Leishmania donovani Tryparedoxin Peroxidase

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
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zu Braunschweig
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Tagungsbeiträge


Dedicated to my parents for their eternal belief in me and for everything else............
This thesis is the culmination of the efforts and prayers of a galaxy of people to whom I owe my gratitude. So, I want to thank God first for providing me the rare chance to be fortunate enough to be associated with such wonderful people and much more.

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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorption</td>
</tr>
<tr>
<td>Abs$_{260}$</td>
<td>Absorption at 260 nm</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosinediphosphate</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>app</td>
<td>Apparent</td>
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<tr>
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<td>Ammonium persulphate</td>
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<td>Adenosinetriphosphate</td>
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<tr>
<td>BCA</td>
<td>Bicinchononic acid</td>
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<td>BCIP</td>
<td>5- Bromo-4-Chloro-3- Indolyolphosphate</td>
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<td>β-gal</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>Bis</td>
<td>N,N-Methylenebisacrylamide</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine Triphosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>Cf16</td>
<td><em>C. fusciculata</em> Tryparaodoxin</td>
</tr>
<tr>
<td>Cf21</td>
<td><em>C. fusciculata</em> Tryparaodoxin Peroxidase</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton-mass unit equivalent to 1/12 of the carbon atom</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>dioxyribonucleotriphosphate</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Description</td>
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<tr>
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<td>-------------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>gm</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>Gravity acceleration</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised Glutathione</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focussing</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton-mass unit equivalent to 1,000 daltons</td>
</tr>
<tr>
<td>kg</td>
<td>kilograms</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LdH6TXNPx</td>
<td>Histidine tagged <em>Leishmania donovani</em> peroxidase</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption/Ionization</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOPS</td>
<td>Morpholinopropanesulfoneacid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide-adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide-adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>OD&lt;sub&gt;578&lt;/sub&gt;</td>
<td>Optical density at 578 nm</td>
</tr>
<tr>
<td>ox</td>
<td>Oxidised</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Poly vinylidene-difluoride</td>
</tr>
<tr>
<td>R</td>
<td>Reverse</td>
</tr>
<tr>
<td>RBS</td>
<td>Ribosome binding site</td>
</tr>
<tr>
<td>red</td>
<td>Reduced</td>
</tr>
<tr>
<td>ROH</td>
<td>Generic Alcohol</td>
</tr>
<tr>
<td>ROI</td>
<td>Reactive Oxygen Intermediates</td>
</tr>
<tr>
<td>ROOH</td>
<td>Generic Hydroperoxide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>[S]</td>
<td>Substrate concentration</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>Tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>TPx</td>
<td>Trypanothione peroxidase</td>
</tr>
<tr>
<td>TR</td>
<td>Trypanothione reductase</td>
</tr>
<tr>
<td>Tricine</td>
<td>N-tris[hydroxymethyl]methylglycine; N-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TrR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TS₂</td>
<td>Oxidised trypanothione</td>
</tr>
<tr>
<td>T(SH)₂</td>
<td>Reduced trypanothione</td>
</tr>
<tr>
<td>TTP</td>
<td>Thymidine triphosphate</td>
</tr>
<tr>
<td>TXN</td>
<td>Tryparedoxin (Cf16)</td>
</tr>
<tr>
<td>TXNpx</td>
<td>Tryparedoxin peroxidase (Cf21)</td>
</tr>
<tr>
<td>U</td>
<td>Unit of enzymatic activity- 1 U is equivalent to the enzyme quantity that converts 1 micromole substrate per min</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridinetriphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet spectroscopy</td>
</tr>
<tr>
<td>V</td>
<td>Maximal rate</td>
</tr>
<tr>
<td>V_{app}</td>
<td>Apparent limiting rate</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>VIS</td>
<td>Visible Spectrometry</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by volume</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximum reaction velocity of an enzyme at substrate saturation</td>
</tr>
<tr>
<td>W</td>
<td>Watts</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight by volume</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-Chloro-3-indolyl-(\beta)-D-Galactoside</td>
</tr>
<tr>
<td>(\mu)g</td>
<td>Microgram</td>
</tr>
<tr>
<td>(\mu)l</td>
<td>Microlitre</td>
</tr>
<tr>
<td>(\mu)M</td>
<td>Micromolar</td>
</tr>
<tr>
<td>(\phi)</td>
<td>Dalziel coefficient</td>
</tr>
<tr>
<td>(\varepsilon)</td>
<td>Molar Absorptions coefficient</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

1.1. Leishmania

In 1903, Leishman and Donovan separately described the protozoan now called Leishmania donovani in splenic tissues from patients in India with the life-threatening disease now called visceral leishmaniasis.

The genus Leishmania is a group of protozoa which are parasites of canines and rodents and which infect humans under certain circumstances, mainly as zoonoses (HOEPRICH et al, 1994). They are digenetic protozoa, which inhabit two highly specific hosts-the sandfly, where they grow as motile flagellated promastigotes in the gut, and the mammalian macrophage, where they survive and grow intracellularly as non-flagellated amastigotes in the phagolysosome (HANDMAN, 1999). Transmitted by the bite of the infected female phlebotomine sandfly-about 2mm long flies, the leishmaniasis are a globally widespread group of parasitic diseases. The sand flies are the main reservoir host, but in anthroponotic forms, i.e. transmission from human to human by the sandfly vector, humans are the sole reservoir host. Actually, humans are the accidental hosts of these flies; natural hosts include a variety of rodents, small mammals, and dogs (ROBERTS et al, 2000). The sandfly vector is usually infected with one species of flagellate protozoa belonging to the genus Leishmania (WHO, May 2000). About 30 species of sand flies can become infected when taking a blood meal from a reservoir host (WHO Report, May 2000).

These protozoa are intracellular as Leishman-donovan bodies (amastigotes) in human, canine and rodent hosts, whereas they are flagellated extracellular microorganisms (promastigotes) in insect hosts (NOBLE et al, 1976; DOENGES, 1988). Promastigotes are found in the gut of phlebotomine sand flies and in the blood stream of vertebrates. The blood stream promastigotes invades macrophages and differentiates to the amastigote.
1.2. Diseases caused by Leishmania

Leishmaniasis in humans takes three main clinical forms, which may occur together or separately - cutaneous, mucocutaneous and visceral (HOEPRIICH et al, 1994; BERMANN, 1997; WHO report, May 2000).

**Visceral Leishmaniasis** (VL), also known as *kala azar*, is the most severe form of the disease, which, if untreated, has a mortality rate of almost 100%. It is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia.

**Mucocutaneous Leishmaniasis** (MCL), or *espundia*, produces lesions, which can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities.

**Cutaneous Leishmaniasis** (CL) can produce large numbers of skin ulcers—as many as 200 in some cases—on the exposed parts of the body, such as the face, arms and legs, causing serious disability and leaving the patient permanently scarred. Diffuse cutaneous Leishmaniasis (DCL) never heals spontaneously and tends to relapse after treatment. The cutaneous forms of Leishmaniasis are the most common and represent 50-75% of all new cases.

These groups of diseases are highly prevalent due to increased migration from rural areas to urban areas and vice versa. Moreover, the *Leishmania/HIV* co-infection is making the spread of the disease even faster. Since 1990, 1,616 cases of visceral Leishmaniasis/HIV co-infection have been reported, mainly from southern Europe, and particularly from Spain, southern France, and Italy (DEDET, 2000), the co-infected patients being mainly young adults belonging to the risk group of intravenous drug users.

The group of leishmaniases is prevalent in 88 countries on five continents—Africa, Asia, Europe, North America and South America—with a total of 350 million people at risk, and has been considered to be one of the six priority diseases of its Special Programme for Research and Training in Tropical Diseases by WHO. The extent to
which this group of diseases has caused damage can be assessed by the fact that in southern Sudan, more than 10% of the population died from visceral leishmaniases over a span of five years (ROBERTS et al, 2000). The incidence of leishmaniases is increasing, with many endemic areas reporting a 500% increase over the past seven years (WHO, 2000). Especially interesting is the case of a 15-month old German child with visceral leishmaniases who had never traveled to any of the endemic areas (BOGDAN, 2001).

1.3. Current state of therapy

The treatment involves chemotherapy with high doses of pentavalent antimony compounds or various formulations, mainly lipid formulations of amphotericin B. The latter treatment is usually expensive and administered parentally (HERWALDT, 1999). In the line of organic pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) are the oldest nonsubstituted first-line drugs against different types of leishmaniases in the world (BERMAN, 1988), the distribution of the drugs depending on excolonial influences; with the former being available in English-speaking countries and the latter being in French and Spanish-speaking countries. These antimonials react by depleting the intracellular ATP levels due to interference of glycolysis and β-oxidation of fatty acids in amastigotes (VAN-VOORHIS, 1990) and by accumulating in the spleen, targeting Leishmania-infected macrophages. Because of the limitation to increase the dosage amount due to toxicity; low dosages and discontinuous drug exposures are the main causes of the increasing number of relapses and resistances to the antimonials.

Pentamidines display higher chemical stability, as it eases administration and contains less toxicity, though enough to preclude this group as second-line chemotherapeutic compounds against Leishmania. The mode of action of this compound has not been thoroughly understood.
Amphotericin B, which belongs to a class of polyene antibiotics, has been used in lipid formulations, being very effective against antimony-relapsed strains of visceral leishmaniasis and kala azar (OLLIARO et al, 1993). The mode of action is based on the peculiar metabolism of sterols of *Leishmania* and fungi. The phosphatidylcholine cholesterol, disterylpalmitidyl glycerol amphotericin B complexes (AmBisome, Vestar) and cholesterol sulfate-amphotericin B liposomes (Amphocil) have been successfully tested, with the former being already commercialized in Western countries, but remains elusive for underdeveloped ones because of high costs.

Allopurinol, the adenosine purine analogue, is effective in combination with antimonial drugs (NYAKUNDI et al, 1995). It has also proved effective against cutaneous leishmaniasis in combination with other oral drugs such as ketoconazole in a patient with pentosastam-induced pancreatitis (HALIM et al, 1993). The side effects include rashes due to Steve-Johnson syndrome, which is life-threatening and results in neutropenia, thrombocytopenia, anemia and renal stones (VAN VOORHIS, 1990; BERMAN, 1988).

Recently, Miltefosine, a phosphocholine analogue that affects cell-signaling pathways and membrane synthesis has been reported (JHA, 1999). But more study has to be done with regard to the side effects of this drug.

HIV patients who contract Leishmaniasis and are treated with the conventional drugs, such as antimonials, amphotericin B, pentamidine and allopurinol, result in more than 40% relapse or have persistent chronic infections, demonstrating the importance of the immune response during chemotherapy (BERGER et al, 1992).

There is a great need for research therapy and vaccine research because of increasing drug resistance and the tendency of patients to relapse after an initially successful regimen of chemotherapy. Also, the limitations of most of the presently used drugs due to toxicity, difficulty of administration, undesirable side effects or the high costs warrant the urgent need of a drug, which is effective, minimum or no side effects and are affordable. To date, there is no recombinant vaccine reagent available for use in humans.
1.4. Immunology

*Leishmania* and some other Trypanosomatids like *Trypanosoma cruzi* reside intracellularly in the immunologically competent macrophages. To avoid the immune response effector mechanisms of the hosts, they gain entry through several receptor-ligand systems and multiply within the macrophages (ALEXANDER et al, 1992). It has been also shown that *Leishmania major* metacyclic promastigotes are preferentially taken up by the CR1 receptors of the human macrophages which do not activate the respiratory burst as much as the CR3 receptors (DA SILVA et al, 1989).

The two classic models of experimental mice react differently to *Leishmania*. While BALB/c is susceptible, C57BL/6 is resistant mounting an effective immune response against *L. major* and controls the infection at an early stage. This difference has been attributed to the differential expansion of distinct CD4+ T-cell subsets- Th1 being biased to producing interleukin 2 (IL-2) and gamma interferon (IFN-γ), and Th2 towards the production of IL-4, IL-5, IL-6, and IL-10 (MOSMANN, 1989).

Killed *Corynebacterium parvum* which has been shown to modulate the expression of some interleukins (SCOTT, 1991) was used as an adjuvant with the *L. major* promastigote culture filtrate proteins (CFP) by WEBB et al, 1998 to study the extent of protection provided in BALB/c mice. Protection resulted and further investigation into the immunogenic components resulted in the discovery of a *L. major* TSA protein having significant homology to the eukaryotic thiol-specific-antioxidant proteins.

1.5. Oxidative stress

*Leishmania* are intracellular protozoan parasites of macrophages. The main steps of infection include the invasion of tissue macrophages by the parasite, the avoidance of killing mechanisms, and the subsequent intracellular replication of the parasite, with the eventual spread of the organism to adjacent macrophages (KANE, 2000).
*Leishmania* and for that matter most of the Trypanosomatids have been found to be aerotolerant or microaerophilic and also able to consume oxygen to some extent. So, they produce H$_2$O$_2$ as a product of their metabolism in certain cases, and are highly susceptible to reactive oxygen species. Moreover, because they are intracellular, they are naturally exposed to the toxin metabolites generated by effector immune cells of the host, like the macrophages. So, they would be expected to have strong mechanisms of free radical detoxification. Instead, they have been found to lack the regular detoxification enzymes.

ROS (Reactive oxygen species) are produced during normal cellular function by the host macrophage and also endogenously by the parasites. They are chemically highly reactive and lead to DNA fragmentation, lipid peroxidation and oxidation of proteins. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. When ROS generation is increased to an extent that overcomes the cellular antioxidants, the result is oxidative stress. Primary ROS include hydrogen peroxide (H$_2$O$_2$), the superoxide radical (O$_2^\cdot$) and the hydroxyl radical (HO$_2^\cdot$), which are produced as a result of normal metabolism. Secondary and tertiary ROS, such as alkyl hydroperoxides and lipid peroxides result from the interaction of primary ROS with cellular targets (McGONIGLE et al, 1998). Superoxide dismutase (SOD) catalyzes the dismutation of O$_2^\cdot$ to H$_2$O$_2$ + O$_2$. H$_2$O$_2$ in turn is converted to H$_2$O + O$_2$ by catalase, peroxidase, and glutathione peroxidase, which uses glutathione (GSH) as the reducing agent (MEHLOTRA, 1996). Trypanosomatids contain appreciable superoxide dismutase activity, the gene for iron-containing superoxide dismutases being cloned for *Trypanosoma cruzi* and *Leishmania* (ISMAIL et al, 1994; ISMAIL et al, 1997; PARAMCHUK et al, 1997; TEMPERTON et al, 1996; DEY et al, 1994), though they do not contain catalase and the glutathione peroxidase/glutathione reductase system (MEHLOTRA, 1996; DOCAMPO, 1990.).

Out of the two forms of *Leishmania*, the promastigotes were found to be highly susceptible to H$_2$O$_2$ (MURRAY, 1981), whereas the amastigotes were found to be fairly resistant. The continuous generation of H$_2$O$_2$ by glucose-glucose oxidase suppressed promastigote proliferation, and the addition of catalase completely abrogated the growth-inhibitory effect (REINER et al., 1982). In the case of amastigotes, normal mouse peritoneal macrophages exerted no activity. They
survived equally well within the *in vivo*-activated macrophages as well. MURRAY in 1982 also demonstrated that the amastigotes had threefold more catalase, had 14-fold more glutathione peroxidase and were four times more resistant to enzymatically generated H$_2$O$_2$. Also, transformation and competitive binding studies suggest that the difference could be explained by the qualitative and quantitative differences in the distribution of surface ligands, such as the mannose/fucose receptor involved in binding the parasite to the macrophage membrane. Also, catalase was detected in amastigotes only. Glutathione peroxidase activity was undetectable in either form (CHANNON et al, 1985).

