with the drug entering via at least three transporters: the P2 amino-purine transporter, a high affinity pentamidine transporter (HAPT1) and a low affinity pentamidine transporter (LAPT1) [33]. *Leishmania* parasites are related to trypanosomes, however it was previously shown in *L. mexicana* [55] that pentamidine does not enter the parasites by a nucleoside transporter. Moreover, *Leishmania* sp. do not have
a functional equivalent of the *T. brucei* P2 amino-purine transporter [57,58].

Biochemical evidence shows that pentamidine resistance in *L. donovani* is associated with reduced accumulation of the drug in the resistant promastigotes. Wild-type *L. donovani* accumulated 5.4-fold more pentamidine than resistant cells. The uptake of pentamidine into the cells is carrier dependent. Metabolic inhibitors including KCN, azide and oligomycin all decreased pentamidine uptake. A relatively high affinity (with an apparent $K_m$ of around 6 μM) transporter could be measured in both wild-type and resistant parasites.

The affinity for pentamidine shown by its transporter in *L. donovani* is unaltered in resistant parasites. A similar situation has been noted in resistance to pentamidine in *L. mexicana* [55] and resistance to isometamidium in *Trypanosoma congolense* [59]. Resistance in the latter case was concluded not to associate with alterations at the level of a plasma membrane transporter, but with changes in the mitochondrial membrane potential. Isometamidium rapidly enters the *T. congolense* mitochondrion, where it binds tightly to kinetoplast DNA. The decrease in apparent $V_{max}$ in resistant cells correlated to diminished capacity to accumulate drug intracellularly [59].

Diamidines are organic cations and like isometamidium they have a high affinity for DNA. Ultrastructural changes to kinetoplast DNA and the mitochondrion are observed in parasites exposed to these drugs [26,27,60]. DB99, a fluorescent analogue of pentamidine accumulates in the kinetoplast of sensitive strain of *L. donovani* but not in the resistant promastigotes, suggesting that the mitochondrion is a site of accumulation of pentamidine, as reported in previous study done with *L. mexicana* where DAPI, another fluorescent diamidine, was used. When cells were treated with digitonin to make their membranes permeable, the kinetoplasts of both the wild-type and resistant strains fluoresced indicating that it is access to the kinetoplast, rather than their binding abilities to kinetoplast DNA, that differentiates between sensitive and pentamidine resistant strains. Previous reports have pointed to a decrease in mitochondrial transmembrane potential in pentamidine resistant sp. [18,55]. In this study we have also shown that there is a decrease in accumulation of rhodamine 123 in the resistant promastigotes and in pentamidine treated sensitive parasites, possibly reflecting a decrease in the mitochondrial membrane potential. A decrease in rhodamine 123 fluorescence could also reflect a decrease in the volume of the compartment where it accumulates. That nonylacridine orange fluorescence was unaffected by a mitochondrial permeant indicates a constancy of the mitochondrial biomass. Rhodamine 123 is also a substrate for P-glycoprotein efflux [61]. However, we noted no overexpression of efflux pumps in the resistant strain suggesting that enhanced efflux of this marker does not explain its reduced intracellular accumulation. Rh123 is a lipophilic cation whose cellular accumulation is generally considered to be via passive diffusion. However, we cannot definitively rule out that pentamidine leads to diminished Rh123 accumulation in wild-type cells by competing with uptake of this compound at one or more membrane. Uptake of rhodamine 123 is diminished in resistant lines indicative of reduced mitochondrial membrane potential, although again it cannot be formally excluded that a transporter that carries pentamidine and Rh123 is lost at the mitochondrial membrane. A decrease in mitochondrial membrane potential, however, remains a likely reason for the decrease rhodamine 123 accumulation in resistant parasites.

The studies of drug localization implicated the mitochondrion, and subsequently the kinetoplast of *L. donovani* in accumulation and mode of action of pentamidine. We further analyzed the role of mitochondrial membrane potential on the uptake of pentamidine in wild-type and resistant parasites. Studies on the accumulation of pentamidine reveal that in addition to the transporter that appears to be saturable over a 2 min uptake period in resistant cells, an additional non-saturable component can be measured in wild-type cells. This secondary component can be abolished by interfering with the mitochondrial membrane potential with agents, such as CCCP. Two minutes was required to give significant counts of accumulated pentamidine, and this appeared to fall within the linear range of uptake. However, it cannot be ruled out that this time period does not actually represent initial uptake rates involving simple accumulation at the plasma membrane. It is clear from studies using the fluorescent analogue, DB99, that within this time period drug also commences its accumulation into the mitochondrion. In this event, we can surmise that a single plasma membrane transporter is operative and unaltered in resistant parasites. However, in wild-type cells, but not in resistant cells, accumulation of drug into the mitochondrion removes drug from the cytosol and maintains a concentration gradient across the plasma membrane, allowing continuous uptake in wild-type cells. In this case, the second non-saturable component represents uptake into the mitochondrion. In resistant cells, loss of uptake into the mitochondrion, ensures that equilibrium is reached, and thus over the 2 min time frame uptake appears saturable. Net uptake into *L. donovani* is significantly lower over these time frames than into *L. mexicana* [55]. The concentration within the *L. donovani* cell would be equivalent to the 1 μM external concentration assuming the intracellular volume of *Leishmania* was around 1.5 μl per 10⁸ cells, close to that previously measured [37] indicating that in the resistant line it could be approaching a transmembrane equilibrium between cytosol and extracellular fluid. The drug is clearly accumulated in wild-type cells, probably within the mitochondrion.

In *L. mexicana*, a minor, but significant efflux activity for pentamidine at the plasma membrane was noted [55]. Inhibitors of P-glycoprotein pumps, including PCP, TFP and verapamil, all enhanced net pentamidine uptake into resistant *L. mexicana* and also partially reversed the drug resistance phenotype. In the case of *L. donovani*, none of these agents had any impact on either net drug uptake or drug resistance. This indicates that P-glycoprotein mediated efflux of pentamidine is not operative in *L. donovani* as it is in *L. mexicana*. This result is important as it clearly distinguishes the two Leishmania species, with *L. mexicana*, but not *L. donovani* apparently having an efflux system for pentamidine. In spite of this difference in apparent P-glycoprotein mediated efflux, part of the mechanism of drug resistance in both *L. mexicana* and *L. donovani* appears to be the same. Namely that uptake of drug at the mitochondrial membrane is diminished in resistant cell lines and this leads to
diminished net uptake of drug in both species. Resistance to isometamidium in *T. congolense* [39] and to pentamidine in both *L. mexicana* and *L. donovani* thus appears to relate to diminished mitochondrial membrane potential and this mechanism of drug resistance with respect to cationic drugs appears to be widespread in this group of organisms. Further work is required to elucidate the genetic basis of the alteration of mitochondrial membrane potential. The decrease in mitochondrial transmembrane potential correlates with the decrease in the activities of numerous mitochondrial dehydrogenases and also the F1F0 ATPase. The basis of this coordinated downregulation in expression of several enzymes in relation to the selection of resistance is not known, but it would be of great interest if all of these enzymes are under coordinated regulation activities in both treated and resistant promastigotes.

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**References**


Glyoxalase I from *Leishmania donovani*: A potential target for anti-parasite drug

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Abstract

Glyoxalases are involved in a ubiquitous detoxification pathway. In pursuit of a better understanding of the biological function of the enzyme, the recombinant glyoxalase I (LdGLOI) protein has been characterized from *Leishmania donovani*, the most important pathogenic *Leishmania* species that is responsible for visceral leishmaniasis. A 24 kDa protein was heterologously expressed in *Escherichia coli*. LdGLOI showed a marked substrate specificity for trypanothione hemithioacetal over glutathione hemithioacetal. Antiserum against recombinant LdGLOI protein could detect a band of anticipated size ~16 kDa in promastigote extracts. Several inhibitors of human GLOI showed that they are weak inhibitors of *L. donovani* growth. Overexpression of GLOI gene in *L. donovani* using *Leishmania* expression vector pspa hygro, we detected elevated expression of GLOI RNA and protein. Comparative modelling of the 3-D structure of LDGLOI shows that substrate-binding region of the model involves important differences compared to the homologues, such as *E. coli*, specific to glutathione. Most notably a substrate-binding loop of LDGLOI is characterized by a deletion of five residues compared to the *E. coli* homologue. Further, a critical Arg in the *E. coli* variant at the substrate-binding site is replaced by Tyr in LDGLOI. These major differences result in entirely different shapes of the substrate-binding loop and presence of very different chemical groups in the substrate-binding site of LDGLOI compared to *E. coli* homologue suggesting an explanation for the difference in the substrate specificity. Difference in the substrate specificity of the human and LDGLOI enzyme could be exploited for structure-based drug designing of selective inhibitors against the parasite. © 2005 Elsevier Inc. All rights reserved.

Keywords: *Leishmania donovani*; Recombinant glyoxalase I; Overexpression; Inhibitors; Structural analysis

*Leishmania donovani*, a flagellated protozoan parasite, is the causative agent of visceral leishmaniasis. Sandflies transmit promastigote forms of the parasite to the mammalian host, where they invade macrophages and transform into amastigotes. Pentavalent antimonials are the standard first-line treatment for leishmaniasis [1,2], although resistance is a growing problem [3]. The aromatic diamidine pentamidine represents a second line of treatment [4]. Current chemotherapeutic agents are unsuitable, in part because of their high toxicity and the emergence of drug resistance. Thus, identification of novel chemotherapeutic targets is of tremendous economic and medical importance. Leishmanial patient's refractoriness to existing drugs and the availability of a limited repertoire of drugs have become rapidly growing problems. Hence there is an urgent need for the development of new drugs against leishmaniasis.