There are several theories as to how Leishmania evades intracellular killing, but it has not been completely understood till now. The lipophosphoglycan of *L. donovani* has been shown to play a role in the survival of the parasite in the macrophage, as it inhibits macrophage protein kinase C activity, an important enzyme that is believed to be responsible for initiating the oxidative burst (MCNEELY et al, 1987; DESCOTEAUX et al, 1991 & 1992).

### 1.6. Potential drug targets

Of special interest have been the glycolytic enzymes of the parasite that are found partially compartmentalized in both promastigotes and amastigotes. The first enzymes of the glycolytic pathway are engulfed in a special organelle called a glycosome, a feature common to all trypanosomatids including *Leishmania* spp (BALANA-FOUCE et al, 1998). KNIGHTON et al in 1994 unraveled the three-dimensional structure of a bifunctional DHFR-thymidylate synthase from *Leishmania major* that was based on the inhibition of the trypanosomal glycolysis. They designed a compound -2'-deoxy-2'-(3-methoxybenzamido) adenosine that was 45 times more potent than adenosine and thus had more affinity for the trypanosomal enzyme than the human equivalent of glyceraldehyde-3-phosphate dehydrogenase (GADPH).
Another interesting target for chemotherapeutic agents is the synthesis of sterols in *Leishmania* spp. Lanosterol demethylation produces cholesterol as the major sterol in mammalian hosts; whereas in *Leishmania* they are ergostane sterols-episterol and 5-OH-dehydroepisterol. The similarity of these sterols to the fungus major sterol, episterol has formed the basis of the fungicide drugs such as polyene antibiotics and azoles (ketoconazole and itraconazole). The major drawback of this kind of treatment is the incorporation of the host cholesterol by the amastigote, leading to parasite resistance to the drug.

The differences in purine metabolism of the parasite from its mammalian counterpart have been exploited as yet another target for chemotherapy. Their incapability to synthesize de novo the purine ring from metabolic precursors leads them to obtain it from the host. *Leishmania* amastigotes' enzyme hypoxanthine guanine phosphoribosyl transferase massively uses the aberrant inosine allopurinol as substrate and transforms it into its corresponding nucleoside. This leads to the action of allopurinol being able to block the purine salvage pathway causing purine starvation and parasite cell death. On the other hand *Leishmania* do synthesize pyrimidine de novo. Thymidylate synthase that also displays the activity of dihydrofolate reductase has been targeted by the anticancer drug methotrexate, the antimalarial agent pyrimethamine and aromatic diamidines.

To defend the organism against oxidative stress, trypanosomatids have evolved a unique system called the trypanothione-metabolizing system. Among the potential drug targets for the diseases caused by the group Trypanosomatidae, probably Trypanothione reductase (TR) is one of the best studied. Trypanothione reductase, the enzyme responsible for maintaining trypanothione in its reduced form, is thought to be central to the redox defense systems of Trypanosomatids. KRIEGER et al in 2000 showed that trypanosomes lacking Trypanothione reductase showed increased sensitivity to oxidative stress. DUMAS et al. (1997) attempted to create TR-knockouts in *L.donovani* and *L.major* to investigate the physiological role of TR in *Leishmania*. All their attempts to obtain TR-null mutants failed. Trivalent arsenic drugs (melarsen oxide), substituted naphthoquinones, nitrofurans and 1,3-bis-2 (chlooroethyl)nitrosourea have been reported as powerful inhibitors of this enzyme.
Polyamine synthesis inhibitors also inhibit trypanothione synthesis by depleting parasite spermidine pools.

Cysteine proteases have been eyed as yet another target for the synthesis of anti-trypanosomal drugs. They play a key role in parasitic infections and lack redundancy compared to mammalian systems (MCKERROW et al, 1993 & 1995). *Leishmania* has three cysteine protease gene families (COOMBS et al, 1997). Several cysteine protease inhibitors have been tested in tissue culture models of parasite replication and also *in vivo* and proven to be effective. There is a lot of research going on in both academic centers and industry to provide more effective and specific protease inhibitor, with the development of orally bioavailable inhibitors the most recent one (OLSON et al, 1999; THOMPSON et al, 1997).

Ultimately, however, the *Leishmania* genome project will identify genes responsible for parasite virulence, which should reveal potential targets for therapeutic drugs and vaccines (ROBERTS et al, 2000).

### 1.7. Trypanothione metabolism

Usually, glutathione protects the parasites by metabolizing the primary ROS. *Leishmania* parasites lack glutathione disulfide and contain low concentrations of reduced glutathione. Instead, Trypanothione [N¹, N⁸-bis (glutathionyl)spermidine] is synthesized by the condensation of the tripeptide glutathione with the polyamine spermidine by two ATP-dependent conjugating reactions that form N1 and N8 isomers of glutathionyl spermidine intermediates (FAIRLAMB et al, 1992). Two distinct enzymes, glutathionylspermidine synthetase and trypanothione synthetase are required.

In parasitic trypanosomatids, which typically lack the efficient peroxide detoxifying enzymes catalase and glutathione peroxidase (BOVERIS et al, 1980), the peroxiredoxin represents the terminal peroxidase of a unique cascade of
oxidoreductases that reduces hydroperoxides at the expense of NADPH (NOGOCEKE et al, 1997; FLOHÉ et al, 1999). The reducing equivalents of NADPH are transferred to the cyclic disulfide form of trypanothione [N1,N8-bis-(glutathionyl) spermidine; TS2] by the flavoprotein trypanothione reductase (TR). Trypanothione [T(SH)2] reduces tryparedoxin (TXN), a small protein that is remotely related to thioredoxin and characterized by an WCPPC reaction center (NOGOCEKE et al, 1997; GOMMEL et al, 1997; MONTEMARTINI et al, 1998b; GUERRERO et al, 1998; FLOHÉ et al, 1999). Reduced TXN finally is the specific donor substrate of the trypanosomal peroxiredoxin (NOGOCEKE et al, 1997; STEINERT et al, 2000) that therefore is addressed to as tryparedoxin peroxidase (TXNPx). The relevance of this redox cascade to vitality and virulence of trypanosomatids has recently been demonstrated by an elegant inverse genetics approach in Trypanosoma brucei. In absence of TR, proliferation is inhibited and the sensitivity to H2O2 is increased by two orders of magnitude (KRIEGER et al, 2000). Recently, WILKINSON et al, 2003 exhibited by tetracycline-inducible RNA interference the distinct involvement of a cytoplasmic peroxiredoxin and a non-selenium glutathione-dependent peroxiredoxin in the survival of of the bloodstream form of Trypanosoma brucei. Another interesting development in this field has been the indication of a separate hydroperoxide metabolizing system in T. brucei which is also based on the unique trypanothione metabolism (HILLEBRAND et al, 2003).

Therefore, TR as well as the downstream enzymes of the cascade may therefore be considered attractive molecular targets for the development of trypanocidal drugs (FLOHÉ, 1998; FLOHÉ et al, 1999, SCHMIDT et al, 2002).

1.8. Peroxiredoxins and Peroxidases

Peroxiredoxins are a unique and widespread family of proteins, ranging from being present in bacteria to complex organisms as humans (CHAE et al, 1994; RHEE et al, 1999). Most of the peroxiredoxins are peroxidases. The first example of this novel type of peroxidases was the “thiol-specific antioxidant protein” of yeast (KIM et al, 1988) that was later reclassified as thioredoxin peroxidase (CHAE et al, 1994;
Related thioredoxin peroxidases were also discovered in mammals where they are believed to modulate signaling cascades triggered by growth factors and cytokines (Jeong et al., 1999; Kang et al., 1998a; Rhee et al., 1999). The protein family further comprises the alkyl-hydroperoxide reductases of bacteria (Ellis et al., 1997a; Sherman et al., 1999; Tartagalia et al., 1990), a mammalian non-selenium glutathione peroxidase (Fisher et al., 1999) previously named hORF 6 (Choi et al., 1998), and a large variety of proteins spread over the entire living kingdom whose biological function remains to be established (Montemartini et al., 1998a; Rhee et al., 1999). Previously, several enzymes have been identified and characterized in Trypanosomes that belong to this category of peroxidases (Montemartini et al., 1998a; Levick et al., 1998; Lopez et al., 2000; Guerrero et al., 2000; Webb et al., 1998). Peroxiredoxins are classified on the basis of the Cysteines they possess in the two conserved motifs at the N-terminal and the C-terminal end. The N-terminal end Cysteine is highly conserved among the peroxiredoxin family, but there is some variation in the C-terminal end of the genes. Recently, Castro et al. (2002a) described two Leishmania infantum peroxiredoxins, one of which is a mitochondrial peroxiredoxin and has a Ile-Pro-Cys sequence instead of the common Val-Cys-Pro sequence in the C-terminal motif. They further demonstrated (Castro et al., 2002b) that this mitochondrial peroxiredoxin is a tryparedoxin peroxidase.

Therefore based on the conserved sequences in the N-terminal and C-terminal motifs, peroxiredoxins are classified as 1-Cys or 2-Cys peroxiredoxin (McGonigle et al., 1998). Most of the trypanosomal peroxidases identified so far are 2-Cys peroxiredoxins.
2. MATERIALS AND METHODS

2.1. MATERIAL

2.1.1. Leishmania donovani DNA

Leishmania donovani genomic DNA was obtained from Dr. Joachim Clos (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany) and L. donovani strain LV9 was used, a gift from D. Zilberstein. Promastigotes were routinely cultivated under logarithmic growth conditions (5 \times 10^5 to 2 \times 10^7 cells per ml) at 25 °C in supplemented M199 medium (Krobitsch et al., 1998). Cell growth was monitored using a Schaerfe System CASY pulse resistance cell counter. Genomic DNA was prepared from late logarithmic promastigote cultures (1 \times 10^9 cells) following the protocol by Blin and Stafford (1976) using SDS lysis and protease digestion followed by phenol extraction and ethanol precipitation. DNA was collected by spooling over a blunt glass rod and resolubilized in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) over 76 hours.

2.1.2. Trypanothione

Trypanothione used was from BACHEM Biochemica GmbH and was obtained as a TFA-salt. It was prepared at a concentration of 7 mg/ml in d.w and stored at -20°C till further use. Before use, it was always brought to RT by placing it on ice.

2.1.3. Trypanothione Reductase

The Trypanosoma cruzi Trypanothione Reductase containing clone was obtained from Prof. Krauth-Siegel. The gene encoding TR was excised and subcloned by Mrs. Karin
Planck-Schumacher and expressed in *E. coli* and the protein purified on FPLC system from Pharmacia based on Ni-chelate chromatography. The resin was His.Bind resin from Novagen. Purified TR protein was stored at 4°C in aliquots until further use.

### 2.1.4. Tryparedoxin

C-Terminal His-tagged Tryparedoxin 2 (TXN 2 H6) used was from *Crithidia fasciculata* TXN2 gene cloned into pET 24a by Montemartini et al (1998B). It was heterologously expressed in *E. coli* and purified by Dr. Ulrich Menge and Mrs. Karin Planck-Schumacher and stored at 4°C.

### 2.1.5. Chemicals

Chemicals were from Merck, Sigma, Fluka, Roth, Riedel-de-Haen and Serva unless otherwise specified.

The reagents for culture medium were Bacto Trypton, Bacto Yeast extract and Bacto Agar that were from Difco.

Biodyne membranes for Southern and Colony hybridization were from PALL.

PD-10 columns for desalting and concentrating proteins were from Amersham Pharmacia Biotech.

IPTG was from BIOMOL Feinchemikalien GmbH.
2.1.6. Instruments

Applied Biosystems                  DNA-sequencing machine

Biometra                             Protein Gel electrophoresis instrument
                                          (Minigel twin)

Biotech International                Shaking incubator

Duran                                Glassware

Eppendorf                            Table-top centrifuge 5417, Thermomixer
                                          5431, Pipettes

Gesselschaft Fur Labortechnik GmbH   Waterbath

GIBCO                                DNA eletrophoresis instrument (Horizon
                                          58 and Horizon 11 14)

Heidolph                             Vortex

Heraues                               Clean bench, Incubator, Megafuge,
                                          Biofuge fresco, Biofuge 28 RS

Herolab                              Video Scan-System

Sorvall                              RC5C Centrifuge
2.1.7. Commercial kits

Commercial kits used are listed below:

- QIAGEN DNA miniprep kit (Qiagen)
- QIAquick Gel Extraction Kit (Qiagen)
- JETQUICK Plasmid Miniprep Spin Kit (Genomed)
- JETSORB Gel Extraction Kit (Genomed)
- One Shot™ competent cells (Invitrogen)
- MicroAmp reaction tubes (Perkin Elmer)
- Themocycler Gene Amp PCR System 9600 (Perkin Elmer)
- PCR DIG-Probe synthesis kit (Roche Molecular Biochemicals)
- DIG-Nucleic acid detection kit (Boehringer Mannheim)
- Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems)
- His.Bind Kits (Novagen)
- TOPO TA Cloning Kit (Invitrogen)

2.1.8. Antibodies

LdTXNPx Antibodies were ordered from Eurogentec Bel S.A (Belgium). The antibodies were produced in rabbit. The pre-immune serum was supplied with the antibody serum to serve as the negative control for the Western Blots. Upon arrival, they were checked for any background reading resulting from contamination either in the antibodies or for the non-specific antibodies present which might react with some of the cell lysate proteins and give erroneous results. A Western blot was done with purified recombinant Cf21 protein as a control.
2.2. METHODS

2.2.1. Construction of an \textit{L. donovani} genomic library

2.2.1.1. Western Blot

Western Blot is a technique used to identify proteins immunologically on a membrane (mostly PVDF or nitrocellulose) after they are transferred from a gel. A primary antibody binds to the immobilized protein on the membrane. The secondary antibody binds to the primary one that is linked to an enzymatic molecule. The proteins are detected by incubating the membrane on the enzyme-specific substrate.

So, a Western blot was done on the total \textit{Leishmania donovani} protein lysate to confirm the presence of a similar protein. SDS-PAGE was performed as described in section 2.2.3.3. Semi-dry kind of blotting was used. After the gel was run, it was equilibrated in blotting buffer for 5 min. Meanwhile, the polyvinylidene fluoride (PVDF) transfer membrane (Millipore) was briefly immersed in methanol and then transferred to the blotting buffer and further equilibrated in blotting buffer for 5 mins.

The blot apparatus (from the GBF workshop) was assembled and the graphite plates were wetted with the blotting buffer and four pieces of Whatman 3MM paper wetted with blotting buffer were assembled onto the apparatus with the gel and the PVDF membrane. The transfer was done at 100mA for 1 hr.