The glyoxalase system is a ubiquitous detoxification pathway that protects against cellular damage caused by methylglyoxal, a mutagenic and cytotoxic compound that is mainly formed as a by-product of glycolysis. It is also formed during catabolism of amino acids via aminoacetone
and hydroxyacetone [5]. The glyoxalase system comprises of two enzymes, glyoxalase I (GLOI) (lactoylglutathione lyase, EC 4.4.1.5) and glyoxalase II (GLOII) (hydroxyacylglutathione hydrolase, EC 3.1.2.6). Glyoxalase I catalyses the formation of S-d-lactoyl glutathione from the hemithioacetal formed nonenzymatically from methylglyoxal and glutathione. Glyoxalase II converts S-d-lactoyl glutathione to lactate and free glutathione [6]. Thus, glutathione acts as a cofactor in the overall reaction pathway. The glyoxalase system is present in the cytosol of cells and cellular organelles particularly mitochondria. It is found throughout biological life and is thought to be ubiquitous [7]. The widespread distribution suggests it fulfills a function of fundamental importance to biological life. Glyoxalase has a distinct role in cell proliferation and maturation [8]. Glyoxalase enzyme activities have been reported as the earliest phenotypes expressed in embryogenesis [8]. In tumor tissues, high activities of glyoxalase I has also been reported [9]. The main source of energy for uncontrolled cell division and proliferation in tumor tissues is glycolysis that produces methylglyoxal, which in turn is detoxified by glyoxalase system. However, despite its ubiquitous distribution little is known about its function. Inhibitors of glyoxalase I have been reported to be selectively toxic to proliferating cells, which could be due to increased accumulation of methylglyoxal that could lead to inhibition of DNA synthesis [10,11]. Glyoxalase I inhibitors have also been reported to have antimarialial [12] and antitrypanosomal activities [13]. The glyoxalase I activity has been reported in Leishmania braziliensis [14] but very low levels of GLOI and GLOII activities were detected in lysates using glutathione as the substrate [15]. Glyoxalase system of the pathogenic kinetoplastids has been recently reported to be unique, as a consequence of these protozoa possessing an unusual thiol metabolism. In these organisms, instead of glutathione, the major low molecular mass thiol is trypanothione \(N^2, N^3\)-bis(glutathionyl)spermidine [16]. It has been recently reported that the GLOI system in Leishmania major uses trypanothione as the substitute for glutathione [16]. The metal cofactor is zinc in eukaryotes and nickel in Escherichia coli [17,18] and L. major [16]. Thus, both the substrate and cofactor of leishmanial glyoxalase are different from those of mammalian glyoxalases. The difference in cofactor dependence is reflected in differences between the active sites of the human and Leishmania enzymes, suggesting that the latter may be a target for antimicrobial therapy [19,20].

While biological phenomena characterized in one Leishmania species are frequently taken as pointers to similar activity in other species of the genus, it is important to actually study each individual species. Crucial differences between species exist in this case and this is clearly manifest in pharmacological responses to drug. In this paper, we describe the characterization of glyoxalase I from L. donovani, the most important pathogenic Leishmania species that is responsible for visceral leishmaniasis in India. Using the comparative modelling approach we also examined the plausible structure of LdGLOI based on the available crystal structure of human, yeast, and E. coli GLOI. The 3-D model generated on the basis of the close homologue from E. coli enabled identification of major changes in the shape and residues in the putative active site of LdGLOI compared to E. coli variant thus providing a possible explanation for the different substrate specificity.

Materials and methods

Parasite and culture condition. Leishmania donovani AG83 (MHOM/IN/1983/AG83) promastigotes and strain 2001, a field isolate of L. donovani, were cultured at 22 °C in modified M199 medium (Sigma, USA) supplemented with 100 U/ml penicillin (Sigma, USA), 100 μg/ml streptomycin (Sigma, USA), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco/BRL, Life Sciences Technology, UK).

Axenic amastigotes were obtained after transformation of promastigotes to amastigotes and were grown in RPMI-1640 medium (pH 5.5) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 20% heat-inactivated serum in a CO2 incubator (5% CO2) at 37 °C. Axenically grown amastigotes of the cloned wild type strains of L. donovani were maintained by weekly passages in RPMI 1640 (pH 5.5) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% heat inactivated serum in a CO2 incubator (5% CO2) at 37 °C. From a starting inoculum of \(5 \times 10^5\) amastigotes/ml, cell densities in the range of \(2 \times 10^6 \times 2 \times 10^5\) parasites/ml were obtained on day 7. The population of axenically grown amastigotes appeared homogeneous, round to ovoid, amastigote, and immobile [21].

Nucleic acid isolation, pulse field gradient gel electrophoresis (PFGE), and hybridization analysis. Genomic DNA was isolated from \(-2 \times 10^9\) L. donovani AG83 promastigotes by standard procedures [22], digested with different restriction endonucleases, and subjected to electrophoresis in 0.8% agarose gels. The fragments were transferred to nylon membrane (Amersham Pharmacia Biotech) and subjected to Southern blot analysis. Total RNA was isolated from \(2 \times 10^8\) L. donovani wild type promastigotes and from GLOI overexpressing strain using TRI reagent (Sigma). For Northern blot analysis, 15 μg of total RNA was fractionated by denaturing agarose gel electrophoresis and transferred onto nylon membrane following standard procedures.

Leishmania chromosomes were separated by PFGE in which low melting agarose blocks, containing embedded cells (10^6 log phase promastigotes/ml), were electrophoresed in a contour clamped homogenous electric field apparatus (CHEF DRILL, Bio-Rad) in 0.5x TBE with buffer circulation at a constant temperature of 14 °C. Saccharomyces cerevisiae chromosomes were used as size markers. Pulse field gel electrophoresis (PFGE) running conditions were as follows: initial switch time, 60 s; final switch time, 120 s; run time; 24 h; current 6 V/cm; including angle 120°. Following the transfer of DNA, RNA, and chromosomes onto nylon membranes, the membranes were rinsed in 2x SSC. The nucleic acids were UV cross-linked to the membrane in a StrataGen UV cross-linker. Prehybridization was done at 65 °C for 4 h in a buffer containing 0.5 M sodium phosphate; 7% SDS; 1 mM EDTA, pH 8.0, and 100 μg/ml sheared denatured salmon-sperm DNA. The blots were hybridized with denatured [x-32P]cIPT-labelled DNA probe (PCR probe described for the L. donovani GLOI coding region) at 10⁵ cpm/ml, which was labelled by random priming (NEB Blot Kit, New England Biolabs). Membranes were washed sequentially as follows: 2x SSC, 0.1% SDS; 1x SSC, 0.1% SDS; 0.5x SSC, 0.1% SDS; 0.2x SSC, 0.1% SDS for 10 min each at 65 °C until the non-specific counts had substantially reduced. Membranes were air-dried and exposed to imaging plate. The image was developed by PhosphorImager (Fuji film FLA-5000, Japan) using Image Quant software.

Cloning of glyoxalase I gene from L. donovani. A 426 bp DNA fragment was amplified from genomic DNA, using a sense primer with a flanking BamHI site, 5'-CGCGGATCCATGCGGTTCTGATG-3', the 3' end for the amplification sequence MPSRMM at position 1-18, and the antisense primer with a flanking HindIII site,
1 mM) (Bachem) was reduced with 3 mM DTT at 20 °C. The assay mixture contained, in a final volume of 0.5 ml; crude Leishmania cell lysate. For kinetic studies, the same assay mixture as used for the Glyoxalase I assay was also used for comparative analysis. This is because several crystal structures are available for comparison.

**Expression and purification procedure.** Expression from the construct pET30a-LdGLOI was induced at 0.6 of OD with 0.5 mM IPTG (Sigma) at 37 °C for different time periods. Bacteria were then harvested by centrifugation and the cell pellet was resuspended in binding buffer (50 mM sodium phosphate buffer, pH 7.5, 10 mM imidazole, pH 7.0, 300 mM sodium chloride, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 30 µl protease inhibitor cocktail). The protein was eluted with increasing concentrations of imidazole, pH 7.0. The imidazole was removed by dialysis in 20 mM sodium phosphate buffer, pH 7.5. The purified protein was aliquoted and stored at −80 °C.

**Cross-linkage of subunits.** The recombinant GLOI protein was cross-linked to 0.1%, 0.25%, and 0.025% glutaraldehyde in phosphate-buffered saline (pH 7.0) [20]. The reaction mixture was incubated for 20 min at 37 °C and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel. The protein samples were subjected to gel electrophoresis and transferred to nitrocellulose membranes. The Western blot analysis was performed using polyclonal antibodies raised against recombinant GLOI protein (15 µg) with incomplete adjuvant at two-week intervals to produce the polyclonal antibodies against the recombinant GLOI protein. The mAbs were bled after 2 weeks after the second booster and sera were collected and used for Western blot analysis.

**Preparation of crude lysate of *L. donovani* for glyoxalase activity.** 1 × 10^9 promastigotes of *L. donovani* were harvested in the late log phase by centrifugation, at 1500 g, at 4 °C for 15 min, washed with phosphate-buffered saline with 1% glucose (PBSG), pH 7.4. The cell pellet was resuspended in lysis buffer (20 mM Mops, pH 7.2, 1 mM DTT, 2 mM PMSF, 5 µl protease inhibitor cocktail) and incubated on ice for 10 min. The cells were lysed by freeze-thaw in liquid nitrogen. The lysate was centrifuged at 20,000 g for 30 min at 4 °C and the supernatant was used for GLOI assay as mentioned below.

**Glyoxalase I assay.** The activity of recombinant purified glyoxalase I was assayed spectrophotometrically at room temperature by measuring the initial rate of formation of S-O-lactoyl trypanothione at 240 nm as described by Racker with slight modification [26]. Trypanothione diulide (1 mM) (Bachem) was reduced with 3 mM DTT at 60 °C for 20 min before the assay. The resulting reduced trypanothione was used for GLOI assay. The assay mixture contained, in a final volume of 0.5 ml; 100 mM MOPS buffer, pH 7.2; 400 µM methylglyoxal (Sigma); 300 µM reduced trypano­thione and 20 µM NiCl2 [16]. The assay mixture was incubated for 10 min followed by the addition of either purified recombinant GLOI protein or crude Leishmania cell lysate. For kinetic studies, the same assay mixture as mentioned above contained either varying concentrations of reduced trypanothione or methylglyoxal with concentrations ranging from 0 to 600 µM each. Trypanothione hemithioacetal concentration was calculated by using the published K_d value of 3 mM for the methylglyoxal-glutathione equilibrium [27]. The value for the S_meso was taken as 2.86 mM M^−1 cm^−1 for the isomerization of trypanothione hemithioacetal of methylglyoxal [28]. All assays were performed in triplicate.

**Antibody production and Western blot analysis.** The purified recombinant GLOI protein (20 µg) was subcutaneously injected in mice using Freund’s complete adjuvant, followed by two booster doses of recombinant GLOI protein (15 µg) with incomplete adjuvant at two-week intervals to produce the polyclonal antibody against the recombinant GLOI protein. The mAbs were bled after 2 weeks after the second booster and sera were collected and used for Western blot analysis.

**Transformation and overexpression of glyoxalase I gene in *Leishmania donovani*.** The GLOI ORF was amplified by PCR using a sense primer with a flanking *SalI* site, 5'-TCTCCTAGATGCGCCCTCTCGCTATG-3’, which coded for the amino acid sequence MSPRRM at position 1-18, and the antisense primer with a flanking *HindIII* site, 5'-CCAGCTTTAGCTGCGCCCTCTCGCT-3’, which is homologous to amino acid residues EQGTA including the stop codon, at position 409-426. The amplified DNA fragment, 426 bp (LdGLOI), was also cloned into the BamHI-*HindIII* site of pET-30a vector (Novagen). The recombinant construct was transformed into BL21 (DE3) strain of *E. coli*.

**Protein determination.** The protein concentration was determined by the method of Bradford using bovine serum albumin as standard [25].
of human GLOI bound to analogues of the substrate are available and also because both human and E. coli homologues show specificity to glutathione while LdGLOI shows specificity to trypanothione.

A 3-D model of LdGLOI has been generated using the suite of programs encoded in COMPOSER [30,31] and incorporated in SYBYL (Tripos, St. Louis). The structurally conserved regions, which are largely a-helical and β-strand regions, in template structures are extrapolated to LdGLOI sequence. The rest of the regions that show high divergence from the sequence of the template structures were modelled by identifying a suitable segment from a dataset of non-identical protein structures. This has been done by a template matching approach, wherein a search is made for the loop segments with required number of residues and that match with the end to end distances of the structurally conserved regions across the three 'anchor' C's on either side of the loop. The hits so obtained are then ranked [32]. The best ranking loop with no short contact with the rest of the structure has been fitted using the ring closure procedure of F. Eisenmenger (unpublished results). Side chains are modelled on the equivalent positions as seen in template structure wherever appropriate or by using rules derived from analysis of known protein structures [33]. The model thus obtained was subject to energy minimization to relieve the short contacts if any.

The model generated using COMPOSER has been subjected to energy minimization using the AMBER force field [34] encoded in the SYBYL software. In the initial rounds of energy minimization, the side chain atoms were allowed to move keeping the backbone position fixed in order to first sort out the short contacts amongst the side chain atoms. In the further rounds, the restriction on the movement of backbone atoms while minimization was also lifted. In the final cycles of minimization, an electrostatic term has been included in the force field. This approach ensured that the LdGLOI model generated is free of short contacts and bad geometry.

Results

Sequence analysis and genomic organization

In order to clone the gene encoding glyoxalase I (GLOI), PCR was performed using specific oligonucleotides, whose sequence was based on Leishmania Genome Sequencing Project of Leishmania infantum (www.ebi.ac.uk/parasites/LGN/). The sense primer was 5’-CGGGATCCATGGCCGTCCTGTGATG-3’, that corresponded to amino acid sequence EQGT A and incorporated in SYBYL program. The amino acids are numbered to the left of the respective sequences. Residues that are identical or similar with other glyoxalases are indicated in black showing complete identity and gray when they are conserved in at least three sequences. The symbol * indicate amino acids that are responsible for metal binding in the human and E. coli.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession No.</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. coli</td>
<td>NP_079650</td>
<td>SYBYL</td>
</tr>
<tr>
<td>L. donovani</td>
<td>AAU87880</td>
<td>SYBYL</td>
</tr>
<tr>
<td>L. infantum</td>
<td>Lin3352600</td>
<td>SYBYL</td>
</tr>
<tr>
<td>T. cruzi</td>
<td>AAU87880</td>
<td>SYBYL</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>Lin1352600</td>
<td>SYBYL</td>
</tr>
<tr>
<td>M. musculus</td>
<td>Lin3352600</td>
<td>SYBYL</td>
</tr>
<tr>
<td>P. putida</td>
<td>Lin3352600</td>
<td>SYBYL</td>
</tr>
</tbody>
</table>

Fig. 1. Multiple sequence alignment of glyoxalase I sequences from L. donovani (AAU87880), L. major (NP_079650), L. infantum (Lin3352600), T. cruzi (Te00.104703513074.70), P. putida (WZPSLP), E. coli (NP_753939), Synechoccus sp. WH102 (NP_898436), H. sapiens (Swiss-Prot Accession No. P78375), and M. musculus (NP_079650) using CLUSTALW program. The amino acids are numbered to the left of the respective sequences. Residues that are identical or similar with other glyoxalases are indicated in black showing complete identity and gray when they are conserved in at least three sequences. The symbol * indicate amino acids that are responsible for metal binding in the human and E. coli.
was obtained, cloned, and sequenced. A single open reading frame consisting of 426-bp was isolated (Leishmania donovani glyoxalase I gene, GenBank Accession No. AY739896) showing a 96% identity to L. major trypanothione-dependent glyoxalase I (GLOI) sequence (GenBank Accession No. AY604654) and 73% identity to Trypanosoma cruzi, lactoylglutathione lyase-like protein, putative (Tc00.1047053510743.70).

The open reading frame encoded for putative polypeptide of 141 amino acids, with a predicted molecular mass of 16.3 kDa, which is very similar to L. major (141 amino acids), L. infantum (141 amino acids), and T. cruzi putative lactoylglutathione lyase-like protein (141 amino acids) enzymes but slightly smaller than the human (184 amino acid) and Pseudomonas putida (163 amino acid) enzymes (Fig. 1). The predicted isoelectric point (pI) of L. donovani GLOI was determined to be, pH 4.97, which is comparable to those of proteins from L. major, L. infantum, and T. cruzi. There was only 33% identity between human GLOI (Swiss-Prot Accession No. P78375) and Leishmania donovani GLOI (GenBank Accession No. AAU87880) sequences (Fig. 1). The L. donovani GLOI protein sequence was found to be 53% identical to Synechococcus sp. WH 8102 (GenBank Accession No. NP_898436), 50% identical to Salmonella typhimurium (GenBank Accession No. AAC44877), and 49% identical to E. coli CFT073 (GenBank Accession No. NP_753939).

A phylogenetic tree has been constructed (Fig. 2) using the L. donovani GLOI sequence and other representative GLOI sequences. The tree indicates close evolutionary relationship of L. donovani and T. cruzi among the kinetoplastid protozoa. The kintoplastid GLOI sequences are closer to E. coli and Synechococcus sp. enzymes in phylogenetic analysis but have no similarity with human, M. musculus and P. putida.

To determine the L. donovani GLOI gene copy number, Southern blot studies were performed as described under Materials and methods using the 426-bp PCR product as a probe. A single band was obtained (Fig. 3A), revealing that it is a single copy gene. Chromosomal location analysis revealed that L. donovani glyoxalase I gene is placed at a single chromosomal band ~2.2 Mb (Fig. 3B). These data concur with the Leishmania genome sequencing project findings, according to which glyoxalase I gene has been identified on chromosome 35 (2.2 Mb) in L. infantum (www.ebi.ac.uk/parasites/LGN/ chromosome 35.html).

![Fig. 2. Phylogenetic tree using the amino acid sequences of glyoxalase I from L. donovani and other organisms. The tree view program under the CLUSTALW program viewed the phyletic trees derived from the multiple alignments.](image-url)
Northern blotting of total \textit{L. donovani} RNA and PCR-generated 426-bp gene probe revealed a single transcript of ~3.8 kb (Fig. 3C). The presence of a single RNA band in the corresponding Northern blot analysis indicated further the existence of a single encoding gene.

\textit{Over-expression and purification of full-length \textit{L. donovani} GLOI enzyme in E. coli}

In order to characterize the recombinant protein, the encoding \textit{L. donovani} GLOI sequence was cloned frame in pET-30a expression vector with its own start ATG codon. The resultant pET-30a-\textit{L. donovani} GLOI construct was transformed into \textit{E. coli} and protein overexpression induced as described under Materials and methods. A protein with a molecular weight that matched the estimated ~24 kDa according to amino acid composition of \textit{L. donovani} GLOI with His tag and S-tag present at its N-terminal end was induced (Fig. 4A). The recombinant protein was purified on Ni$^{2+}$-NTA affinity chromatography column (Fig. 4B). Purification of His-tagged \textit{L. donovani} GLOI by metal affinity chromatography yielded ~5 mg of pure protein from a 1-L bacterial culture.