\textbf{BLOTTING BUFFER:}

50mM Tris
38mM Glycine
0.037\% (w/v) SDS
20\% Methanol

After the transfer, the blot was rinsed with 1X PBS (10mM Sodium phosphate buffer, pH 7.4, 138 mM NaCl, 3 mM KCl) and incubated between 4 hrs- O/N (4° C) in 10\% FCS/PBS and then incubated with anti-Cf21 antibodies at a 1:500 dilution in 2 \%FCS/
PBS for 2 hrs (RT). The blot was then rinsed 3X with 1X PBS and then incubated with secondary antibodies -anti-rabbit antibodies (BIORAD) at a dilution of 1:20,000 in 2%FCS/PBS and incubated for 1.5 hrs. At the end of the antibody incubation, the blot was rinsed with PBS three times and then incubated in the substrate (Western Blue Stabilized Substrate for Alkaline Phosphatase, PROMEGA). Rinsing the blot in water stopped the reaction.

### 2.2.1.2. PCR on genomic DNA

PCR (Polymerase Chain Reaction), which was invented by Cary Mullis, is a very powerful method that amplifies selective regions of any gene by using primers that bind to both the ends of the gene. Heating separates the two strands of the DNA molecule and the part of the gene is amplified with the help of a DNA Polymerase. The Polymerase is heat-stable and oftentimes proofreading to minimize the errors due to replication.

The polymerase chain reaction on the *L. donovani* genomic DNA was done in 0.2 ml MicroAmp thin walled reaction tubes (Perkin Elmer) in Themocycler Gene Amp PCR System 9600 (Perkin Elmer). A typical 100 µl PCR reaction was set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. donovani</em> genomic DNA (0.37 µg/µl)</td>
<td>1</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>10</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>12</td>
</tr>
<tr>
<td>Forward Primer (CL1For)</td>
<td>1 (100pmol)</td>
</tr>
<tr>
<td>Reverse Primer (CL2 Rev)</td>
<td>1 (100pmol)</td>
</tr>
<tr>
<td>10 mM dNTP-Mix</td>
<td>8</td>
</tr>
<tr>
<td>AmpliTaq DNA Polymerase (5U/µl)</td>
<td>0.5</td>
</tr>
<tr>
<td>d.w (sterile)</td>
<td></td>
</tr>
</tbody>
</table>

up to a final volume of 100 µl
The cycling parameters were as follows

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Start</td>
<td>2 mins</td>
<td>96°C</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 min</td>
<td>96°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>1 min</td>
<td>50°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Elongation</td>
<td>3 mins</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>10 mins</td>
<td>72°C</td>
<td>1X</td>
</tr>
</tbody>
</table>

10 μl of the PCR reaction was taken and analyzed on a 1 % agarose gel.

2.2.1.3. TOPO cloning

2.2.1.3.1. Ligation

Ligation is a process where two separate DNA fragments can be joined with the help of a T4 DNA ligase. The vector and the insert are both linearized and before ligating them, the vector is dephosphorylated to avoid self-ligation and give rise to "empty clones". Before ligating, the vector and the insert DNA are run separately on the gel to check the purity and concentration of both the DNAs by the intensity of the fluorescence on an agarose gel. The insert DNA band is usually less intense as the fragments are smaller than the vector DNA, even though the concentration may be same for both.

The principle of a TOPO vector (Invitrogen) is based on the assumption that freshly PCR-ed products usually have overhanging adenosine ends, which can be used to clone into a vector that has T ends. As the overhanging A ends tend to disintegrate with time the PCR product has to be fresh when it is set up for ligation with the vector.
To this effect, 4 µl of the PCR product from step 2.2.1.2. was taken for ligation into the pCR 2.1 vector.

The ligation reaction was done strictly according to the manufacturer’s instructions. The composition of the ligation reaction was

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product</td>
<td>4 µl</td>
</tr>
<tr>
<td>10 X Ligation buffer</td>
<td>1 µl</td>
</tr>
<tr>
<td>pCR 2.1 vector (25 ng/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>2 µl</td>
</tr>
<tr>
<td>T4 DNA ligase (4 Weiss units)</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>10 µl</strong></td>
</tr>
</tbody>
</table>

The ligation reaction was then incubated overnight at 14°C in a water bath.

### 2.2.1.3.2. Transformation

One vial of One Shot™ competent cells (Invitrogen) and 0.5 M β-mercaptoethanol were thawed on ice. 2µl of the ligation reaction was added to the competent cells and mixed with the tip and then incubated on ice for 30 mins for the cells to sufficiently cool. The cells were given a heat-shock at 42°C for 30 seconds and then the vial was placed on ice for 2 mins so that the competent cells would take up the DNA added. 250 µl of SOC medium without antibiotics was added and then the tube was placed in a shaker for 1 hr at 300 rpm at 37°C to allow for the sufficient growth of the transformed cells. 50µl of the transformation was plated on LB agar plates containing 50µg/ml of Ampicillin and 40 µl each of 100 mM IPTG and 40 mg/ml X-Gal preadsorbed on them. This was done in order to identify the positive clones from the non-insert containing clones, as only cells which had the insert would have antibiotic resistance.
The plates were incubated overnight at 37°C and next day typically about 10 colonies from each transformation were inoculated into 5 ml of LB medium containing Ampicillin (50 µg/ml). Ampicillin was taken in the medium at this stage to avoid contamination. 4 ml of the culture was taken for plasmid DNA minipreps and 1 ml of the culture was taken for stock culture preparation as described later on. Stocks were numbered and carefully stored for later reference.

**SOB Medium:**
2 % (w/v) Trypton
0.5 % (w/v) Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM MgCl₂
10 mM MgSO₄

MgCl₂ and MgSO₄ were prepared in a 100X concentration, sterile filtered and added to the autoclaved medium in a ratio of 1:100.

**SOC Medium:**
SOB Medium with 0.4 % Glucose.
Glucose was prepared as a 40 % solution, sterile filtered and added to the SOB medium at a ratio of 1:100.

### 2.2.1.3.3. Restriction Analysis and sequencing of insert

#### 2.2.1.3.3.1. Plasmid DNA minipreps

Plasmid DNA was isolated using the QIAGEN DNA miniprep kit. It is essentially a
QIAGEN-tip 20 and was used for the isolation of plasmid from 4 ml of the culture. For larger quantities, QIAGEN-tip 100 was used. This was done according to the capacity of the resin in the tips to bind DNA.

**Buffer P1 (Resuspension Buffer)**
50 mM Tris, pH 8.0
10 mM EDTA
100 µg RNase/ml

**Buffer P2 (Lysis Buffer)**
200 mM NaOH
1 % (w/v) SDS

**Buffer N3 (Neutralization Buffer)**
3 M Sodium Acetate, pH 5.5

**Buffer QBT (Equilibration Buffer)**
750 mM NaCl
50 mM MOPS, pH 7.0
15 % (v/v) Ethanol
0.15 % (v/v) Triton X-100

**Buffer QC (Wash Buffer)**
1 M NaCl
50 mM MOPS, pH 7.0
15 % (v/v) Ethanol

**Buffer QF (Elution Buffer)**
1.25 M NaCl
50 mM Tris, pH 8.5
15 % (v/v) Ethanol

**Storage Buffer**
10 mM Tris-HCl, pH 8.5

Buffers P1 and P3 were stored at 4°C and all the others were stored at RT. P1 was stored at 4°C because it contains RNase and P3 because it has acetate.

The overnight cultures from 37°C were centrifuged at 1000-x g for 10 min at 4°C. The bacterial cell pellet was then suspended in 250 µl of buffer P1. It was made sure that no clumps were remaining. 250 µl of Buffer P2 was added and the tube inverted 4-6 times and then incubated for 5 min at RT. Buffer P3 was then carefully added and the tube again inverted 4-6 times to ensure uniform mixing and then centrifuged at 12,000 g for 10 min to collect the chromosomal DNA at the bottom of the tube.

After it was made sure that the pellet was a tight one so that when the supernatant was taken, it made it impossible to get chromosomal DNA contamination, the supernatant was carefully taken and applied to a QIAGEN-tip 20 and centrifuged for 30-60 sec.

After discarding the flow-through, the QIAprep spin column was washed with 0.5 ml of buffer QBT and centrifuged for 30-60 sec. The flow-through was then discarded. 0.75 ml of Buffer QC was added and the column was centrifuged again for 30-60 sec.

The flow-through was discarded and the column centrifuged again for 1 min to remove the residual wash buffer. The QIAprep spin column was placed in a 1.5-ml centrifuge tube and 50 µl of Buffer QF was added to the center of the column and centrifuged it for 1 min to elute the DNA.
2.2.1.3.3.2. Restriction Digest of the plasmid DNA

The plasmid DNA isolated was then set up for restriction digest, as pCR2.1 vector has Eco R1 sites close to the insert site. *Eco* R1 enzyme was taken to check for the insert-containing clones and eliminate empty ones. The composition of the digest reaction was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (0.2 μg/μl)</td>
<td>10 μl</td>
</tr>
<tr>
<td>10 X <em>Eco</em> R1 Buffer (NEB)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td><em>Eco</em> R1 Enzyme, 10U/μl (NEB)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>d.w.</td>
<td>2.5 μl</td>
</tr>
<tr>
<td><strong>Total reaction volume</strong></td>
<td><strong>15 μl</strong></td>
</tr>
</tbody>
</table>

The reactions were then incubated overnight at 37°C. The next morning 10 μl of each digestion was loaded on a 1.5 % agarose gel using 1 kb and 100 bp DNA ladders as reference standards to check clones containing insert and the size of the insert.

2.2.1.3.3.3. Sequencing of insert

The sequencing of the insert was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer Cetus. The kit comprises of the Fluorescent marked terminators and the AmpliTaq DNA-Polymerase<sup>FS</sup>.

**Terminator-Premix**
- Tris, pH 9.0
- MgCl₂
Thermostable Pyrophosphatase
AmpliTaq DNA-Polymerase

Loading buffer
20 % 25 mM EDTA, pH 8.0
80 % Formamide

The sequencing reaction composition was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (0.2 µg/µl)</td>
<td>14 µl</td>
</tr>
<tr>
<td>DMSO</td>
<td>1 µl</td>
</tr>
<tr>
<td>Premix</td>
<td>4 µl</td>
</tr>
<tr>
<td>M13 Forward (-20) primer or M13 Reverse Primer</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>20 µl</strong></td>
</tr>
</tbody>
</table>

The sequencing reaction was performed in a DNA-Thermal Cycler (GeneAmp PCR System 9600, Perkin Elmer Cetus) and the reaction parameters were:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Start</td>
<td>2 mins</td>
<td>96°C</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>15 sec</td>
<td>96°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>20 sec</td>
<td>55°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Elongation</td>
<td>4 mins</td>
<td>60°C</td>
<td></td>
</tr>
<tr>
<td>Last incubation</td>
<td>Forever</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

After the amplification, 2 µl of 3 M sodium acetate, pH 5.2 and 50 µl of 95 % ethanol was added to each 20 µl of sequencing reaction. The mixture was vortexed briefly and incubated on ice for 30 min. At the end of the incubation period, the mixture was centrifuged at 13,000 rpm for 45 min at 4°C and the supernatant was discarded. 250
µl of 70% ethanol was added and centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was discarded and the pellets were air-dried for about 20 min. The DNA pellet was then dissolved in 3 µl of the loading buffer.

The gel electrophoresis of the sequencing samples was performed on a DNA-Sequencer 373 A (Applied Biosystems).

2.2.1.4. DIG-Labeling

A PCR DIG-Probe synthesis kit (Roche Molecular Biochemicals) was used and the reaction performed in a thermal cycler (Perkin Elmer GeneAmp PCR System 9600).

The reaction conditions were:

<table>
<thead>
<tr>
<th></th>
<th>Sample Tube</th>
<th>Control Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>1.0 µl (0.37 µg)</td>
<td>-</td>
</tr>
<tr>
<td>10X PCR DIG probe</td>
<td>5.0 µl</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>synthesis mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10X PCR buffer with</td>
<td>5.0 µl</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>MgCl2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL1 For Primer</td>
<td>1.0 µl</td>
<td>-</td>
</tr>
<tr>
<td>CL2 Rev Primer</td>
<td>1.0 µl</td>
<td>-</td>
</tr>
<tr>
<td>DIG Primer mix</td>
<td>-</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Enzyme mix (Expand TM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Fidelity</td>
<td>0.75 µl</td>
<td>0.75 µl</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>36.25 µl</td>
<td>29.25 µl</td>
</tr>
<tr>
<td>Control DNA</td>
<td>-</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
About 10 µl of the reaction was run on a 0.8% agarose gel to see the purity of the DIG-labeled PCR product to make sure there were no unspecific-labeled products. In cases where this was not the case, the PCR reaction was run on a gel and the expected sized product purified from the gel. To distinguish the labeled from the unlabeled product a PCR reaction was also done without the DIG probe synthesis mix.

2.2.1.5. Genomic DNA Restriction digest

*L. donovani* genomic DNA was taken for restriction digestion with different restriction enzymes, namely *Bam* H1, *Eco* R1, *Hind* 111, *Pst* 1, *Sal* 1 and *Xho* 1.

The reaction components were:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. donovani</em> genomic DNA (0.37 µg/µl)</td>
<td>20 µl</td>
</tr>
<tr>
<td>Restriction enzyme buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>Restriction Enzyme</td>
<td>5 µl</td>
</tr>
<tr>
<td>BSA (in case of <em>Bam</em> H1, <em>Sal</em> 1, <em>Xho</em> 1)</td>
<td>1 µl</td>
</tr>
<tr>
<td>d.w</td>
<td>19 µl in <em>Bam</em> H1, <em>Sal</em> 1 and <em>Xho</em> 1 digestions and 20 µl in others.</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

The eppies containing the reaction components were briefly spun to collect all the components at the bottom and then incubated at 37°C overnight so as to achieve complete digestion.

The next morning, the restriction digests were run along with DIG labeled markers from Roche Molecular Biochemicals and total *L. donovani* genomic DNA on a 0.8 % submerged agarose gel for nearly 1 hour using TAE Buffer with Ethidium Bromide.
2.2.1.6. Southern Hybridization

Southern hybridization was done to transfer the restriction digested separated *L. donovani* genomic DNA fragments to a nitrocellulose membrane so as to facilitate detection of the hybridizing fragment(s).

2.2.1.6.1. Denaturation, Neutralization and Blotting

After the electrophoresis was over, the gel was submerged in depurination solution (in order that the DNA is depurinated and is cut into smaller, easier to transfer fragments) for 10 minutes with shaking at room temperature and then rinsed with d.w briefly to prepare the gel for the next step i.e. denaturation. The membrane was submerged in denaturation solution twice for 15 min each at RT on a shaker. After this step, the gel was again rinsed with d.w and submerged twice in neutralization solution for 15 min each at RT.

Now, the gel and the membrane were prepared for capillary transfer. For this, the nitrocellulose membrane was cut to the size of the gel and several pieces of Whatman 3MM) cut to the size of gel were placed on a raised platform in the order - 2 layers of Whatman 3MM wicks connected to the lower tank of 20X SSC, agarose gel, nitrocellulose membrane, and then several layers of Whatman 3MM paper (approx. 10 cm thickness). A weight (approx. 2 kgs) was placed on the setup. This setup was left overnight at RT.