In order to determine the number of subunits in the recombinant glyoxalase 1, the homogeneous protein was cross-linked with the bifunctional reagent glutaraldehyde (0.1\%, 0.05\%, and 0.025\% respectively) prior to electrophoresis on a 10\% polyacrylamide gel in the presence of SDS (Fig. 4C). Lane 1 shows recombinant GLOI without glutaraldehyde showing a band size of ~23.44 kDa. The results in lane 2, 3, and 4 show recombinant GLOI cross-linked with 0.1\%, 0.05\%, and 0.025\% of glutaraldehyde, respectively. Bands corresponding to GLOI dimer of ~46 kDa can be seen (Fig. 4C). Lysozyme (14.4 kDa) from chicken egg white, a known monomer when cross-linked with the bifunctional reagent glutaraldehyde on electrophoresis on a 10\% polyacrylamide gel in the presence of SDS appeared as a band of ~14 kDa (data not shown).

Recombinant GLOI was used to raise polyclonal antibody in BALB/c mice as described under Materials and methods. The antiserum recognized ~24 kDa fusion protein on Western blot of purified recombinant \textit{L. donovani} GLO-I fusion protein (Fig. 5A). A Western blot using size-fractionated parasite protein, the antiserum could detect a band of anticipated \textit{L. donovani} GLOI size ~16 kDa in promastigote extracts, which is in agreement with the value calculated from the predicted sequence (Fig. 5B). A Western blot using promastigote (50 \mu g) and amastigote extracts (50 \mu g) did not show any detectable difference with the polyclonal antiserum (Fig. 5B). Expression of LdGLOI protein in promastigotes from AG83 strains varies during growth in culture (Figs. 5C, D, and E). Protein accumulation increased somewhat on a per cell basis from 24 h of growth (early log phase) to reach a maximum at 96 h (a late log phase) after which a slight decrease was observed as the cells reached stationary phase (120 h).

\textit{Leishmania donovani} glyoxalase 1 activity

The kinetic parameters of recombinant \textit{L. donovani} glyoxalase 1 were determined with trypanothione hemithioacetal as substrate. The effect of both the substrates namely reduced trypanothione (at fixed concentration of methylglyoxal) and methylglyoxal (at fixed concentration of reduced trypanothione) on glyoxalase 1 activity was studied using nickel as a cofactor. Increase in the concentration of either reduced trypanothione or methylglyoxal showed similar \(K_m\) values towards trypanothione hemithioacetal.
Overexpression of glyoxalase I in *L. donovani*

To evaluate the consequences of GLOI overexpression, GLOI protein was measured in wild type and GLOI overexpressors. Western blot analysis of wild and GLOI overexpressing cell extract demonstrated a marked increase in GLOI protein in the overexpressers (Fig 6A). Northern blot analysis of wild and GLOI overexpressing cells showed overexpression of GLOI transcript (Fig. 6B).

**Glyoxalase I inhibitor profiles**

The IC50 values of known inhibitors of human and yeast glyoxalase I were obtained for both the wild type and overexpressing *L. donovani* strains (Table 1). There was no difference in the IC50 values between the wild type and overexpressing strains with hydroxynaphthoquinone derivative lapachol (IC50 ~ 94 μM) and quercetin (IC50 ~ 26 μM). Purpurogallin, another known inhibitor of human and yeast glyoxalase I, has an IC50 of 70 μM for the wild type and 132 μM for the GLOI overexpressing *L. donovani*. Flavones have been reported to be potential inhibitors of glyoxalase I [35]. In the present study, the IC50 value of flavone was found to be ~56 μM for the wild type *L. donovani* strain and ~70 μM for the GLOI overexpressing *L. donovani*. Glyoxalase I overproducer exhibited significant resistance to purpurogallin and flavone. In contrast, the IC50 values of wild type and GLOI overproducer for lapachol and quercetin were equivalent (Table 1).

**3-D model of *L. donovani* glyoxalase I**

The sequence of LdGLOI could be fitted comfortably onto the fold of *E. coli* GLOI with all the regular secondary structure elements conserved. The regions of alignment between the template and the model sequences involving insertions/deletions of residues have been modeled using the database searching mentioned under Materials and methods. The energy minimization resulted in stereochemically sound model with no short contacts between non-bonded atoms. Fig. 7 shows the superposition of the Cα traces of the model and the template structure with some of the functionally important residues shown.

Several crystal structures of human GLOI are available bound to ligands which give an opportunity to understand the structural basis of substrate specificity. It is known that both *E. coli* and human homologues of GLOI form dimers. Ligand bound complex structure of human GLOI (Fig. 8) shows that the two subunits interact closely with residues from both the subunits participating in the ligand recognition. The available structural knowledge about such ligand binding is extrapolated to the structural model of LdGLOI in order to understand the different substrate specificity of LdGLOI compared to *E. coli* GLOI.

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as substrate ($K_m$ 28.4 ± 3 μM). The *L. donovani* glyoxalase I showed a marked preference for trypanothione hemithioacetal as substrate over glutathione hemithioacetal (data not shown). Recombinant *L. donovani* glyoxalase I had specific activity of 340 × 10^4 nmol min⁻¹ mg⁻¹ protein and that of the native enzyme from the crude Leishmania lysate was 340 nmol min⁻¹ mg⁻¹ protein using trypanothione hemithioacetal as substrate.

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**Fig. 4.** Overexpression and purification of *L. donovani* glyoxalase I protein. (A) Coomassie blue staining of SDS–PAGE showing overexpression of full-length *L. donovani* glyoxalase I protein in *E. coli*. The pET-30a bacterial extract before induction (lane 1) and after induction (lanes 2–6) at 15, 30 min, 1, 2, and 3 h, respectively with 0.5 mM IPTG. Arrow shows the induced recombinant glyoxalase I protein. Broad range protein MW marker (Bio-Rad) was used to identify the size of the recombinant protein. (B) Purification of glyoxalase I protein on Ni²⁺ affinity resin. Lane 1, glyoxalase I without Ni²⁺ affinity resin; lanes 2–4, eluted fractions showing purified glyoxalase I protein from the affinity column; lane 5, supernatant from the crude lysate; lane 6, broad range protein MW marker (Bio-Rad). (C) Analysis of subunit structure of glyoxalase I protein. Protein samples were run on a 10% SDS–polyacrylamide gel. Lane 1, glyoxalase I without cross-linkage treatment; lanes 2, 3, and 4 glyoxalase I cross-linked with 0.1%, 0.05%, and 0.025% glutaraldehyde respectively; lane 5, broad range protein MW marker (Bio-Rad).
Fig. 5. Western blotting using anti-His-GLOI antibody. (A) Western blot analysis of different concentrations of purified GLOI-His fusion recombinant protein (lanes 1–4 containing 0.15, 0.30, 1.5, and 3.0 µg of recombinant protein, respectively). Prestained broad range protein molecular weight marker (Bio-Rad) was used to identify the size of the recombinant protein on the Western blot. (B) Western blot analysis of cell extracts of promastigotes (lane 1) and amastigotes (lane 2) reacted with anti-His-GLOI antibody. Arrow shows the position of the L. donovani GLOI. (C) Abundance of GLOI protein during the growth of L. donovani promastigotes. Promastigotes of strain AG83 were harvested at different times during growth in culture and proteins were separated by SDS-PAGE. Western blot shows 0.1 µg of purified His-GLOI fusion protein (lane 1); a leishmanial promastigote cell extract (50 µg per lane) at 0 h (lane 2); 24 h (lane 3); 48 h (lane 4); 72 h (lane 5); 96 h (lane 6); and 120 h (lane 7). (D) Densitometric scanning of the Western blot in (C). The bands were quantified by scanning on a densitometer and signal intensities relative to the zero point control are plotted. (E) Growth of wild type L. donovani. Parasites were enumerated every 24 h by counting in a hemocytometer. All these experiments were repeated thrice with essentially similar results.

Table 1

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>IC₅₀ values (µM)</th>
<th>GLOI overexpressor</th>
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<tbody>
<tr>
<td>Purpurogallin</td>
<td>70 ± 10</td>
<td>132 ± 4</td>
</tr>
<tr>
<td>Flavone</td>
<td>56 ± 2.6</td>
<td>69 ± 5.3</td>
</tr>
<tr>
<td>Quercetin</td>
<td>26 ± 1.8</td>
<td>27 ± 3.5</td>
</tr>
<tr>
<td>Lapachol</td>
<td>93 ± 3.5</td>
<td>96 ± 5.3</td>
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IC₅₀ represents the concentrations of the drugs, which inhibited the growth of wild type and overexpressors by 50%. Each value is the mean of three independent experiments.

Discussion

All organisms require systems to shield them from chemical stress, such as the antioxidant enzymes that detoxify endogenous oxidants and the enzymes that metabolize exogenous toxins. However, endogenous toxins such as the reactive α-oxoaldehyde, methylglyoxal, are also byproducts of metabolism [5]. Methylglyoxal reacts rapidly with both proteins and nucleic acids and thus is both toxic and mutagenic [3,36]. Methylglyoxal is formed mainly by the degradation of triose phosphates, and also...
Fig. 7. Superposition of Cα traces of the crystal structure of E. coli GLOI (red) [20] and the model of LdGLOI (blue). Clear difference in the size and shape of the substrate-binding loop (near Arg to Tyr substitution) can be seen. Side chains of two of the critical residues (Arg and Tyr in LdGLOI) and another residue proximal to the functional site (His in LdGLOI) are also shown. This figure has been prepared using Setor software [45]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Fig. 8. Close-up of the substrate-binding site in the crystal structure of human GLOI bound to nitrobenzyloxycarbonylglutathione [19]. The Cα traces of the two closely interacting subunits of the dimeric human GLOI are shown in green and yellow. The transition-state analogue is shown in blue. The side chains of the three critical functional residues Arg 37 and Arg 103 (according to the residue numbering of human GLOI) from one of the two subunits and Arg 122 from the other subunit are shown. Arg 122 of the human GLOI corresponds to Tyr in LdGLOI. This figure has been prepared using Setor software [45]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)
with the bacterial enzyme (Pseudomonas putida) is 55% and with yeast enzyme between residues 1-182 and 183-326 (S. cerevisiae) is 47% suggesting that glyoxalase I of different origins may have arisen by divergent evolution from a common ancestor.