**Depurination Solution:**
0.25 M HCl

**Denaturation Solution:**
0.5 N NaOH
1.5 M NaCl
Neutralization Solution:
0.5 M Tris HCl, pH 7.5
3 M NaCl

20 X SSC:
3 M NaCl
300 mM Sodium Citrate, pH 7.0

2.2.1.6.2. Fixation

At the end of the transfer, the DNA was fixed to the membrane by UV irradiation at 120,000 µJoules in a UV Strata Crosslinker 2400 (Stratagene). The membrane was finally rinsed in sterile d.w to remove any pieces of membrane and salt sticking to it and then the membrane was air-dried.

2.2.1.6.3. Prehybridization & Hybridization

The membrane was then incubated in 20 ml prehybridization (standard hybridization solution) solution (100 cm$^2$ of the membrane surface area) in a plastic bag (autoclave bag). The bag with the membrane and the solution was then put in a shaking water bath preheated at 68°C. The bag was made sure to be free of any air-bubbles and that the bag was fully submerged in the water bath. The prehybridization was allowed to proceed for about 3 hrs.

At the end of prehybridization, the solution was discarded and the hybridization solution containing 200 ng/ml of the DIG-labeled probe was added. The bag was sealed again and made sure to be free of air bubbles and placed in the preheated water bath at 65°C. The hybridization was allowed to proceed overnight.
**Standard hybridization buffer:**

5X SSC  
0.1 % N-Lauroylsarcosine  
0.02 % SDS  
5 % Blocking reagent (from the 10 % Blocking reagent stock solution)

### 2.2.1.6.4. Detection

Detection was done as chemiluminescent detection, which produces a light signal on the site of the hybridized probe. The signal was recorded on X-Ray films, requiring only very short exposure times.

The membranes were first treated with Blocking Reagent to remove any non-specific attraction of the antibody to the membrane. So, after the hybridization and the post-hybridization washes, the membrane is equilibrated in washing buffer for 1 min. Meanwhile, the chemiluminescent substrate that was CSPD (DIG Luminescent Detection kit, Roche Biochemicals) was allowed to come to room temperature. The membrane is blocked by agitating it in blocking solution for 1 hr after which it is placed in the Anti-Digoxigenin-AP solution for 30 min, which is at a concentration of 1:10,000 in blocking buffer. After the incubation, the antibody solution is discarded and the membrane washed twice, 15 min per wash, in washing buffer.

After the washings, the membrane was equilibrated in detection buffer for two min and then the filter-batching method of applying substrate was used. The substrate was at a concentration of 1:100 in detection buffer. 10 ml of the CSPD solution is pipetted into the center of a dish and the membrane is placed in the dish until the membrane seems to be thoroughly saturated. After incubating the filter for 5 min the membrane is removed from the substrate and the excess liquid allowed to drip off. The damp membrane is placed carefully avoiding any air bubbles between two polythene sheets (autoclave sheets were used). The same method was applied to the rest of the membrane discs. The membranes were then incubated for 30 min at 37°C to allow the
substrate to come to a steady state after which they are taken to the dark room for exposure to X-ray film.

The X-Ray films (KODAK Biomax MR) were placed in an X-ray film cassette with the membranes and then after every 10 min, 30 min, 1 hr and 3 hr; the films are exposed and developed in an automatic film developer (Protec M45, INTAS).

**2 X SSC Wash Solution**

2 X SSC
0.1 % SDS

**0.1 X SSC Wash Solution**

0.1 X SSC
0.1 % SDS

**Maleic acid buffer**

0.1 M Maleic Acid, pH 7.5
0.15 M NaCl

**Wash buffer**

0.3 % (v/v) Tween-20 in Maleic Acid buffer

**Block Solution**

1 % Blocking reagent in Maleic acid buffer

**Detection buffer**

0.1 M Tris, pH 9.5
0.1 M NaCl
2.2.1.7. DNA elution from gel

**JETSORB Gel-extraction Kit (GENOMED)**
The cut agarose gel segment with the DNA fragment embedded in it is excised under UV light and the weight was determined. 300 µl of buffer A1 and 10 µl of JETSORB-suspension was added per 100 mg of the gel and incubated at 50°C for 15 min. The suspension was mixed every 3 min. After the incubation, the suspension is centrifuged at 20,800-x g for 30 sec and the supernatant is discarded. The pellet was again resuspended in 300 µl of buffer A1 and then washed twice with buffer A2. The pellet is finally dried at RT and suspended in 20 µl of TE Buffer. The suspension is then dried at 50°C and centrifuged at 20,800-x g for 1 min. The supernatant containing the DNA is then stored at -20°C.

**QIAquick Gel Extraction Kit (QIAGEN)**
The DNA fragment was excised from the gel under UV light and the gel slice weighed. 3 volumes of buffer QG was added to one volume of gel and incubated at 55°C for 10 min. After checking for the complete dissolution of the gel, the color was checked for optimal pH. 1 gel volume of isopropanol was added to the mix and the mix applied to a QIAquick spin column and centrifuged for 1 min. The flow-through was discarded and 0.5 ml of buffer QG was added and again centrifuged for 1 min. Washing is done by adding 0.75 ml of buffer PE and centrifuging for 1 min. The flow-through is again discarded and the column is again centrifuged for 1 min more at 13,000 rpm. The column is placed in a microcentrifuge tube and 30 µl of elution buffer (10 mM Tris-HCl, pH 8.5) is added to the center of the column and allowed to stand for 1 min after which it is centrifuged again for 1 min to collect the dissolved DNA.
2.2.1.8. pUC 18 cloning

2.2.1.8.1. Digestion of pUC 18

*L. donovani* genomic DNA fragments were cloned into the *Sal I* digested pUC 18. To avoid self-ligation of the restriction-digested plasmid ends, the 5' ends of the vector DNA were dephosphorylated with SAP (Shrimp Alkaline Phosphatase).

The reaction composition of the restriction digestion was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (pUC 18)</td>
<td>30 µl</td>
</tr>
<tr>
<td><em>Sal I</em> Enzyme (NEB)</td>
<td>3 µl</td>
</tr>
<tr>
<td><em>Sal I</em> Buffer (NEB)</td>
<td>5 µl</td>
</tr>
<tr>
<td>BSA (10 mg/ml)</td>
<td>1 µl</td>
</tr>
<tr>
<td>d.w</td>
<td>11 µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50 µl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C overnight. After stopping the reaction by heating it to 65°C for 10 min, the vector was prepared for dephosphorylation.

2.2.1.8.2. Dephosphorylation

The dephosphorylation reaction was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA digested</td>
<td>16 µl</td>
</tr>
<tr>
<td>10 X SAP buffer (Amersham USB)</td>
<td>2 µl</td>
</tr>
<tr>
<td>SAP enzyme (1 U/ml, Amersham USB)</td>
<td>2 µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The reaction tube was incubated at 37°C for 90 min and then incubated at 75°C for 15 min to stop the reaction i.e. inactivate the Alkaline Phosphatase enzyme.
After the dephosphorylation reaction, a phenol/chloroform extraction was done to desalt and purify the digested plasmid DNA from the proteins. After making up the volume of the reaction above to 50 μl with d.w, 50 μl of TE-saturated Phenol was added, mixed thoroughly by vortexing, and then centrifuged at 20,800 x g for 30 sec. The supernatant was taken in a new eppie and then 50 μl of Phenol/Chloroform (1:1) was added, mixed again by vortexing and then centrifuged same way as before. Finally, 50 μl of chloroform was added, vortexed and centrifuged. The supernatant contains the purified DNA which was the taken for Ethanol precipitation.

**Ethanol precipitation:** As the DNA was diluted, it has to be concentrated for the next step i.e. ligation.

For the precipitation, 1/10 volume of 3 M sodium acetate, pH 5.2 and 3 volumes of 100 % ethanol was added and incubated on ice for 2 hrs after which it was centrifuged at 4°C at 13,000 rpm for 45 min and the supernatant was discarded. The pellet was washed with 70 % ethanol, air-dried and then suspended in 20 μl of d.w and stored at -20°C.

**2.2.1.8.3. Ligation**

Cohesive-end ligation of the *L. donovani* DNA fragments and *Sal I*-digested pUC 18 plasmid ends was brought about by T4 DNA ligase which catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA.

Linear dephosphorylated vector DNA and genomic DNA fragments were added in equimolar amounts. The approximate concentration of both the DNAs was determined by running an agarose gel. The insert DNA shows a weaker band under UV as the vector DNA because the insert is smaller in length as the vector DNA.
The reaction composition:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. donovani</em> genomic DNA</td>
<td>13 μl</td>
</tr>
<tr>
<td><em>Sal I</em> digested pUC 18 DNA</td>
<td>3 μl</td>
</tr>
<tr>
<td>10 X ligase buffer (NEB)</td>
<td>2 μl</td>
</tr>
<tr>
<td>T4 DNA ligase enzyme (4U/μl, NEB)</td>
<td>2 μl</td>
</tr>
<tr>
<td><strong>Total reaction volume</strong></td>
<td>20 μl</td>
</tr>
</tbody>
</table>

The reaction was allowed to proceed in a 14°C water bath overnight.

As a control to check the extent of self-ligation of the vector, a reaction was set up where no *L. donovani* genomic DNA was added and the other components were exactly the same. When the transformation was not performed immediately, the ligation reaction was stored at -20°C.

10 X Ligation Buffer:

- 500 mM Tris-HCl, pH 7.5
- 100 mM MgCl₂
- 100 mM DTT
- 10 mM ATP
- 250 μg/ml BSA
2.2.1.8.4. Transformation

2.2.1.8.4.1. Preparation of competent cells

LB medium with appropriate antibiotics was inoculated with a single colony and the culture was grown at 37°C with shaking at 180 rpm until the O.D at $A_{600}$ was 0.25-0.5. The culture was transferred to centrifuge tubes and the tubes were chilled on ice for 15 mins. The culture was then centrifuged at 2500 rpm for 15 mins at 4°C. After discarding the supernatant, the pellet was dissolved in Competency Buffer 1 corresponding to one-third of the original culture volume. After one-hour incubation on ice, they were centrifuged at 3000 rpm for 15 mins at 4°C. The pellet obtained was resuspended in Competency Buffer 2 corresponding to 1/25 of the original culture volume. The cells were incubated on ice for 15 mins and finally 0.2 ml of the cells were aliquoted in 2 ml eppies. The eppies were then quick-frozen and stored at -70°C for later use.

Competency Buffer 1 (200 ml)

Add

20 ml 1M KCl
1.2 ml 5 M Potassium acetate
12 ml 1 M CaCl$_2$
30 ml glycerol
to 100 ml d.w

Mix and titrate to pH 5.8 with 0.2 M acetic acid.

The volume was made up to 200 ml with d.w, filter-sterilized and stored at 4°C.
Competency buffer 2

Add
4 ml 0.5 M MOPS
2 ml 1 M KCl
15 ml 1 M CaCl₂
30 ml glycerol
to 100 ml d.w. Mix and titrate to pH 6.8 with 10 N NaOH.

The volume was made up to 200 ml with d.w and filter-sterilized. The buffer was stored at 4°C.

2.2.1.8.4.2. Heat-shock Transformation

For each transformation, one tube of *E.coli* SURE competent cells was taken, placed on ice and 30 μl of the reaction mix was added after which it was mixed gently and then incubated for 30 min on ice. The tube is then incubated for 45 sec at 42°C, then again chilled on ice for 2 min. 1 ml of SOC medium was then added to the transformed cells and mixed gently. Incubation at 37°C for 2 hours was done with shaking at 180 rpm. 250 μl of the culture is then plated on Ampicillin-containing agar plate on which X-gal and IPTG have been plated. The plates are then incubated at 37°C overnight to allow the transformed cells to grow.
2.2.1.8.5. Blue-white screening

To check the presence of an insert in the clones and to eliminate the "empty" clones i.e. containing no insert a blue-white screening is performed.

There is a short segment of the *E.coli* DNA that contains regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (lac Z). This portion of the gene has a polycloning site that does not disrupt the reading frame, but results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of β-galactosidase. So, vectors containing this fragment are used in host cells that code for the carboxy terminal of the β-galactosidase gene. Neither the host-encoded nor the plasmid-encoded fragments are themselves active but they can associate to form an enzymatically active protein. This type of complementation in which deletion mutants of the operator-proximal segment of the lac Z gene are complemented by β-galactosidase- negative mutants that have the operator proximal region intact, is called α-complementation (Ullmann et al, 1967). The Lac + bacteria that result form blue colonies when chromogenic substrate 5-bromo-4-chloro-3-indoly1-β-D-galactoside (X-gal) is present (Horwitz et al, 1964). White colonies result when a foreign DNA is inserted into the polycloning site of the plasmid, because it results in the production of an amino-terminal fragment that is not capable of α-complementation.

So, the colonies were screened using LB plates that were previously plated with about 40 µl of X-gal (20mg/ml) and 4 µl of IPTG (0.2g/ml).

**X-gal Stock solution**

20 mg/ml solution in DMF is made and then stored at -20°C.
IPTG stock solution

2 g of IPTG is dissolved in 8 ml of d.w and then the volume is adjusted to 10 ml. The solution is filter-sterilized by passing it through a 0.22- micron filter. 1 ml aliquots were made and stored at -20°C.

2.2.1.9. Colony Hybridization

Colony hybridization was done to check the clone containing the gene of interest in a heterologous background.

The bacterial colonies were transferred to a nylon membrane after which alkaline treatment was given to lyse the colonies. The denatured DNA is then immobilized on the membrane, followed by a proteinase K treatment to digest the interfering proteins. The DIG-labeled probe was then used for hybridization. Detection was carried out by a chemiluminescent immunoassay.

2 layers each of Whatman 3 MM paper were soaked with denaturation solution, neutralization solution and 2 X SSC. The agar plates containing the colonies were pre-cooled for approx. 30 min at 4°C. The membrane discs (PALL) were carefully placed onto the surface, avoiding air bubbles. The membrane was left on the plate and the orientation marked so as to identify the positive colonies after detection. The membrane was briefly blotted on a dry Whatman 3 MM paper. The membrane discs were placed on the filter paper soaked with denaturation solution for 15 min with the colonies side facing up after which the membrane was briefly blotted on dry filter paper. The membrane discs were then placed on the filter papers soaked with neutralization solution for 15 min after which they were again placed briefly on dry filter paper followed by placing them on the filter paper soaked in 2 X SSC and the DNA crosslinked by UV light by placing it in a Stratagene crosslinker after which
they were placed on a piece of aluminium foil and 0.5 ml of 2 mg/ml Proteinase K was pipetted onto each membrane disc.
The Proteinase K incubation was done at 37°C for 1 hr. Using filter paper fully wetted with d.w the membranes were blotted between the filter paper several times to remove all the cellular debris and proteins sticking to the membrane, after which the remaining steps were exactly as in steps 2.2.1.6.3. and 2.2.1.6.4.

Denaturation Solution
0.5 N NaOH
1.5 M NaCl

Neutralization Solution
1 M Tris-HCl, pH 7.5
1.5 M NaCl

20 X SSC Buffer
3 M NaCl
300 mM sodium citrate, pH 7.0

Proteinase K
2 mg/ml Proteinase K in 2 X SSC buffer
2.2.1.10. DNA Purification and Sequencing

The positive clone containing the gene of interest was picked up carefully and grown in a 3 ml LB medium containing Ampicillin at 37°C overnight with shaking at 180 rpm and then grown in larger culture i.e. 20 ml after which the DNA was isolated for future sequencing and amplification of the gene.

The DNA was purified on a QIAGEN -tip 100. 20 ml of the overnight culture was centrifuged at 5000-x g for 15 min at 4°C. The bacterial cell pellet was dissolved in 4 ml of Buffer P1 and further Buffer P2 is added, mixed thoroughly and incubated at RT for 5 min. After adding 4 ml of buffer P3, the tube was placed on ice for 15 min. The lysate was then centrifuged at 13,000 rpm for 30 min at 4°C to collect the entire chromosomal DNA into a tight pellet. The supernatant is placed on a previously equilibrated (with 4 ml of buffer QBT) QIAGEN -tip 100. The tip was washed twice with 10 ml buffer QC. The plasmid DNA is then eluted with 5 ml buffer QF and precipitated through the addition of 3.5 ml isopropanol. After centrifuging the tube at 13,000 rpm for 30 min at 4°C, the pellet is washed with 70 % Ethanol, dried and dissolved in 100 μl of TE Buffer.

**Buffer P1 (Resuspension Buffer)**
50 mM Tris, pH 8.0
10 mM EDTA
100 μg RNase/ml

**Buffer P2 (Lysis Buffer)**
200 mM NaOH
1 % (w/v) SDS
Buffer P3 (Neutralization Buffer)
3 M Sodium Acetate, pH 5.5

Buffer QBT (Equilibration Buffer)
750 mM NaCl
50 mM MOPS, pH 7.0
15 % (v/v) Ethanol
0.15 % (v/v) Triton X-100

Buffer QC (Wash Buffer)
1 M NaCl
50 mM MOPS, pH 7.0
15 % (v/v) Ethanol

Buffer QF (Elution Buffer)
1.25 M NaCl
50 mM Tris, pH 8.5
15 % (v/v) Ethanol

TE Buffer
10 mM Tris-HCl, pH 8.0
1 mM EDTA

Buffers P1 and P3 were stored at 4°C and all the others were stored at RT.

The DNA was sequenced in the same way as described in section 2.2.1.3.3.3., except that the primers used were CL1For and CL2 Rev to sequence the full-length gene.
2.2.2. Cloning the full-length gene into an expression vector

2.2.2.1. Amplification of gene

After the full-length gene was sequenced along with its flanking regions, the open reading frame was amplified from the positive clone containing the gene. The gene was amplified using a forward primer overlapping the 5'-end of the gene but containing a His-tag and a Nde 1 site (Forward Nde1 His) and a reverse primer overlapping the 3' end of the gene but containing Xho 1 site (Reverse Xho 1 end) to facilitate cloning into the pET22b (+) vector. Restriction sites on the primers were flanked by 3-4 spacer nucleotides at the 5'-end to facilitate efficient digestion. To decrease the risk of introducing mutations in the gene, a proofreading polymerase was used (Pwo polymerase) and high quantities of primers and DNA was used. The numbers of PCR cycles were reduced too.

The PCR reaction composition was as follows:

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18-LdTXNPx (0.5 µg/µl)</td>
<td>2</td>
</tr>
<tr>
<td>Primers (Forward Nde1 His, Reverse Xho 1 end)</td>
<td>1 µl each</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>2 µl each</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>10 µl</td>
</tr>
<tr>
<td>10 X reaction buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>Pwo Polymerase (5 U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>d.w.</td>
<td>67 µl</td>
</tr>
</tbody>
</table>

Total reaction volume 100 µl

The PCR reaction parameters were
<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Start</td>
<td>2 mins</td>
<td>96°C</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 min</td>
<td>96°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>1 min</td>
<td>50°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Elongation</td>
<td>3 mins</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>10 mins</td>
<td>72°C</td>
<td>1X</td>
</tr>
</tbody>
</table>

10µl of the PCR reaction was taken and analyzed on a 1% agarose gel to check for the correct size of the gene amplified and to check for the purity of PCR fragments amplified.

2.2.2.2. pET22b(+) cloning

2.2.2.2.1. Double-restriction digest

The vector DNA pET22b (+) has an Ndel and XhoI site, which was used to clone the gene of interest.
The components were taken in the following amounts:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET22b(+) plasmid DNA</td>
<td>30 µl</td>
</tr>
<tr>
<td>XhoI enzyme (NEB, 20 U/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NdeI enzyme (NEB, 20 U/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NEB Buffer 4</td>
<td>5 µl</td>
</tr>
<tr>
<td>BSA</td>
<td>1 µl</td>
</tr>
<tr>
<td>d.w</td>
<td>10 µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50 µl</td>
</tr>
</tbody>
</table>

The reaction was incubated overnight at 37°C. The next day, the reaction was stopped by incubating it at 65°C for 10 min and 10 µl of the reaction was analyzed on an agarose gel to check for complete digestion. After it was checked for a single band, the total reaction digest was run on a gel and then agarose gel portion corresponding to the single digested linear band was cut and DNA purified out of the gel. The agarose gel was run long enough to separate the linear form from the nicked and supercoiled, if present. A sample of uncut plasmid DNA was run alongside the digested to identify the undigested from the linearized plasmid. This was also done to ensure that no fragments corresponding to the intervening sequence was present.

NEBuffer 4

50 mM potassium acetate  
20 mM Tris-acetate  
10 mM magnesium acetate  
1 mM dithiothreitol (pH 7.9).

Similarly, the LdTXNPx gene fragment amplified in section 2.2.2.1. was double-digested with NdeI and XhoI. But in this case, one NdeI digestion was done, and the DNA gel-purified and then the second digestion i.e. XhoI digestion was done. The
DNA was again gel-purified. As the DNA here was diluted, it was concentrated by doing an ethanol precipitation.

2.2.2.2. Ligation

The ligation of *NdeI* and *XhoI* digested pET22b (+) DNA to the LdTXNPx gene was carried out 37°C overnight. Equimolar amounts of both were taken. A control reaction without insert DNA was taken to check for the non-recombinant background.

2.2.2.3. BL21 (DE3) transformation

Competent BL21 (DE3) cells were from Novagen. The competent cells were obtained as a singles kit i.e. the cells were obtained as 50 μl aliquots of cells. The cells were then thawed on ice for 10 min and then mixed gently to ensure that the cells are evenly suspended.

1 μl of the ligation reaction was added directly to the cells and stirred gently to mix. The tube was placed on ice for 5 min and then the cells were given a heat shock by placing the tubes in a 42°C water-bath for exactly 30 sec, after which they were again placed on ice. 250 μl of room temperature SOC medium was added. The tubes were then placed in a shaking incubator at 37°C for 30-60 min prior to plating them on Ampicillin-incorporated agar plates.
2.2.3. Recombinant protein expression

2.2.3.1. Induction of cultures

A single colony was taken for expression of LdTXNPx which was cloned into pET22 (+) and transformed into BL21 (DE3). First, 100 ml LB medium with Ampicillin in a flask was inoculated with a single colony of the transformed BL21 (DE3). The culture was allowed to grow at 37°C overnight in a shaking incubator at 180 rpm. Next morning, 1000 ml of LB/Amp medium was inoculated with the previous growing culture to an OD_{600} of 0.1. This was allowed to grow at 37°C at 180 rpm for 3 hrs (OD_{600} approx.0.5) after which IPTG was added to a final concentration of 1 mM to induce the culture. The induced culture was then allowed to grow overnight for the protein to be over-expressed.

2.2.3.2. Sonication

For sonication, the culture was centrifuged to collect the cell pellet and this pellet was dissolved in 4 ml binding buffer (Ni^{2+} chelate chromatography)/100 ml of original culture volume. The cells were sonicated in the Sonoplus HD 200 for 1 min each five times with one-minute intervals. If the culture volume was small i.e. 1 ml or 500 µl, sonication was done only once for 1 min. Throughout sonication, the cells were placed on ice and the sonicator was at 50 W.
2.2.3.3. SDS-PAGE electrophoresis

2.2.3.3.1. Coomassie-Blue staining

After the gel run was over, Coomassie Blue staining, the sensitivity of which is 0.3-1 μg protein was done for about 1 hr and the destaining for about 2 hr or till the bands are clearly visible.

0.2 % Coomassie Blue stock solution
1 tablet PhastGel Blue R (Pharmacia) was dissolved in 80-ml d.w and then 120 ml of methanol was added.
The stock solution was stored at 4°C.

Destaining solution
Methanol: Acetic acid: d.w was at a ratio of 3:1:6.

Staining Solution
The Coomassie Blue stock solution was diluted 1:10 with the destaining solution.

2.2.3.3.2. Silver Nitrate staining

Silver staining of gels enables us to see even 10 ng of protein. After the gel run, the proteins were fixed onto the gel by incubating it in fixing solution for 1 hour. Great care was taken to keep the tray and other things free of any proteins, keeping in mind the sensitivity of staining. After fixing, the gel was washed twice for 5 min each in wash solution after which it was incubated in Farmer's solution for 10 min till the gel takes the yellow color. After that the gel was washed repeatedly in water till it becomes transparent. The gel is then incubated in the silver nitrate solution for 20 min in dark. The gel is then given a quick wash in water and treated with the developing
solution. After the brown bands start appearing, the reaction is stopped by the addition of stop solution.

Fixing solution
50% Methanol
12 % Acetic Acid
made in d.w

Wash Solution
10 % Ethanol
5 % Acetic Acid
Made in d.w.

Farmer's Solution
150 mg Potassium Ferricyanide
300 mg Sodium thiosulphate
50 mg Sodium Carbonate
Made in 100 ml d.w

Silver Nitrate Solution
0.1 % Silver Nitrate in d.w

Developing Solution
0.25 M Sodium Carbonate
0.05 % Formaldehyde

Stop Solution
1 % Acetic Acid in d.w
2.2.3.3. Preservation of Protein gels

The acrylamide protein gels were preserved for later reference. There were two methods of preserving them-glycerin method in which the gels were incubated with shaking in glycerin for about 1 hr and then pressed between two damp sheets of films. The sheets with the gel in between them were clamped onto a gel-drying cassette and allowed to stand overnight for drying.

The second method was submerging the gel in technical Ethanol and then pressing the gel between two damp sheets of films and clamping it into a gel drying cassette and allowed to stand overnight.

2.2.4. Protein purification

2.2.4.1. Metal chelation chromatography

For purifying proteins, metal chelation chromatography was used. His.Bind resin (Novagen) enables a rapid one-step purification of proteins containing a His Tag sequence, which binds to Ni\textsuperscript{2+} cations, which are in turn immobilized on the His.Bind resin. After the unbound proteins are washed away, the target protein is recovered by imidazole elution.
2.2.4.1.1. Preparation of cell-extract

As the LdTXNPx protein was relatively highly expressed, for the Quick Columns i.e. small-scale purification, about 100 ml of the culture was taken and for large-scale about 500-1000 ml of the culture was taken.

The cells were harvested by centrifugation at 5,000-x g for 10 min. After decanting the supernatant and making sure the cell pellet was dry as completely as possible, the cells were suspended in 4 ml ice-cold 1X Binding Buffer. After this, the cells were sonicated as described in Section 2.2.3.2.

After sonication, the cell lysate was centrifuged at 20,000-x g for 30 min to get a clear supernatant containing the protein.

2.2.4.1.2. Small-scale protein purification

For small-scale purification of proteins Quick columns (Novagen) were used which are supposed to yield up to 5 mg of protein. The column was pre-wetted with 15 ml 1X Binding Buffer. The cell extract was loaded onto the column and then washing was done with 30 ml 1X Binding Buffer. The second wash was done with 15 ml 1X Wash Buffer and the protein was eluted with 10 ml 1X Elute Buffer or 10 ml 1X Strip Buffer. The columns were attached to peristaltic pumps where the flow-rate was adjusted to 5-10 ml/min for pre-wetting and washings and 1-3 ml/min for loading and eluting.

8X Binding Buffer
40 mM Imidazole
4 M NaCl
160 mM Tris-HCl, pH 7.9
8X Wash Buffer
480 mM Imidazole
4 M NaCl
160 mM Tris-HCl, pH 7.9

4X Elute Buffer
4 M Imidazole
2 M NaCl
80 mM Tris-HCl, pH 7.9

4X Strip Buffer
400 mM EDTA
2 M NaCl
80 mM Tris-HCl, pH 7.9

8X Charge Buffer
400 mM NiSO₄

2.2.4.1.3. Large-scale protein purification

For large scale purification of protein, a litre of the culture was harvested and treated as described in section 2.2.4.1.1. and then the pellet was resuspended in 4% of the original volume in Buffer A. After sonication, and centrifugation to remove cellular debris, the supernatant was diluted with one volume of the same Buffer A. This was then applied to His-Bind column (5×3.7 cm³ from Novagen). The column was washed with 10 column volumes of Buffer A and then eluted with a gradient of 0-80% of Buffer B.
Buffer A
20 mM Tris (pH 7.9)
500 mM NaCl
5 mM Imidazole

Buffer B
20 mM Tris, pH 7.9
500 mM NaCl
500 mM Imidazole

2.2.4.2. Measurement of protein concentration

The protein concentration was measured by two methods- the Bradford method (Bradford, 1976) and the BCA method.

Bradford method- The protein concentration in a solution was determined by Coomassie Brilliant Blue-G dye that was obtained as Bradford reagent from Biorad. The Bradford reagent was diluted 1:5 with water. For the concentration determination, 200 µl of the diluted Bradford reagent was mixed with 50 µl of the protein sample and incubated at 37°C for 15 min and the absorption measured at 595 nm in a Microtiterplate reader 3550-UV (Biorad). The protein content was calculated using a calibration curve of BSA in water in the range of 1 to 10 µg/ml.

BCA method- BCA reagent from Pierce was used for this determination of protein concentration. 50 parts of Reagent A was mixed thoroughly with 1 part of Reagent B and vortexed to ensure complete mixing. The reagent was brought to RT before taking it for measurement. Different quantities of the protein solution was taken and mixed with water (10-100 µl) and mixed with 2 ml of the reconstituted reagent mixture and then measured in a spectrophotometer which already had a BSA standard curve.
(protein concentration 200-1000 µg/ml) stored for reference. The extinction measurements were done 562 nm.

2.2.4.3. Gel Filtration

For desalting the protein solution after purification, PD-10 columns from Amersham Pharmacia Biotech was used. They are prepackaged disposable columns containing Sephadex G-25 M for rapid desalting and buffer exchange.

The protein solution was exchanged with water, so after pouring off the excess liquid and removing the bottom cap, the gel bed was equilibrated with 25 ml of d.w. The sample was added in a total volume of 2.5 ml. When the sample has run into the column, it was eluted with 3.5 ml of d.w.

2.2.4.4. UV-VIS spectroscopy

The UV-Visible spectrum of LdTXNPx was recorded using a SPECORD spectrophotometer (Zeiss) adjusted for 45.9 ms of integration time and accumulation value of 10 and wavelengths between 200.95-450.13 nm. The temperature of the measurements was 25°C. The protein was at a concentration of 0.9 µg/µl. The chromatographic buffers were used as reference for the measurements.