In this paper, we describe the molecular cloning and characterization of glyoxalase I from L. donovani, the most important pathogenic Leishmania species that is responsible for visceral leishmaniasis in India. The gene of glyoxalase I is located on chromosome 35. Comparison of the glyoxalase I from L. donovani and those of L. major, L. infantum, and T. cruzi protein showed 70% identity to T. cruzi lactoylglutathione lyase-like protein, 97% identity with L. major glyoxalase I, and 98% identity with L. infantum glyoxalase I protein. As in the case of L. major glyoxalase I the enzyme is trypanothione-dependent rather than glutathione-dependent. We have successfully cloned glyoxalase II (GLOII) from L. donovani (GenBank Accession No. AY851655) and there is only 30% identity between human GLOII and L. donovani GLOII sequence. Glyoxalase II recombinant fusion protein has a molecular mass of ~38 kDa and is a monomer. Glyoxalase II strongly prefers thioesters of trypanothione, instead of glutathione, as substrate (data not shown). Further characterization of glyoxalase II is presently going on in the laboratory.

Phylogenetic tree analysis showed a close evolutionary relationship of L. donovani and T. cruzi among the kinetoplastid protozoa. The kintoplastid GLOI sequences are closer to E. coli and Synechococcus sp. enzymes in phylogenetic analysis but no similarity with human, M. musculus, and P. putida.

Western blot analysis of whole cell lysates of promastigote of L. donovani using the polyclonal antibody against GLOI enzyme shows a single band of approximately 16-kDa and the same antibody recognized the recombinant protein of about 24 kDa expected size of the GLOI-His tag fusion protein. Cross-linking studies established that the recombinant GLOI is a dimer of equal subunits. Expression of LdGLOI protein in promastigotes from AG83 strains varies during growth in culture. Maximum protein accumulation was observed at 96 h (a late log phase). Sequence analysis of L. donovani glyoxalase I protein for metal binding showed high degree of conservation of the metal-binding residue between the trypanosomatid and E. coli GLOI enzymes. Recombinant parasite glyoxalase I enzyme required nickel for its activity thereby further confirming its close relationship to E. coli glyoxalase I enzyme. Earlier studies have shown that recombinant L. major glyoxalase I is unique among eukaryotic enzymes since it uses nickel as a cofactor, a property seen in E. coli enzyme [16]. L. donovani GLOI showed high level of specificity for trypanothione hemithioacetal and little activity with glutathione-hemithioacetal.

Previous studies have shown enhanced levels of glyoxalase I protein in human tumor tissues when compared to corresponding normal tissues [43]. In an attempt to study the significance of this overexpression, we transfected the L. donovani with glyoxalase I gene. Overexpression of glyoxalase I in transfectants was observed both by Northern and Western blot analysis. Inhibitors of glyoxalase I are known to be potential anticancer and antimalarial agents [43,12]. In this report, we evaluated, effect of several of the compounds that have been previously reported to have adverse effect on the human and yeast glyoxalase I enzyme, on both wild type and overexpressing L. donovani cells [44]. The IC50 values of several known inhibitors of human and yeast glyoxalase I showed that the compounds tested were weak inhibitors of L. donovani growth (lapachol, IC50 = 100 μM and quercetin IC50 of 26 μM in L. donovani compared to 0.35–0.7 μM for lapachol and IC50 of 10 μM for quercetin in human). Weak response of L. donovani to known inhibitors of glyoxalase I is a highly significant observation and could be related to the structural differences between the parasite and the host enzyme. When the cells were treated with purpurogallin or flavone, known inhibitor of human and yeast glyoxalase I, the transfectant cell line exhibited approximately 1.8- and 1.2-fold resistance, respectively, to the cytotoxic effects of these inhibitors when compared to control cell lines. However, hydroxynaphthoquinone derivatives lapachol and quercetin did not show any difference in the IC50 values between the control and overexpressing cells.

While there are a number of important residues involved in the ligand binding to human GLOI, there are three critical residues essential for the function and these residues are also proximal to the substrate. Following the residue numbering of LdGLOI, the residues in the human homologue, Arg 8 and Asn 63 from a subunit in the dimer and Arg 101 in the other subunit, are the critical residues involved in function and also in ligand binding. These residues correspond to positions 37, 103, and 122, respectively, according to the numbering followed in the crystal structure of human GLOI complexed to a transition state analogue, Nitrobenzoxycarbonylglutathione [19]. A close-up of the structure of Nitrobenzoxycarbonylglutathione bound to the human GLOI is shown in Fig. 8. Out of the three crucial residues Arg 8 and Asn 63 (following the numbering of LdGLOI) are absolutely conserved in the members of the family (Fig. 1). Interestingly, Arg 101 is conserved or conservatively substituted by Lys in all homologues except in those from Leishmania organisms. In the homologues from Leishmania organisms, this Arg residue is replaced by Tyr. Remarkably, Tyr in this position is also present in the GLOI of T. cruzi. Thus, it appears that presence of Tyr in this alignment position is possibly a property of those homologues which are known to be or expected to be specific to trypanothione. Presence of very different residues, Arg (positively charged) and Tyr (aromatic, uncharged polar), present, respectively, in glutathione-specific and trypanothione-specific GLOI can be expected to play a crucial role indicating the specificity apart from other key changes as discussed below.
The critical Tyr 101 of LdGLOI model is located in a region of insertions/deletions (Fig. 1) corresponding to the substrate-binding loop. Considering this loop region there are two key distinguishing features between glutathione and trypanothione-specific homologues. First, as also noted by Vickers et al. [16], the number of residues in various glutathione-specific enzymes is more than that in the substrate-binding loop of trypanothione-specific homologues (Fig. 1). This results in a substantially long and bulky loop in the case of glutathione-specific enzymes compared to trypanothione-specific homologues. This change in the loop size also results in entirely different conformations and overall shapes of these loops. Fig. 7 shows the superposition of the crystal structure of E. coli GLOI and the modeled structure of LdGLOI. The clear changes in size and shape of the substrate-binding loop can be appreciated.

Second, as can be seen in Fig. 1, the substrate-binding loop of glutathione-specific GLOI sequences such as homologues from human and E. coli contains a substantial number of positively charged residues apart from a few acidic residues. However in the case of trypanothione-specific GLOI sequences such as those from L. donovani and T. cruzi, the substrate-binding loop is generally devoid of positively charged amino acids. This difference can also possibly influence the substrate-specificity.

Finally, in view of the uniqueness of L. donovani glyoxalase I enzyme, it could be exploited for structure-based drug design of selective inhibitors against the parasite. Further work related to gene-knockout of glyoxalase I is presently going on to elucidate the importance of this enzyme in L. donovani.

Acknowledgments

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Characterization of the gene encoding glyoxalase II from Leishmania donovani: a potential target for anti-parasite drugs

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The glyoxalase system is a ubiquitous detoxification pathway that protects against cellular damage caused by reactive oxoaldehydes such as methylglyoxal which is mainly formed as a by-product of glycolysis. The gene encoding GLOI (glyoxalase II) has been cloned from Leishmania donovani, a protozoan parasite that causes visceral leishmaniasis. DNA sequence analysis revealed an ORF (open reading frame) of ~888 bp that encodes a putative 295-amino-acid protein with a calculated molecular mass of 32.5 kDa and a predicted pI of 6.0. The sequence identity between human GLOI and LdGLOI (L. donovani GLOI) is only 35%. The ORF is a single-copy gene on a 0.6-Mb chromosome. A ~38 kDa protein was obtained by heterologous expression of LdGLOI in Escherichia coli, and homogeneous enzyme was obtained after affinity purification. Recombinant L. donovani GLOI showed a marked substrate specificity for trypanothione hemithioacetal over glutathione hemithioacetal.

INTRODUCTION

The glyoxalase system catalyses the conversion of 2-oxoaldehydes into the corresponding 2-hydroxy acids [1–4]. The process involves two consecutive reactions mediated by two enzymes, GLOI (glyoxalase I) (lactoylglutathione lyase, EC 4.4.1.5) and GLOII (glyoxalase II) (hydroxyacylglutathione hydrolase, EC 3.1.2.6). GLOI catalyses the formation of S-b-lactoylglutathione from the hemithioacetal formed non-enzymatically from methylglyoxal and glutathione. GLOII converts S-b-lactoylglutathione into lactate and free glutathione [1–4]. Thus glutathione acts as a cofactor in the overall reaction pathway. The glyoxalase system is present in the cytoplasm of cells and cellular organelles, particularly mitochondria. The two distinct enzymes have been purified from a wide variety of species, including prokaryotes, plants and mammals [5–11]. The widespread distribution suggests that the system fulfils a function of fundamental importance to biological life, and glyoxalase has a distinct role in cell proliferation and maturation [6]. Glyoxalases seem to be ubiquitous, in accord with their proposed role of detoxifying methylglyoxal, which is a physiological substrate, protecting against cellular damage. Methylglyoxal reacts rapidly with both proteins and nucleic acids and thus is both toxic and mutagenic [4,12,13]. Methylglyoxal is produced mainly as a by-product of glycolysis. It is formed mainly by the degradation of triose phosphates, and also by metabolism of ketone bodies, threonine degradation and the fragmentation of glycated proteins [14,15]. GLOI is part of the glyoxalase system present in the cytosol and it prevents the accumulation of these reactive α-oxoaldehydes and thereby suppresses α-oxoaldehyde-mediated glycation reactions [16]. It is a key enzyme of the anti-glycation defence. Genes encoding GLOI and GLOII have been characterized from several species [8–10,17–20].

Leishmania donovani, a flagellated protozoan parasite, is the causative agent of visceral leishmaniasis. Quinvariant antimonials are the standard first-line treatment for leishmaniasis [21,22], although resistance is a growing problem. The aromatic diamidine pentamidine represents a second line of treatment [23]. Leishmanial patients' refractoriness to existing drugs and the availability of a limited repertoire of drugs has become a rapidly growing problem. Hence, there is an urgent need for the development of new drugs against leishmaniasis. The GLOI activity has been reported in Leishmania braziliensis [24], but very low levels of GLOI and GLOII activity were detected in lysates using glutathione as the substrate [25]. The glyoxalase system of the pathogenic kinetoplastids has been reported recently to be unique, as a consequence of these protozoa possessing an unusual thiol metabolism [25,26]. In these organisms, instead of glutathione, the major low-molecular-mass thiol is trypanothione \([N^1,N^4\text{-bis(glutathionyl)spermidine}]\) [25]. It has been recently reported that the GLOI system in Leishmania major uses trypanothione as the substrate for glutathione [25]. In Trypanosoma brucei, GLOII strongly prefers thiocysteine instead of glutathione as substrate [27].

The above findings prompted us to characterize GLOI from Leishmania donovani. In the present paper, we report molecular cloning, expression and characterization of GLOII from Leishmania donovani, the parasite protozoan that is responsible

Antiserum against recombinant LdGLOII protein could detect a band of anticipated size ~32 kDa in promastigote extracts. By overexpressing the GLOI gene in Leishmania donovani using Leishmania expression vector pspahygro, we detected elevated expression of GLOII RNA and protein. Overexpression of the GLOI gene will facilitate studies of gene function and its relevance as a chemotherapeutic target. This is the first report on the molecular characterization of glyoxalase II from Leishmania spp. The difference in the substrate specificity of the human and Leishmania donovani glyoxalase II enzyme could be exploited for structure-based drug design of selective inhibitors against the parasite.

Key words: drug design, glutathione, glyoxalase II, Leishmania donovani, parasite inhibitor, trypanothione.

Abbreviations used: GLOI, glyoxalase I; GLOII, glyoxalase II; LdGLOII, Leishmania donovani GLOII; Ni-NTA, Ni\(^2+\)-nickel nitrotriacetate; ORF, open reading frame; PFGE, pulse-field gradient gel electrophoresis.

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The nucleotide sequence data reported for the Leishmania donovani glyoxalase II gene has been deposited in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY831655.
for visceral leishmaniasis. This is the first report on the characterisation of GLOII from Leishmania species. The difference in the substrate specificity of the human and Leishmania donovani GLOII suggests that the latter may be a target for antimicrobial therapy.

EXPERIMENTAL

Materials

Trypanothione disulphide was obtained from Bachem. Restriction enzymes and Pfu Taq DNA polymerase were obtained from MBI Fermentas. All other chemicals were of analytical grade and were available commercially.

Parasite and culture conditions

L. donovani AG83 (MHOM/IN/1983/AG83) promastigotes were cultured at 22°C in modified M199 medium (Sigma) supplemented with 100 units/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 10% heat inactivated foetal bovine serum (Gibco/BRL, Life Technologies).

Cloning of GLOII gene from L. donovani

An ~888-bp DNA fragment was amplified from genomic DNA, using a sense primer with a flanking BamHI site (underlined), 5′-CGCGGATCCATGCCTAATCTGCACA-3′, that coded for the amino acid sequence MRNYCT at positions 1–18, and the antisense primer with a flanking HindIII site (underlined), 5′-CCCAAGCTTTAGTCGACGCCTTTG-3′, which corresponded to amino acid residues YNACD including the stop codon, at positions 872–888. PCR was performed in 50 μl reaction volumes containing 100 ng of genomic DNA, 25 pmol each of the gene-specific forward and reverse primers, 200 μM of each dNTP, 2 mM MgCl₂, and 5 units of Pfu Taq DNA polymerase. The conditions for PCR were as follows: 94°C for 10 min, then 35 cycles of 94°C for 1 min, 57°C for 45 s and 72°C for 45 s. Final extension was carried out for 10 min at 72°C. A single ~888-bp PCR product was obtained and subcloned into pGEM-T vector (Promega) and subjected to automated sequencing. Sequence analysis was performed by DNastar, whereas comparisons with other sequences of the database were performed using the search algorithm BLAST [28]. Multiple alignment of amino acid sequences was performed using the ClustalW [http://www.ebi.ac.uk/ clustalw/] program. The phylogenetic tree was constructed using the BLAST algorithm and subjected to automated sequencing. Sequence analysis was performed by DNastar, whereas comparisons with other sequences of the database were performed using the search algorithm BLAST [28]. Multiple alignment of amino acid sequences was performed using the ClustalW [http://www.ebi.ac.uk/ clustalw/] program. The phylogenetic tree was constructed using the BLAST algorithm and subjected to automated sequencing. Sequence analysis was performed by DNastar, whereas comparisons with other sequences of the database were performed using the search algorithm BLAST [28].

Expression and purification procedure

Expression from the construct pET30a-LdGLOII was induced at a D₅₀₀ of 0.6 with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) (Sigma) at 37°C for different time periods. Bacteria were then harvested by centrifugation at 5000 g for 15 min, and the cell pellet was resuspended in binding buffer (50 mM sodium phosphate buffer, pH 7.5, 10 mM imidazole, 300 mM sodium chloride, 2 mM PMSF and 30 μl of protease inhibitor cocktail). Lysozyme (100 μg/ml) was added to the cell suspension, which was kept on a rocking platform for 30 min at 4°C. The resulting cells suspension was sonicated six times for 20 s with 1 min intervals. The lysate was centrifuged at 20000 g for 30 min at 4°C. The resulting supernatant, which contained the protein, was loaded on to pre-equilibrated Ni-NTA (Ni²⁺-nitriilotriacetate)-agarose beads (Qiagen). The mixture was kept on a rocking platform for 2 h at 4°C. It was centrifuged at 400 g for 30 min at 4°C. The supernatant was discarded and pellet was washed three times with wash buffer (50 mM sodium phosphate buffer, pH 7.5, 50 mM imidazole, 300 mM NaCl, 2 mM PMSF and 30 μl of protease inhibitor cocktail). The protein was eluted with increasing concentrations of imidazole, pH 7.0. The imidazole was removed by dialysis in 20 mM sodium phosphate buffer, pH 7.5. The purified protein was ρ > 95% pure as judged by SDS/PAGE. The purified protein was divided into 200 μl aliquots and stored at −80°C.

Cross-linkage of subunits

The recombinant GLOII protein was cross-linked with 0.025% (w/v) glutaraldehyde in PBS (pH 7.0) [29]. The reaction mixture was incubated for 20 min at 37°C and analysed by SDS/PAGE using a 10% gel with known standards. The protein samples were mixed with an equal volume of loading buffer containing 100 mM Tris/HCl (pH 6.8), 0.4% SDS, 20% (v/v) glycerol and 0.001% (w/v) Bromophenol Blue and subjected to boiling in a water bath for 5 min.

Nucleic acid isolation, PFGE (pulse-field gradient gel electrophoresis) and hybridization analysis

Genomic DNA was isolated from ~2 × 10⁶ L. donovani AG83 promastigotes at late exponential phase by standard procedures [30], digested with different restriction endonucleases and subjected to electrophoresis in 0.8% (w/v) agarose gels. The fragments were transferred on to nylon membrane (Amersham Biosciences) and subjected to Southern blot analysis. Total RNA was isolated from 2 × 10⁶ L. donovani wild-type promastigotes and from GLOII-overexpressing strain using TRI reagent™ (Sigma). For Northern blot analysis, 15 μg of total RNA was fractionated by denaturing agarose gel electrophoresis and transferred on to nylon membrane following standard procedures.

Leishmania chromosomes were separated by PFGE in which low-melting agarose blocks containing embedded cells (10⁷ exponential phase promastigotes/ml) were electrophoresed in a contour-clamped homogeneous electric field apparatus (CHEF DRIII, Bio-Rad). Saccharomyces cerevisiae chromosomes were used as size markers. PFGE running conditions were as follows: 6 s initial switch time; 120 s final switch time; 24 h run time; 6 V/cm current; 120° including angle. Following the transfer of DNA, RNA and chromosomes on to nylon membranes, the nucleic acids were UV-cross-linked to the membrane in a Stratagene UV cross-linker. Prehybridization was carried out at 65°C for 4 h in a buffer containing 0.5 M sodium phosphate, 7% (w/v) SDS, 1 mM EDTA, pH 8.0, and 100 μg/ml sheared denatured salmon sperm DNA. The blots were hybridized with denatured [α-32P]dCTP-labelled DNA probe (PCR probe described for the L. donovani GLOII-coding region) at 10°C c.p.m/ml, which was labelled by random priming (NEB Blot® Kit, New England Biolabs). Membranes were washed, air-dried and exposed to an imaging plate. The image was developed by Phospholmage (Fuji FLA-5000) using ImageQuant software (Amersham Biosciences).

GLOII assay

The activity of recombinant purified GLOII was determined at room temperature (25°C) by measuring the hydrolysis of the thioester in a coupled assay. The assays were performed in a total volume of 0.5 ml of 100 mM Mops buffer (pH 7.2), various
concentrations of methylglyoxal (0-600 μM) (Sigma) and 300 μM reduced trypanothione [25,27]. Trypanothione disulfide (1 mM) (Bachem) was reduced with 3 mM dithiothreitol at 60 °C for 20 min before the assay. The resulting reduced trypanothione was used for the assay. The assay mixture was incubated for 10 min followed by the addition of purified recombinant L. donovani GLOI protein. Lactotrypanothione was produced by adding 0.3 nmol of recombinant L. donovani GLOI, and the reaction was monitored at 240 nm. At the end of the reaction, 0.3 nmol of recombinant LdGLOII was added, and the rate of decrease in absorbance at 240 nm was measured. Glutathione-dependent GLOII was also measured under similar conditions. The value for Δε240 was taken as 6.5 mM⁻¹·cm⁻¹ for S-D-lactotrypanothione [27]. The experiment was repeated twice with similar results.