2.2.4.5. Concentrating protein

After purification, the protein was concentrated and desalted using Amicon's centrifugal concentrators (Millipore). They provide a fast, convenient, high-recovery alternative to dialysis. Salt transfer across the membrane is efficient and independent
of biomolecule concentration or size. The sample can also be concentrated in the same device.

Centriprep YM-10 columns were used up to 15 ml of the protein solution could be concentrated. The columns were placed in a rotor that could hold 50 ml tubes. 3000-x g was the maximum centrifugal force used. The protein solution was bought down to about 1 ml after which it could be loaded on the Centricon YM-10 columns. 5000-x g was the maximum speed used for the Centricon.

The tubes were inverted and centrifuged at 1000-x g to elute the concentrated protein solution.

2.2.5. Enzyme Kinetics

2.2.5.1. Trypanothione-dependent Peroxidase System assay

The trypanothione-dependent assays were performed in a UV/VIS Spectrophotometer Biochrom 4060 (Pharmacia) and in Specord Spectrophotometer (Zeiss) at a wavelength of 340 nm. The NADPH oxidation was measured with an Absorption coefficient according to $\varepsilon_{340}(\text{NADPH}) = 6.3 \text{ mM}^{-1}\text{cm}^{-1}$.

2.2.5.1.1. Specific Activity measurements

The Specific Activity of the protein (LdTXNPx) was measured essentially as described in Montemartini et al 1999 under the following conditions: The test system comprised of 375 $\mu$M NADPH, 1 U ml$^{-1}$TR, 130 $\mu$M trypanothione, 18.75-75 $\mu$M TXN2, and 73 $\mu$M t-BOOH in 50 mM HEPES, 1 mM EDTA, pH 7.6 in a final
volume of 500 μl. the reaction was preincubated for 15 min and started by the
addition of the peroxide.

2.2.5.1.2. Steady-State Kinetics of LdTXNPx in the Trypanothione-dependent Peroxidase System

Steady State kinetics were analyzed essentially according to the procedure described
above in section 2.2.5.1.1. The kinetic data were analyzed as described in Dalziel
(1957).

2.2.5.2. Glutathione assay

The glutathione assay was performed for the Y44H mutant protein. The assay buffer
was MOPS buffer at 37°C. 80 mM Glutathione, 300 μM NADPH, 0.5 μM
Glutathione Reductase, 78 μM t-BOOH and 100 μl of 312.25 μg/ml Y44H protein
was taken in a total volume of 500 μl. Other conditions were identical to the
Trypanothione-dependent peroxidase assay. PfGPx was taken as a positive control.
2.2.6. Site-directed Mutagenesis

2.2.6.1. Primary PCR

For mutant construction, pET22b (+) plasmid containing the LdTXNPx gene cloned into it with an N-terminal His tag was taken. The PCR was performed essentially as described in section 2.2.2.1. Pwo DNA polymerase (Boehringer Mannheim) that is a proofreading polymerase was taken to avoid the introduction of any undesired mutations into the gene. For every mutation i.e. C52S, T49V, T49S, W87R, W87F, C173S, W177L, Y44H, Y44F and R128D, the corresponding forward primers with the dismutated base/bases was taken along with the T7 terminator primer and the reverse primer was taken with the T7 promoter primer for the PCR. The primer sequences are given in the Appendix section.

After the PCR was over, 10 µl of the PCR reaction was taken to analyze the amplification of a single fragment and the yield of the PCR reaction. The whole PCR reaction was loaded on a 1.5 % agarose gel to separate any unused primers and to elute the amplified partial gene. The DNA was eluted using the QIAGEN DNA elution kit and used for the next step i.e. the secondary PCR.

2.2.6.2. Secondary PCR

For the secondary PCR, the partially amplified gene fragments were mixed to get a complete gene amplification i.e. the corresponding fragments were taken. T7 promoter and T7 terminator primers were taken and there was a portion of the gene that was common to both the fragments that anneal during the process. The other conditions were the same as described for the primary PCR.
After the PCR, an aliquot of the reaction was analyzed on a 1.5 % agarose gel after which the PCR amplification reaction was loaded onto a common large well of a 2 % agarose gel and DNA eluted as described before.

2.2.6.3. Restriction Enzyme digestion

After the gene is amplified with the desired mutation in it, it has to be "cleaned" of the extending fragments on both the 5' and the 3' end and the restriction site made available for ligation into a pET22b(+) plasmid. The digestion was done basically as described in Section 2.2.2.2.1. with *XhoI* and *NdeI* enzymes. After the digestion was complete, the DNA was eluted.

2.2.6.4. pET-22b(+) cloning

The mutated DNA was cloned into pET22b(+) basically as described before and transformed into BL21(DE3) so as to be expressed and the protein made available for further analysis and protein activity measurements.

2.2.6.5. Protein Expression, Purification and Concentration

For the expression of the mutated proteins, different temperatures were tried for induction as some mutants expressed at 30°C and others at RT. Moreover, the yield of soluble protein varied for different mutants. The mutants were purified according to Sec.2.2.4.1.2. for small-scale purification and Sec.2.2.4.1.3. for large-scale purification. Whenever needed, proteins were concentrated according to Sec.2.2.4.5.
2.2.6. 6. Mutant enzyme kinetic assay

The mutants were analyzed kinetically according to the procedure described in Section 2.2.5.1.1.

2.2.7. Computer Programs used

Vector Nti was used for selection of primers and for checking the restriction sites in any plasmid.

GCG Wisconsin-Package 9.1 for UNIX and DNASIS for Windows was used for checking the sequence homology.

BLAST and FASTA were used for submitting the gene sequences to the gene bank.

The graphics for kinetic studies were done using SigmaPlot 3.0 and ORIGIN.

The kinetic assays were analyzed using SWIFT for Reaction Kinetics.
3. RESULTS

3.1. Presence of a *C. fasciculata*-homologous peroxidase

The trypanothione-mediated hydroperoxide system has already been elucidated in *Crithidia fasciculata* (NOGOCEKE et al, 1997) which is the non-human insect pathogen belonging to Trypanosomatids. It is supposed to comprise of three key enzymes-Tryparedoxin peroxidase, Tryparedoxin and Trypanothione Reductase. The presence of all three has been confirmed in *Crithidia*. To establish the presence of a similar system in the human parasite *Leishmania donovani*, the presence of at least one of the enzyme equivalents have to be shown in *Leishmania*. To this effect, a Western Blot was done with the total *L. donovani* cell lysate and probed with *Crithidia* Tryparedoxin peroxidase i.e., Cf 21 and Tryparedoxin, i.e. Cf16 antibodies.

![Western Blot Image](image)

Figure 1: Western blotting of proteins Cf 16 (*C. fasciculata* Tryparedoxin) and Cf 21 (*C. fasciculata* Tryparedoxin Peroxidase) alongwith the total protein lysate of *Leishmania Donovani*. Lane 1: Protein marker, Lane 2: *L. donovani* total protein lysate, Lane 3: Recombinant Cf21 (i.e. protein purified from the expression of a
pET24a/BL21 (DE3) with the TXNPx gene cloned into it). Lane 4: Native Cf21 (protein purified from total Crithidia lysate as described in NOGOCEKE et al (1997). Lane 5: Recombinant Cf16 (i.e. protein purified from the expression of a pET24a/BL21 (DE3) with the TXN gene cloned into it). Lane 6: Native Cf16 (protein purified from total Crithidia lysate as described in NOGOCEKE et al (1997).

The Western Blot of the total protein lysate with *C.fasciculata* Tryparedoxin Peroxidase and Tryparedoxin antibodies was done so as to know if homologous proteins exist in *L.donovani*. The antibodies were used at dilutions 1:500 as this concentration was determined to be the dilution at which the native and the recombinant proteins showed up. In this case, a native protein implies that the protein was purified directly from a *C.fasciculata* culture as described in NOGOCEKE et al (1997) and the recombinant proteins denote the cloned (into pET24 a), expressed and purified protein as described in MONTEMARTINI et al (1998). The Western Blot was not very revealing, showing no major protein bands in the region of these proteins. Attempts to excise the corresponding regions and do protein sequencing were not successful due to the purity of the protein relative to other proteins that may have contaminated while trying to excise the protein. Moreover, it is difficult to determine exactly which band corresponds to the Tryparedoxin Peroxidase of *Leishmania*. As we shall see later, the protein band, which we presumed to be the peroxidase, was not in fact the peroxidase as the *Leishmania* peroxidase turned out to be of a higher molecular weight than the *Crithidia* peroxidase. The relatively small proportion of the relevant proteins in the total protein lysate might have made it difficult to establish the presence of a reaction in this Western Blot. Therefore, a highly sensitive method i.e. PCR was done as is described in the next section.
3.2. *L. donovani* genomic DNA PCR

![Image of gel electrophoresis](image)

**Figure 2**: PCR amplification of *L. donovani* genomic DNA with the primers from the conserved regions.

Lane 1 & 5: 1 kb DNA marker (GIBCO), Lane 2: *L. donovani* total DNA PCR product using CL1For and CL4Rev primers, Lane 3: PCR negative control, Lane 4: *L. donovani* total DNA PCR product using CL1For & CL2 Rev primers. Description of the primers is given in the appendix.

Figure 2 shows the PCR amplification results using two different primer sets. The idea was to get a PCR product so as to have a probe to start screening a genomic library. Also, this would confirm the presence of a similar peroxidase gene in *Leishmania*. At that time since it was not yet confirmed whether there is a similar gene present in any *Leishmania* species, this would give something to start with. These primers were designed from the highly conserved regions of the *Crithidia* gene with other closely related peroxidases. The two conserved VCP surrounding motifs were taken for the primer design. The sequence of the primers was CL1 For (**5'**-GACTTCACCTTCGTCGGCCGAGCGCCG-**3'**)) and CL2 Rev (**5'**-GTTGGGTGATGATGAGACGGCGCG-**3'**).
CL4Rev primer in conjunction with CL1For gave two distinct bands and therefore was not considered suitable for cloning into the TA cloning vector, as the manufacturer suggests that the PCR product be a clear distinct single band for the high-efficiency cloning of the PCR product. Moreover we got a desired single band with the second set of primers. CL1For and CL2Rev primers on the other hand when used on *L. donovani* genomic DNA gave a distinct and clear single band which was taken for cloning into pCR2.1 and which shows that the regions from where these primers are designed are highly conserved in all the copies of the peroxidase gene in *L. donovani*. Not surprisingly these are the highly conserved regions including the two VCP clusters, which was been indeed found to be highly conserved in most of the peroxidases. The size of the single band was about 270 bp, which is what would be expected if this PCR were performed on the *C.fasciculata* Tryparedoxin Peroxidase. This suggests that a similar gene having a similar sequence is definitely present in *Leishmania*. This was the first evidence ever reported for the presence of a similar gene in a human pathogen for Leishmaniasis.

### 3.3. Cloning of the 270-bp PCR fragment

![Figure 3](image.png)

**Figure 3:** Different pCR2.1 clones after ligation with the 270 bp PCR fragment. Figure 3 shows the different clones of pCR2.1 after ligation with the 270 bp PCR fragment done according to the manufacturer's suggestions. The vector has
overhanging nucleotides that anneal to the nucleotides of the insert that has been amplified by PCR. The insert can be further popped out by digestion with Eco RI because the vector has these sites on either side of the insert.

Because blue-white screening was possible with these clones, it was thought that the clones could be picked and checked for the insert. The digestion shows only some clones had the desired fragment although they appeared to be positive i.e. they were white on the plate. Majority of the clones were positive but some false positives were present. The digestion with EcoRI was done because there are two EcoRI sites flanking the insert in pCR2.1 vector. The correct size of the insert is thus confirmed along with the 1kb DNA ladder as a reference standard.
3.4. **Labeling of SK lig 2.7.**

![Image of gel with labeled bands](image)

**Figure 4:** DIG-Labeling of SK lig 2.7 insert by PCR DIG Probe synthesis kit. Lane 1 & 5: 1 kb DNA ladder (GIBCO), Lane 2: Control labeled DNA, Lane 3: Digested SK lig 2.7 plasmid, Lane 4: DIG-labeled fragment.

The plasmid (pCR2.1) containing the 270 bp PCR fragment was used as the template for obtaining a DIG-labeled probe for further hybridization. DIG-labeling PCR gave several amplified bands but the fragment slightly higher than 270 bp (because of DIG incorporated into it) was the major amplified band. To avoid background hybridizations, the band was cut and purified from the agarose gel.
3.5. *L. donovani* genomic DNA restriction digest and Southern Blotting

![Image of Southern Blotting](image)

**Figure 5:** Southern Blotting of the total *Leishmania donovani* genomic DNA digested with different enzymes and then probed with the DIG-labelled 270-bp PCR DNA fragment.

Lane 1: DNA M.W marker III, DIG-Labeled (Boehringer Mannheim), Lane 2: *Xho* I digestion, Lane 3: *Sal* I digestion, Lane 4: *Pst* I digestion, Lane 5: *Hind* III digestion, Lane 6: *Eco* R I digestion, Lane 7: *Bam* H1 digestion, Lane 8: *L. donovani* total genomic DNA

The total *L. donovani* genomic DNA digested with different restriction enzymes and probed with the 270 bp DNA fragment reveals a lot of interesting results. Basically it shows that the *Sal* I digestion has three fragments that hybridize very distinctly, leading to the fact that there are at least three copies of the peroxidase gene in the genome which is not unexpected knowing that there are multiple copies of the gene in the Leishmania and for that matter other trypanosomatids. *Xho* I digested products also shows that there are at least two copies of the gene in the genome. *Pst* I digestion reveals two smaller bands showing that these sites are present in the middle of the
gene somewhere, so they cannot be used for cloning as what we are aiming is for a total gene. Hybridization of the Hind III digestion reveals a large band of about 20 kb which is impractical for cloning purposes, though it leads to the conclusion that the three genes are scattered on an area of about 20 kb. Eco R1 and Bam H1 digested genomic DNA shows a similar fragment but also a smaller fragment that may a fragment from one of the genes itself revealing the presence of these sites in at least one copy of the gene.