**Protein determination**

Protein concentration was determined using the method of Bradford [31] using BSA as standard.

**Antibody production**

The purified recombinant GLOI protein (20 μg) was subcutaneously injected in mice using Freund’s complete adjuvant, followed by two booster doses of recombinant GLOI protein (15 μg) with incomplete adjuvant at 2-week intervals to produce the polyclonal antibody against the recombinant GLOI protein. The mice were bled 2 weeks after the second boost, and sera were collected and used for Western blot analysis.

**Transfection and overexpression of the GLOII gene in L. donovani**

The GLOII ORF (open reading frame) was amplified by PCR using a sense primer with a flanking XbaI site (underlined), 5’-TCTCTCTAGAATGCGCAACTACTGCACA-3’, that coded for the amino acid sequence MRNYCT at positions 1-18, and the antisense primer with a flanking HindIII site (underlined), 5’CCCAAGCTTTCAGTCGCAGGCGTTGT3’, which corresponded to amino acid residues YNACD including the stop codon, at positions 872-888. The amplified DNA fragment (GLOII) was cloned into the XbaI/HindIII site of pspHzg9ro L. donovani shuttle vector (kindly provided by Dr Marc Ouellette, Université Laval, Québec, Canada) to create pspHzg9ro-LdGLOII containing the hygromycin phosphotransferase gene. The construct (20 μg) was transfected into L. donovani promastigotes by electroporation [2 mm gap cuvettes, 450 V, 500 μF (BTX Electro Cell Manipulator 600)]. Transfectants were selected for resistance with hygromycin (40 μg/ml) as described previously [32].

**Western blot analysis**

Promastigotes were lysed by sonication on ice four times with a 10 s pulse at 1 min intervals, and cell supernatants were prepared by centrifugation at 20,000 g protein (50 μg) from promastigotes was fractionated by SDS/PAGE, and blotted on to nitrocellulose membrane using electrophoretic transfer cell (Bio-Rad). Western Blot analysis was carried out using the ECL® (enhanced chemiluminescence) kit (Amersham Biosciences) according to the manufacturer’s protocol. Polyclonal antibody (1:100 dilution) against purified recombinant L. donovani GLOII generated in mice was used for the Western blot analysis. The mouse monoclonal antibody anti-β-tubulin (human) (Santa Cruz) was used as a control. The results shown are from a single experiment typical of at least three giving similar results.

**RESULTS**

**Sequence analysis and genomic organization**

In order to clone the gene encoding GLOII, PCR was performed using specific oligonucleotides whose sequence was based on the Leishmania Genome Sequencing Project of Leishmania infantum (http://www.ebi.ac.uk/parasites/LGN/). Genomic DNA from L. donovani AG83 (MHOM/IN/1983/AG83) promastigotes was used as a template. A single ~888-bp PCR product was obtained, cloned, and sequenced. A single ORF consisting of ~888-bp was isolated (LdGLOII gene, GenBank® accession number AY515655) showing 96% identity with L. major trypanothione-dependent GLOII sequence (LmjF12.0220) [2-hydroxyacylglutathione hydrolase, putativeLeishmania majorchr 12], 99% identity with L. infantum (LinJ12.0200), 53% identity with Trypanosoma cruzi putative lactoylglutathione lyase-like protein (NCBI accession number AAL96759), 51% identity with T. brucei brucei (NCBI accession number CAD37800), 32% identity with Arabidopsis thaliana (NCBI accession number O24496) and 35% identity with human GLOII (NCBI accession number AAM2503) [32]. E. coli probably hydroxacylglycylglutathione hydrolase (NCBI accession number Q47677) shows 28% identical residues (Figure 1).

The ORF encoded a putative polypeptide of 295 amino acids, with a predicted molecular mass of ~32.5 kDa, which is very similar to that of L. major (295 amino acids), L. infantum (295 amino acids) and T. cruzi (putative lactoylglutathione lyase-like protein) (299 amino acids) enzymes, but slightly larger than the human (260 amino acids) enzyme (Figure 1). The predicted pl of LdGLOII was determined to be pH 6.0 which is comparable with those of proteins from L. major, L. infantum and T. cruzi. There was only 33% identity between human GLOII and Leishmania donovani GLOII (NCBI accession number AAW52503) sequences (Figure 1). Metal-binding motif THXXHDXH, common to all GLOIIs consisting of five histidine and two aspartate residues that link directly to two metal ions [33], is highly conserved in LdGLOII sequence (Figure 1). A phylogenetic tree has been constructed (Figure 2) using the LdGLOII sequence and other representative GLOII sequences. The tree indicates the close evolutionary relationship of L. donovani and T. cruzi among the kinetoplastid protozoa. The kinetoplastid GLOII sequences have no similarity with the human enzyme in phylogenetic analysis.

To determine the LdGLOII gene copy number, Southern blot studies were performed as described in the Experimental section using the ~888-bp PCR product as a probe. A single band was obtained (Figure 3), revealing that it is a single-copy gene. Chromosomal location analysis revealed that LdGLOII gene is placed at a single chromosomal band of 600 kb (Figure 4). These data agree with the Leishmania Genome Sequencing Project findings, according to which GLOII gene has been identified on chromosome 12 (600 kb) in L. major (http://www.genedb.org/).

Northern blotting of total L. donovani RNA and PCR-generated ~888-bp gene probe revealed a single transcript of ~5 kb (Figure 5). The presence of a single RNA band in the corresponding Northern blot analysis indicated further the existence of a single encoding gene.

**Overexpression and purification of full-length LdGLOII enzyme in E. coli**

In order to characterize the recombinant protein, the encoding LdGLOII sequence was cloned into-frame in a pET30a expression vector with its own start ATG codon. The resultant pET30a-LdGLOII construct was transformed into E. coli and protein...
overexpression was induced as described in the Experimental section. A protein with a molecular mass that matched the estimated ~38 kDa according to the amino acid composition of LdGLOII with a His tag and an S-tag present at its N-terminal end was induced (Figure 6A). The recombinant protein was purified on an Ni-NTA affinity chromatography column (Figure 6B). Purification of His-tagged LdGLOII by metal-affinity chromatography yielded ~5 mg of pure protein from a 1-litre bacterial culture.

In order to determine the number of subunits in the recombinant GLOII, the homogeneous protein was cross-linked with the bifunctional reagent glutaraldehyde (0.025%) before electrophoresis on a 10% polyacrylamide gel in the presence of SDS (Figure 6C). Lane 1 shows recombinant GLOI cross-linked with 0.025% glutaraldehyde showing a band size of ~46 kDa (dimer). Lane 2 shows recombinant GLOI (23 kDa) without glutaraldehyde. Lane 3 shows recombinant GLOII cross-linked with 0.025% glutaraldehyde. Lane 4 shows recombinant GLOII without glutaraldehyde. A band size of ~38 kDa representing GLOII was observed in lanes 3 and 4. These results show that the predominant species observed on SDS/PAGE corresponded to the molecular mass of a dimer in the case of GLOI in *L. donovani*, whereas GLOII corresponded to a monomer. Lysozyme (14.4 kDa) from chicken-egg white, a known monomer when cross-linked with the bifunctional reagent glutaraldehyde on electrophoresis on a 10% polyacrylamide gel in the presence of SDS, appeared as a band of ~14 kDa (results not shown).

Recombinant GLOII was used to raise polyclonal antibody in BALB/c mice as described in the Experimental section. The results in lane 2 show recombinant GLOI (23 kDa) without glutaraldehyde. Lane 3 shows recombinant GLOII cross-linked with 0.025% glutaraldehyde. Lane 4 shows recombinant GLOII without glutaraldehyde. A band size of ~38 kDa representing GLOII was observed in lanes 3 and 4. These results show that the predominant species observed on SDS/PAGE corresponded to the molecular mass of a dimer in the case of GLOI in *L. donovani*, whereas GLOII corresponded to a monomer. Lysozyme (14.4 kDa) from chicken-egg white, a known monomer when cross-linked with the bifunctional reagent glutaraldehyde on electrophoresis on a 10% polyacrylamide gel in the presence of SDS, appeared as a band of ~14 kDa (results not shown).

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Figure 2 Phylogenetic tree using the amino acid sequences of GLOII from L. donovani and other organisms

The TreeView program of the ClustalW software was used to create the phylogenetic trees from the multiple alignments.

Figure 3 Genomic organization of LdGLOII gene

Southern blot analysis of LdGLOII gene. Lanes 1–11, restriction digests of L. donovani genomic DNA with BamHI, HindIII, EcoRI, NdeI, XbaI, XhoI, SalI, XmaI, NotI, BstEII and BglII respectively. The blot was probed with ~888-bp full-length GLOII gene. DNA molecular-mass marker and the sizes (in kb) are indicated on the left of the figure.

antiserum recognized a ~38 kDa fusion protein on a Western blot of purified recombinant LdGLOII fusion protein (20 ng) (Figure 7A). In a Western blot using size-fractionated parasite protein, the antiserum could detect a band of anticipated LdGLOII size ~32 kDa in promastigote extracts (200 µg), which is in agreement with the value calculated from the predicted sequence (Figure 7A). Our attempts to analyse GLOII protein in the mastigote extracts (200 µg) with the same polyclonal antibody failed. This could be due to the nature of the antiserum.

Overexpression of GLOII in L. donovani

To evaluate the consequence of GLOII overexpression, GLOII protein was measured in wild-type and GLOII-overexpressing L. donovani. Western blot analysis of wild-type and GLOII-overexpressing cell extract demonstrated a significant increase in GLOII protein in the overexpressors (Figure 7A). Northern blot analysis of wild-type and GLOII-overexpressing cells showed overexpression of GLOII transcript (Figure 7B).