3.6. Cloning of the L. donovani DNA genomic fragments

![Figure 6: Sal1 digestion of Gene1-Sal1-1-21. Lane1: 1 kb DNA ladder (Gibco), Lane 2: Gene1-Sal1-1-21 digested with Sal1. The hybridizing clone showed an insert of about 3 kb which corresponds to the hybridizing fragment obtained in the Southern hybridization.](image)
3.7. Sequencing

The sequencing of the pUC 18 plasmid containing the LdTXNPx (Leishmania donovani peroxidase) gene was done using the internal primers, CL1 For and CL1 Rev so that overlapping sequences would be obtained leaving no ambiguity about the sequence. Sequencing revealed the full-length sequence of the gene along with a portion of the 3'-end and the 5'-end sequence. The sequencing was done by the dideoxynucleotide chain termination method (SANGER et al., 1977) by automated cycle sequencing on an ABI PRISM 373A sequencer and done in duplicate to confirm the sequence and to avoid any ambiguities in the gene sequence. The sequence is shown below.

```
MSCGNAK
1 gcaagcagctgaagagccggtgccgccttgccccccaccgcc TTCCTCTGGTAAACGCAA

ICPAPPBEEVALMPNGSFK
21 GATCAACTGTCCCGCCGCCCCCTTGAGAGGTGGCTCATGCCCCAAGGCAAGCTTCCAA

KLISLAAKGGKWWVFVLFFFYPLD
81 GAAGACCGCTCAGCAGCAGGCCTACAAAGGGGAAGATGGTGCTGCTCTTCTTCTCACCCGGCTCGA

FTFVCPTEIIIAFSENSEVSRFN
141 CTTCACCTTCTGTGCACAGGACAGATCTCATCTGCTCGGCTCCGAAAAACGTGAGGCTGGCTTCAAA

ELNCEVELACSMDSBEYAHLQW
201 CGAGCTCCAGGTCTCTGGCTGGCTCATGAGACACCCAGTGCAGTGCAGCCACCTGATAGT

TLQRDDKGGGLGAMAIPLMLAD
261 GACGCTGACCAGCGAAGAGGGGGGCTCCGCGCCAGCATGCGGATTCCAAATCTGCTGCGCG

KTISKIARAYGVLKEEEKQGVAAY
321 CAGAGGCAAGACATCTGCTGGCTTGGATGAGGAAACACAGGCGTGCGCCTA

CL2Rev

RGFLIDPNGMVRQITVNDM
381 CGGCGGTCTCTTCTCATCGACCCCAATGGCAATTGTCGGCCAGATCCTCAGCAGCACAT

PVGRCNEVELRRLBALFQFVE
441 GCCGGTGGCGCAAGCTTGAGAGGATTCTGCGCCCTGCTGGAGGCTTTTCTCAGATGGCTGGGA

KHGEVCPANWKKKGAPTMKPE
501 GAAGACCGGCGAGGGTGGCGCCCCGCGACCTGGAAGAACGGCGCCCCGCCATTGAAACGCG

PKASVEGYFSKQ*
561 GCCGAGGCGGTCTGTGCGGAGGATCCTACAGAGACTGAAGAACGGCGCCCCGCCATTGAGC
```
aaagctgaactggccacggcccttcacgtcatggtggcgggtacgctggtcgctcctccagtacacgctggcctgctcgggtccaggtgctacagctccgctcgcctgccatccgctctccatgagaaaggtgtctctcattgtgctgctgctgctgacttgacact

**Figure 7:** Genomic DNA sequence of LdTXNPx. The primers used for PCR are shown.

From the sequence, it can be seen that the two VCP clusters characteristic of peroxidases were present.

### 3.8. Amplification of the full-length LdTXNPx gene

![Image](image_url)  
600 bp

**Figure 8:** Amplification of the full-length LdTXNPx gene.

Lane1: 1 kb DNA marker, Lane 2: Amplification from pUC 18/LdTXNPx using End Xho1 and Start Nde 1 primers, Lane 3: Amplification from pUC 18/LdTXNPx using His start-Nde 1 and stop end-Sal 1 primers, Lane 4: Amplification from pET24a/BL21-cf21 using His start-Nde 1 and stop end-Sal 1 primers, Lane 5: Amplification from pET24a/BL21-cf21 using Start-Nde 1 and stop end-Sal 1 primers.
The gene needed to be amplified to be eventually cloned into a protein expression vector, namely pET 22 b vector. Primers were designed from the 5'-end and 3'-end of the gene and PCR was done to amplify the gene.

3.9. Cloning of LdTXNPx into an Expression Vector, pET22b(+)

The PCR fragment which encoded the whole gene was cloned into the expression vector, pET22b(+) so as to facilitate expression and purification of the protein. pET22b(+) is a commercial vector from Novagen with a 6-His tag at the C-terminal end of the cloning site. However, from previous results from our lab we discovered that for some reason N-terminally his-tagged proteins were expressed at much higher levels than the C-terminally proteins. We verified that by making both N-terminal and C-terminal his-tagged proteins and expressing them both in the E.coli BL21 (DE3) system and running SDS-Page gels stained with Coomassie blue. We found out that the N-terminal His-tag gave much higher levels of expression, so for all further studies we took the N-terminally his-tagged protein for further studies. However, we did not study the level of protein expression enhanced by changing the location of the His-tag.

3.10. Expression of the recombinant protein

The open reading frame encoding the LdTXNPx gene was cloned into an expression vector, which was pET22b (+) in this case as it allows IPTG-inducible expression. In pET vectors, target genes are cloned under control of strong bacteriophage T7 transcription and translation signals, and providing a source of T7 RNA polymerase in the host cell induces expression. T7 RNA polymerase is so selective and active, that when fully induced, almost all the cell's resources were supposed to be converted to target gene expression.
The vector is designed to have a C-terminal His Tag, but the expression levels were minimal with a C-terminal His tag. The reason for this difference is unknown. So, a stop codon was encoded in the reverse primer and a 6 His tag was incorporated in the forward primer so that now it was a N-Terminal His tag leading to a marked increase in the expression of the recombinant protein.

The expression of the protein was achieved to high levels in *E.coli* as can be seen from the figure in the next section.

### 3.11. Purification of recombinant protein

The protein, LdTXNPx was seen to be expressed at high levels and was purified on a His-Bind column from Novagen. The Talon spin column from Invitrogen was tried too, though compared to the His-Bind column it did not give higher yields.
Figure 9: 15% SDS-protein gel showing the expression and purification of LdTXNPx on a His-Bind column. The gel was stained with Coomassie Blue.

1: 10 kDa protein marker (Gibco), 2: pET 22-b/BL21 (DE3) total protein, 3: pET 22-b-LdTXNPx/BL21 (DE3) total protein before induction, 4: pET 22-b-LdTXNPx/BL21 (DE3) total protein after overnight (16 hours) induction, 5: LdTXNPx protein after purification, 6: L. donovani total protein lysate, 7: Purified C.fasciculata peroxidase

The maximal expression of the protein was obtained after an overnight induction. The purified protein did not show very distinct dimer bands but they showed up on a western blot. The Crithidia fasciculata peroxidase is also supposed to be present in dimers. The Western blot with the anti-peroxidase antibodies also showed a leaky expression of the protein before induction which is not unusual.

Figure 10: Western blot. C.fasciculata peroxidase (Cf21) antibodies were used as primary antibody and anti-rabbit goat antibodies (Biorad) as secondary antibody.

The figure below shows a typical profile of purifying the LdTXNPx on a Quick Hist-bind column. It was fast and relatively pure protein was obtained, though there were some losses in the washes. Also, it can be seen that the protein was expressed both as a soluble protein in the supernatant and as an insoluble fraction in the pellet. Only the soluble fraction was taken for purification, as the amount was enough to go on with the studies.

![Image of gel electrophoresis](image)

**Figure 11:** Induction and purification of LdTXNPx on a Quick Hist-Bind column (Novagen).

Lane 1: 10kDa Protein Marker (GIBCO), Lane 2: Pellet of the overnight induced culture, Lane 3: Supernatant of the overnight induced culture, Lane 4: Flowthrough after the first wash (Binding buffer), Lane 5 & 6: Flowthrough after the second and third washes, respectively (Wash buffer), Lane 7: Eluted protein.
When the purification was scaled up to a 100 ml culture, no major difference was found in the yield. This time too, a Novagen His-Bind resin was obtained and used for purification. The fraction nos. 22 to 26 was found to be containing the purified protein. Running quick gels of each fraction checked the purity and the presence of the protein. The LdTXNPPx protein eluted at 34 % buffer B, which is equivalent to 170 mM imidazole. The fractions were run on acrylamide gels and stained with Coomassie to check the purity of the protein. On the whole, these fractions were found to contain pure protein. No other contaminants were found, at least on the Coomassie stained gel. Silver staining was not done.

![Graph](image)

**Figure 12:** A purification profile of the LdTXNPPx on a His-Bind column (Novagen).
### 3.12. UV-VIS spectroscopy

Both the ultraviolet and the visible spectra displayed no UV or visible absorbing chromophores other than the aromatic amino acids as is apparent from the figure below. Also the wavelength absorption characteristic for cofactors such as FAD (ε\textsubscript{450 nm}=11,300 cm\textsuperscript{-1} M\textsuperscript{-1}), haem (ε\textsubscript{380 nm}=5,500 cm\textsuperscript{-1} M\textsuperscript{-1} and ε\textsubscript{530 nm}=3,900 cm\textsuperscript{-1} M\textsuperscript{-1}), or transition metals were absent, showing that only the amino acids residues participate in the catalytic electron transfer from Trypanothione to the hydroperoxide.

![UV and Visible spectra of LdTXNPx](image)

**Figure 13:** UV and Visible spectra of LdTXNPx.
3.13. Mutant Construction

All the 10 mutants were expressed and purified under identical conditions as the wild-type LdTXNPx protein i.e. Novagen His-Bind resin was used to purify after an overnight induction.

The expression of all the mutants revealed variable levels of expression. In case of the T49S mutant, the activity was markedly increased, whereas in case of the T49V mutant it was significantly decreased. The other mutants that showed a decrease in the level of expression were C52S, W87F, W87R, Y44F, Y44H, C173S, R128D and W177L, though in all the cases it was possible to get enough of the mutant to check the specific activity. The relative levels of the soluble and the insoluble fractions also varied. For activity measurements only the soluble fraction was taken. In some cases where the expression was really low; in case of C52S, W87R, C173S and W177L, the protein had to be concentrated using Amicon concentrators. These concentrators do not alter the properties of the proteins in any way.

Expression of some of the mutants is shown in the gels on the next page as an example.
**Figure 14:** Induction of C173S, R128D, T49V, T49S and W87R mutants.

Lane 1 & 13: 10kDa Protein ladder (GIBCO), Lane 2, 3, 4 & 5: C173S mutant, 2 & 3- Uninduced pellet and supernatant, respectively. 4 & 5 - Induced pellet and supernatant,
respectively. Lane 6, 7, 8 & 9: R128D mutant, 6 & 7- Uninduced pellet and supernatant, respectively. 8 & 9 -Induced pellet and supernatant, respectively. Lane 10, 11, 14 & 15: T49V mutant, 10 & 11- Uninduced pellet and supernatant, respectively. 14 & 15-Induced pellet and supernatant, respectively. Lane 16, 17, 18 & 19: T49S mutant, 16 & 17- Uninduced pellet and supernatant, respectively. 18 & 19 -Induced pellet and supernatant, respectively. Lane 20, 21, 22 & 23: W87R mutant, 20& 21- Uninduced pellet and supernatant, respectively. 22 & 23 -Induced pellet and supernatant, respectively. Lane 12 & 24: LdTXNPx protein.

3.14. Kinetic Measurements

The mutants were analyzed kinetically whenever the protein was sufficiently expressed to allow analysis. So, three mutants, namely W87F, T49V and T49S mutants were analyzed. Also, the wild-type protein kinetics was studied. As a substrate, t-butyl hydroperoxide was selected, because its spontaneous reaction with trypanothione and tryparedoxin can be neglected and it did not affect the stability of the protein within the time frame required for activity measurements. The kinetic analysis included studying the initial velocities deduced from the substrate consumption curves. Essentially, ping-pong type of kinetics was revealed with the wild-type protein. Enzyme-normalized double reciprocal primary plots derived at fixed co-substrate (Tryparedoxin in this case) concentrations show straight lines, which deviate insignificantly from being parallel. This shows that the protein is oxidized by the peroxide substrate and then regenerated by reduced Tryparedoxin in two independent steps i.e. at no given time does a ternary complex exist between the enzyme, the hydroperoxide and Tryparedoxin, which is essentially what a ping-pong type of kinetic mechanism is.

When the reciprocal apparent maximum velocities i.e. \( \frac{[E_0]}{V_{app}} \) values, which are obtained as ordinate intercepts of the primary plot were plotted against the reciprocal Tryparedoxin concentrations, a secondary plot was obtained. It basically provides
with $K_m$ values for the substrate and the co-substrate. The kinetic pattern of LdH6TXNPx can be described by the Dalziel equation for multi-substrate kinetics (Dalziel, 1957).

$$[E_0]/v = \phi_0 + \phi_1/[A] + \phi_2/[B] + \phi_{1,2}/[A][B]$$

where $[E_0]$ is the total enzyme concentration, A is the substrate i.e. t-butyl peroxide and B is *Crithidia* TXN2H6. The empirical coefficients $\phi_1$ and $\phi_2$ are simply the reciprocal values of the net forward rate constants characterizing the reactions of the reduced enzyme with hydroperoxide, $k_1'$, and of the oxidized enzyme with reduced tryparedoxin, $k_2'$. In other words, they are the reciprocal apparent rate constants for t-butyl peroxide and B is *Crithidia* TXN2H6-dependent steps.

The non-convergence of the slopes on the plot of $1/[t-BOOH]$ against $E/v$ for diverse concentrations of TXN suggests that the last term of the above equation is zero, bringing us to the equation for two-substrate ping-pong kinetic reactions

$$[E_0]/v = \phi_0 + \phi_1/[A] + \phi_2/[B]$$

All the various parameters are compiled in the table in the last section of results.
Wild type *L. donovani* peroxidase (LdH6TXNPx)

![Graph showing enzymatic activity](image1)

W87F Mutant

![Graph showing enzymatic activity](image2)

**Fig 15 A & 15 B:** Primary plots for the wild-type LdTXNPH6 and the W87F mutant.
Fig 16 A & 16 B: Primary plots for the T49V and the T49S mutants.
**Double Reciprocal Plot of Initial Velocities for LdH6TXNPx (Wild type)**

[Graph showing double reciprocal plot with data points and a linear trend line.]

**W87F Mutant Secondary Plot**

[Graph showing a similar plot with data points and a linear trend line for the W87F mutant.]

**Fig 17 A & 17 B:** Secondary plots for the Wild-type LdTXNPxH6 and the W87F mutant.
Fig 18 A & 18 B: Secondary plots for the T49V and the T49S mutant.
3.15. Mutant Enzymes Activity Measurements

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity (U/mg)</th>
<th>Yields of soluble protein expression (mg/l)</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
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<td>32</td>
</tr>
<tr>
<td>LdH6TXNPx</td>
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<td></td>
</tr>
<tr>
<td>C52S</td>
<td>No activity detected</td>
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</tr>
<tr>
<td>T49V</td>
<td>0.02</td>
<td>13.7</td>
</tr>
<tr>
<td>T49S</td>
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</tr>
<tr>
<td>W87F</td>
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<td>19.4</td>
</tr>
<tr>
<td>W87R</td>
<td>No activity detected</td>
<td>1.6</td>
</tr>
<tr>
<td>Y44F</td>
<td>No activity detected</td>
<td>16.7</td>
</tr>
<tr>
<td>Y44H</td>
<td>No activity detected</td>
<td>11.4</td>
</tr>
<tr>
<td>C173S</td>
<td>&lt;0.02</td>
<td>1.7</td>
</tr>
<tr>
<td>R128D</td>
<td>No activity detected</td>
<td>7.7</td>
</tr>
<tr>
<td>W177L</td>
<td>0.06</td>
<td>0.3</td>
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</tbody>
</table>

Table 1: Specific activities and the amount of soluble protein expressed. Renaturation of mutants was not tried so only the soluble protein was taken into consideration. Data are the means of two independent measurements.
The specific activities of the mutants were measured essentially as described in Section 3.13.