Figure 4 PFGE analysis of L. donovani indicating chromosomal localization of the GLOII gene

(a) Chromosomes of S. cerevisiae and L. donovani separated and visualized with ethidium bromide before blotting. (b) The same blot probed with LdGLOII gene probe. The arrow shows the hybridizing band of L. donovani. Sizes are given in Mbp.

LdGLOII activity

The kinetic parameters of recombinant LdGLOII were determined for S-β-lactoyltrypanothione as a substrate in a coupled reaction as mentioned in the Experimental section. LdGLOII hydrolysed S-β-lactoyltrypanothione with a Vₐₘₐₓ value of 0.14 µmol·min⁻¹·mg⁻¹ and a Kₘ value of 0.039 mM (Table 1). LdGLOII showed a high level of specificity for S-β-lactoyltrypanothione and little activity with S-β-lactoylglutathione. The Kₘ of 187 µM has been reported for the human GLOII with S-β-lactoylglutathione [11]. The preference for trypanothione hemithioacetal over glutathione hemithioacetal by LdGLOII could be due to the absence of three conserved basic residues, namely Arg-249, Lys-252 and Lys-143, which are known to be involved in complexing with the substrate in human GLOII [34]. These residues are highly conserved in all GLOII studied so far, except for kinetoplastids [27].
that does not use glutathione as cofactor. Trypanothione, a unique glutathione-dependent glyoxalase system is present in all organisms analysed so far [35,36]. Recently, Lrsch and Krauth-Siegel characterized the GLOII from T. brucei. It is the first GLOII that does not use glutathione as cofactor. Trypanothione, a unique thiol in the kinetoplastid organisms, replaces glutathione in the glyoxalase system of the African trypanosome T. brucei.

DISCUSSION
All organisms require systems to shield them from chemical stress, such as the antioxidant enzymes that detoxify endogenous oxidants and the enzymes that metabolize exogenous toxins. However, endogenous toxins such as the reactive α-oxoaldehyde, methylglyoxal, are also by-products of metabolism [4]. The glyoxalase system is a ubiquitous detoxification pathway that protects against cellular damage caused by methylglyoxal. The glutathione-dependent glyoxalase system is present in all organisms analysed so far [35,36]. Recently, Irach and Krauth-Siegel [27] characterized the GLOII from T. brucei. It is the first GLOII that does not use glutathione as cofactor. Trypanothione, a unique

Figure 6 Overexpression and purification of LdGLOII protein

(A) Coomassie Blue staining of SDS/PAGE gel showing overexpression of full-length LdGLOII protein in E. coli. The pET30a-bacterial extract before induction (lane 2) and after induction (lanes 3 and 4) at 1 and 4 h respectively with 0.5 mM IPTG (isopropyl β-D-thiogalactoside). The arrow shows the induced recombinant GLOII protein. Broad-range protein molecular-mass marker (Bio-Rad) was used to identify the size (in kDa) of the recombinant protein (Lane 1). (B) Purification of the GLOII protein on Ni-NTA affinity resin. Lane 1, broad-range molecular-mass marker (Bio-Rad). Lanes 2–4 are eluted fractions showing purified GLOII protein from the affinity column: lane 2, fraction eluted with 150 mM imidazole, lane 3, fraction eluted with 80 mM imidazole and lane 4, fraction eluted with 60 mM imidazole. (C) Analysis of subunit structure of GLOII protein. Protein samples were run on a 10% SDS/polyacrylamide gel. Lane 1 shows recombinant GLOII cross-linked with 0.025% glutaraldehyde showing a band size of ~46 kDa (dimer), lane 2 shows recombinant GLOII (23 kDa) without glutaraldehyde, lane 3 shows recombinant GLOII cross-linked with 0.025% glutaraldehyde, and lane 4 shows recombinant GLOII without glutaraldehyde. A band size of ~38 kDa representing GLOII was observed in lanes 3 and 4. Lane 5, broad-range protein molecular-mass markers (Bio-Rad). Sizes in kDa are indicated on the right of the Figure.

Figure 7 Analysis of (A) protein and (B) RNA from promastigotes of wild-type L. donovani (WT) and GLOII overexpressor.

(A) a) Recombinant GLOII protein (GLOII) (20 ng) was used as a control. Polyclonal antiserum against LdGLOII generated in mouse was used to detect GLOII protein in fractionated extracts obtained from cultures harvested after 48 h of growth. Lane 1, 20 ng of rGLOII protein, lane 2, 200 μg of total cell lysate of wild-type AG83 promastigotes, lane 3, 200 μg of total cell lysate from GLOII overexpressor. (A, b) The same blot was reprobed with antibody against β-tubulin protein to normalize the loading on each lane of the gel. (B) Total RNA was isolated from exponential phase culture of L. donovani wild-type promastigotes (lane 2) and GLOII overexpressor (lane 3). Lane 1, molecular-mass markers. Total RNA was electrophoresed, transferred on to a membrane and probed with a ~888-bp full-length GLOII gene as described in the Experimental section. An ethidium bromide-stained RNA gel showing equal loading of the RNA is shown. The results shown are from a single experiment typical of at least three giving similar results.

Table 1 Comparison of kinetic parameters of recombinant L. donovani and human GLOII

<table>
<thead>
<tr>
<th>Parameter</th>
<th>L. donovani</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kₐ (μM)</td>
<td>S-hydroxyacetylglutathione</td>
<td>0.14</td>
</tr>
<tr>
<td>V₉₈ (μmol·min⁻¹·mg⁻¹)</td>
<td>S-hydroxyacetylglutathione</td>
<td>ND</td>
</tr>
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The values of human enzyme are taken from [11]. ND, not detected.

To date, GLOII sequences have been reported in several species, including prokaryotes, plants, mammals, yeast and bacteria [8–11]. The present paper describes the molecular cloning and characterization of LdGLOII. The gene of GLOII is located on a 600-bp chromosome. Comparison of the GLOII from L. donovani and that of L. major, L. infantum and T. cruzi protein showed 51% identity with T. cruzi lactoylglutathione lyase-like protein, 96% identity with L. major GLOII and 99% identity with L. infantum GLOII protein. There was 32% identity with A. thaliana, 35% homology with human GLOII and 28% identity with E. coli probable hydroxyacylglutathione hydrolase. Phylogenetic tree analysis showed a close evolutionary relationship of L. donovani and T. cruzi and T. brucei among the kinetoplastid protozoa.
From an evolutionary perspective, it is interesting to note that the kinetoplastid GLOII had very little similarity with human and A. thaliana enzyme in phylogenetic analysis, thereby ruling out a common evolutionary origin of the two enzymes.

The enzyme is trypanothione-dependent rather than glutathione-dependent. GLOII strongly prefers thiosters of trypanothione instead of glutathione as substrate. LdGLOII showed a high level of specificity for trypanothione hemithioacetal and little activity with glutathione hemithioacetal.

Sequence analysis of LdGLOII showed the conservation of metal-binding motif THXXHDH [33]. This metal-binding motif, along with two histidine residues and an aspartate residue interacts directly with two metal ions and is highly conserved in all glyoxalases studied so far [34]. It has been reported previously that storage of T. brucei GLOII causes a simultaneous decrease of $k_{m}$ and $K_{m}$ [27]. A similar observation was made with GLOII of L. donovani. As in A. thaliana, the reason for this could also be the loss of metal as described for the A. thaliana enzyme [11].

The molecular mass of the enzyme was calculated as 32 500 Da, higher than that of the human enzyme (22 861 Da) and A. thaliana (28 791 Da). GLOII from L. donovani with a predicted pI of 6.0 is more acidic than the human enzyme (pI 8.5). Western blot analysis of whole-cell lysates of promastigotes of L. donovani using the polyclonal antibody against GLOII enzyme shows a single band of approx. 32 kDa, and the same antibody recognized the recombinant protein of the expected size of the GLOII-His$_6$ tag fusion protein of approx. 38 kDa. Cross-linking studies established that the recombinant GLOII is a monomer.

Overexpression of GLOII in transfectants was observed both by Northern and Western blot analysis. The difference observed between the mRNA and protein levels expressed in the transfected cells could be due to post-transcriptional regulation which seems to be the mechanism of choice for gene expression in Leishmania and other lower organisms [37].

The exact biological role of the ubiquitous glyoxalase system is not known. It appears that one of the main roles of the glyoxalase system is glutathione-based detoxification of methylglyoxal. Characterization of the LdGLOII gene and expression of the protein will facilitate studies of the structural and functional aspects of the enzyme.

The glyoxalase system has received a considerable amount of attention as a possible anti-malarial target and for its possible anti-trypansomal activity [38,39]. Inhibitors of GLOII and GLOII have been shown to inhibit the growth of tumour cells [19]. The three-dimensional structure of the human GLOII bound to analogues of the substrate revealed three conserved basic residues, Arg-249, Lys-252 and Lys-143, that are in close proximity and show specificity to glutathione [34]. The glycine carboxylate of the substrate glutathione interacts directly with the side chains of Arg-249 and Lys-252 [34]. These critical residues are not conserved in LdGLOII. The substrate for GLOII in Leishmania is trypanothione thiosteres. These thiosters carry an overall positive charge because of the spermidine moiety, whereas glutathione thiosters carry a negative charge. The active site of the mammalian enzyme therefore cannot accommodate positively charged trypanothione thiosters. A difference in the substrate specificity of the human and LdGLOII enzyme renders the glyoxalase system an attractive target for antileishmanial chemotherapy. Finally, in view of the uniqueness of LdGLOII enzyme, it could be exploited for structure-based drug designing of selective inhibitors against the parasite.

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### REFERENCES


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