The specific activity of an enzyme preparation is defined as the number of enzyme units per milligram of protein (μmol min⁻¹ mg or U/mg of protein). The exact yields of the various mutants are shown in the table. Also, the measurement of specific activity is listed. The expression of the different mutants revealed some very interesting patterns. T49S mutant was found to be expressed at a greater quantity than the wild-type protein but was relatively unstable compared to the wild-type protein. On the other hand, the T49V mutant was expressed at a significantly lower amount than the wild type. Mutants C52S, W87R, C173S, R128D and W177L were expressed as soluble proteins at a significantly lower amount. Some of these mutants expressed as insoluble protein but renaturation of the insoluble fraction was not attempted, as it would give us erroneous results due to structural changes due to renaturation.

The specific activity of the T49S mutant was much higher than the wild-type protein, whereas the substitution of the same threonine with an apolar valine reduced the activity to almost negligible values. The same applies for the substitution of the Tryptophan at position 87. Exchange with a charged Arginine abolished the activity, whereas exchange with a bulky Phenylalanine retained some of the original activity. Substitution of the Tyrosine at position 44 with a Phenylalanine or Histidine abolished the activity completely.

LdTXNPx showed ping-pong kinetic behavior with defined Kₘ values for substrates t-BOOH (Kₘₐ) and Tryparedoxin (Kₘᵦ) as can be seen from Table 3. The pattern complies with the simplified Dalziel equation (DALZIEL, 1957).

\[
\frac{[E_0]}{v} = \phi_0 + \phi_1/[t-BOOH] + \phi_2/[TXN]
\]
The kinetic parameters are compiled in the following table.

<table>
<thead>
<tr>
<th></th>
<th>$\phi_1$</th>
<th>$k_1$</th>
<th>$\phi_2$</th>
<th>$k_2$</th>
<th>$\phi_0$</th>
<th>$k_{cat}$</th>
<th>$K_{mA}$</th>
<th>$K_{mB}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LdTXNPx</td>
<td>4.13</td>
<td>0.24</td>
<td>2.64</td>
<td>0.38</td>
<td>0.10</td>
<td>10.0</td>
<td>41.3</td>
<td>26.4</td>
</tr>
<tr>
<td>T49S</td>
<td>1.43</td>
<td>0.69</td>
<td>2.33</td>
<td>0.43</td>
<td>0.12</td>
<td>8.33</td>
<td>11.9</td>
<td>19.4</td>
</tr>
<tr>
<td>T49V</td>
<td>664.9</td>
<td>$1.5 \times 10^3$</td>
<td>420.6</td>
<td>$2 \times 10^3$</td>
<td>5.4</td>
<td>0.19</td>
<td>123.1</td>
<td>77.9</td>
</tr>
<tr>
<td>W87F</td>
<td>0.71</td>
<td>1.40</td>
<td>2.03</td>
<td>0.49</td>
<td>0.174</td>
<td>5.75</td>
<td>4.0</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Table 2: Dalziel coefficients ($\phi_1$ and $\phi_2$) and apparent second-order rate constants ($k_1$ and $k_2$) for the reactions of the wild-type LdTXNPx and the active mutants with t-butyl hydroperoxide (73 $\mu$M) and different concentrations (75$\mu$M, 50$\mu$M, 37.5 $\mu$M and 18.8$\mu$M) of tryparedoxin 2. Data are the means of two independent measurements.

The kinetic coefficients, $\phi_1$ and $\phi_2$ represent the partial oxidative and reductive reactions of the LdTXNPx with t-BOOH (substrate A) and Tryparedoxin (substrate B). The Michaelis constants, $K_{mA}$ and $K_{mB}$ show estimates for the affinities of substrates A and B, which in this case is t-BOOH and Tryparedoxin. 2.5- fold increase in the $K_{mA}$ value for the T49V mutant shows an increased affinity for t-BOOH. $\phi_0$ is the reciprocal value of the lowest rate constant of the whole catalytic cycle. The reciprocal of $\phi_0$ i.e. $1/\phi_0$ is the $k_{cat}$ value, or in other words it is the molar efficiency of LdTXNPx.
3.16. CC-SS Mutant

A Cysteine double mutant was constructed with the aim of obtaining an X-ray crystal structure of LdTXNPx. The expression levels of this double mutant were high compared to the wild-type, though the protein was not as stable as the wild-type. When concentrated more than 2mg/ml, it precipitated, thus making it impossible to use the protein for crystallization.

Figure 19: Expression of the Cysteine double mutant (CC-SS):
Lane 1: Low Molecular Weight Marker, Lane 2 & 3: LdTXNPx Protein pellet and supernatant respectively after induction, Lane 4 & 5: CC-SS mutant Protein pellet and supernatant respectively after induction.
4. DISCUSSION

4.1. Presence of a Trypanothione-mediated hydroperoxide metabolism in Leishmania donovani

Peroxiredoxins, first described in 1994 by CHAE, are a widespread family ranging from being present from archaea to higher organisms associated with diverse biological functions such as adaptive cellular response to oxidative stress, cell differentiation and cell proliferation; increasing evidence being present for their activity as peroxidases. Also, they share strong homologies between themselves, are highly abundant and also isoforms of these proteins are often observed. They have been found in mammalian systems where they present distinct tissue distribution profiles, implying that they serve specific biological roles (CHAE et al, 1994b; IMMENSCHUH et al, 1995). Peroxidases are frequently found to rely on the use of redox active co-factors such as metal ions, heme, flavin or selenocysteine. However, peroxiredoxins distinguish themselves in utilizing the chemical properties of cysteine for functionality. The simplest system has been found in bacteria, where a peroxiredoxin-related protein, AhpC is reduced by NADPH-dependent flavoprotein (AhpF), which in turn reduces the hydroperoxide (POOLE et al, 1996).

The trypanothione -mediated hydroperoxide metabolism had been characterized in detail in the insect pathogen, *Crithidia fasciculata* belonging to Trypanosomatids (NOGOCEKE et al, 1997; TETAUD et al, 1998). It appears to be common to all members of *Kinetoplastida*. It has been shown to comprise of three enzymes acting in concert to transfer the electrons from NADPH to the final peroxide donor. Tryparedoxin, a thioredoxin-like protein; Tryparedoxin Peroxidase, which is related to the peroxiredoxin family of proteins, and Trypanothione reductase, a well studied enzyme act together with substrates, Trypanothione and NADPH. The presence of a similar system in the human pathogen group, *Leishmania* has long been suspected. This work shows the presence of at least one of the three established enzymes in the trypanothione-mediated hydroperoxide metabolism. Previously, an equivalent
peroxidase had been shown to be present in *Leishmania major* (LEVICK et al, 1998), but never in *Leishmania donovani*, the causative agent of the disease, Kala Azar. Also, trypanothione reductase and trypanothione had been discovered in *Leishmania*. Tryparedoxin peroxidase was never reported. Tryparedoxin has yet to be found. Two isoforms of Tryparedoxin have been reported in *Crithidia fasciculata* (GUERRERO et al, 1999; MONTEMARTINI et al, 1998; TETAUD et al, 1998). Their catalytic site and behavior have been studied (GOMMEL et al, 1997; STEINERT et al, 2000) and has been crystallized (KALISZ et al, 1999). The terminal peroxidase of the trypanothione-dependent hydroperoxide metabolism is highly specific for reduced TXN (Tryparedoxin), not being efficiently reduced by trypanothione [N¹, N⁸-bis (glutathionyl) spermidine](FAIRLAMB et al, 1985); or by trypanothione reductase (NOGOCEKE et al, 1997), or by the homologous human thioredoxin reductase (NOGOCEKE et al, 1997). On the other hand, it unspecifically reduces a wide range of hydroperoxides including peroxidized complex lipids such as phosphatidylcholine hydroperoxide (NOGOCEKE et al, 1997). But it has been found to be comparatively slower than the selenoenzyme phospholipid hydroperoxide glutathione peroxidase; which may not be a factor at all as the efficiency is increased by the increased expression of the peroxidase. The tryparedoxin peroxidase is expressed at high levels in *Crithidia fasciculata* i.e. at 6 % of the total protein (NOGOCEKE et al, 1997).

The identification and characterization of this peroxiredoxin confirms the presence of a thiol-dependent reduction of peroxides, which possibly is responsible for the protection of the parasite against oxidative damage. Oxidative damage in trypanosomatids results primarily from metabolically produced ROS (reactive oxygen species) and from ROS released from host immune effector cells. The first peroxiredoxin to be reported, thioredoxin peroxidase (TPx); also previously called thiol-specific antioxidant, TSA, was detected in *Saccharomyces cerevisiae*, where its expression is induced specifically by exposure to oxidative stress (KIM et al, 1988; KIM et al, 1989). Oxidative stress is the result of ROS generated by the sequential reduction of oxygen to water. Primary ROS include hydrogen peroxide (H₂O₂), the superoxide radical (O₂⁻) and the hydroxyl radical (HO₉) produced as a result of normal metabolism and secondary and tertiary products, such as alkyl hydroperoxides and lipid peroxides, are produced by the interaction of primary ROS with cellular
targets (MCGONIGLE et al, 1998). In S.cerevisiae, peroxidoxins are the second most abundant proteins in erythrocytes, first being hemoglobin (LIM et al, 1994).

Electron microscopy of the immunogold labeled protein, LdTXN Px revealed that it was scattered all over the cytosol and not associated with a single organelle. In the case of the Crithidia fasciculata TXN Px (STEINERT et al, 1999) it was found to be colocalized in the cytosol with Tryparedoxin. Tiny amounts were present in the nucleus and the vesicular structures, the kinetoplast and the mitochondrion being virtually free of the enzymes. Trypanothione reductase has been found to be present in the cytosol of T.brutcei (SMITH et al, 1991) earlier by cell fractionation studies and T. crusi by immunohistochemistry (MEZIANE-CHERIF et al, 1994), although here a minor proportion was seen in the mitochondrion too. This cytosolic localization of all these enzymes constitute an advantage for the organism in that it enables a detoxification of H2O2 and lipoxygenase products generated by phagocytes of the host organism. They may also help in detoxifying the hydroperoxides generated as by-products of the parasite metabolism. Also, STEINERT et al, 1999 did not find any kinetoplastid localization of the genes of this system.

4.2. LdTXN Px belongs to the peroxiredoxin family

The peroxidase described in this work is in many ways similar to most other peroxiredoxin described so far in literature, confirming its rightful place in the peroxiredoxin family. Various peroxiredoxins described so far in literature include thiol-specific antioxidant protein (TSA) of yeast (KIM et al, 1988), natural killer enhancing factor (SHAU et al, 1993), heme-binding protein (IWAHARA et al, 1995), macrophage stress protein (ISHII et al, 1993), proliferation associated gene product (PROSPERI et al, 1993), osteoblast specific factor 3 (KAWAI et al, 1994), MER5 gene product (NEMETO et al, 1990), and a 21 kDa component of the alkyl hydroperoxide reductase (AhpC) in Salmonella typhimurium (JACOBSON et al, 1989; TARTAGLIA et al, 1990). Recently, Tryparedoxin peroxidase congeners from Trypanosoma cruzi (LOPEZ et al, 2000; GUERRERO et al, 2000) and from
Leishmania major (LEVICK et al, 1998) have been cloned and characterized. The highly conserved WCPCPR motif in Tryparedoxins has been shown to be indispensable for catalytic activity in at least Tryparedoxin 2 of Crithidia (STEINERT et al, 2000) by site-directed mutagenesis. Also, they were able to prove that Tryparedoxin cannot be changed into a thioredoxin or glutaredoxin functionally simply by exchanging the active site motifs. This constitutes a distinct advantage in designing Tryparedoxin-based inhibitors for drug design, as it shows a distinct difference from its human homologue. Two L. infantum peroxiredoxins have been recently reported by CASTRO et al (2002a).

The parasites Peroxiredoxins are classified as 1-Cys or 2-Cys enzymes based on the presence of one or two cysteines in their conserved VCP regions (MCGONIGLE et al, 1998). The VCP motif near the N-terminus is strictly conserved, whereas the second VCP motif near the C-terminal end appears to tolerate some sequence variation. The Leishmania donovani Tryparedoxin peroxidase belongs to the 2-Cys family due to the presence of two cysteines. The two peroxidases described in the close relative, Leishmania major (LEVICK et al, 1998; WEBB et al, 1998), are also classified as 2-Cys peroxiredoxin as they have two distinct cysteines in their conserved VCP regions. However, the L. infantum mitochondrial peroxiredoxin has an Ile-Pro-Cys motif in the distal region (CASTRO et al, 2002a).

This novel Leishmania donovani peroxidase may also be involved in some other activities in the cell which have been reported for thioredoxin family; such as refolding (LUNDSTROM et al, 1990), activate receptors (TAGAYA et al, 1989), or modulate the DNA-binding activity of transcription factors (MATTHEWS et al, 1992).
4.3. *Comparison of* L. *donovani peroxidase and* L. *major peroxidase*

LEVICK et al in 1998 have characterized a functional peroxidoxin with a molecular weight of 22.2 kDa in *Leishmania major*. They found it to be a multi-copy gene arranged in a complex tandem array located on the size polymorphic homologues of chromosome 15. Northern analysis showed that it expresses a single 1.6 kb mRNA throughout promastigote development. They also found that the peroxidoxin was a 2-Cys peroxidoxin, which is the case in most of the trypanosomatids characterized so far.

The coding region DNA was G+C rich (61%) and the codon usage was biased for G or C occupancy in the third base position (89%), which is the case in most of the *Leishmania* genes (LANGFORD et al, 1992). In our case, in *Leishmania donovani* Tryparedoxin peroxidase the coding region DNA was 62.3% GC-rich and 92% of the codons had G or C in the third base position. Further evidence for restricted codon usage was that 45% of the amino acids were encoded for by ten codons UUC, CUG, AUC, CCG, AAC, AAG, GAC, GAG, CGC, and GGC in *Leishmania major* and 47% in *Leishmania donovani* peroxidase. In *Leishmania*, the frequency of usage of these ten codons is significantly higher in genes that are abundantly expressed, compared with genes that are not (ALVAREZ et al, 1994). This suggests that this peroxidoxin is an abundant protein in *Leishmania*.

BLAST analysis of the full-length sequence of the *L.major* peroxidase showed that it has the critical motif found in all peroxiredoxins, the N-terminal cysteine containing element around Cys52 (FFYPLDFTFVCPTEV) and a second-cysteine containing element around Cys173 (KHGEVCPA). The only exception in case of the *L.donovani* peroxidase was in the presence of an isoleucine instead of a valine at the end of the N-terminal cysteine element, so that the sequence was FFYPLDFTFVCPTEI instead of FFYPLDFTFVCPTEV. The activity of the both the peroxidases were very close, being 0.39 U mg⁻¹ for *L.major* peroxidase and 0.4 U mg⁻¹ for *L.donovani* peroxidase with t-butyl hydroperoxide as a substrate. In other peroxidases, such as the authentic
and the recombinant TXNPx of *Crithidia fasciculata* (NOGOCEKE et al., 1997; MONTEMARTINI et al., 1998A), the specific activity ranged from 5.83-2.51 U mg\(^{-1}\), whereas for *Trypanosoma cruzi* (GUERRERO et al., 2000), it was 2.9 U mg\(^{-1}\).

The evidence supporting the use of Tryparedoxin Peroxidase as a vaccine candidate comes from studies by WEBB et al in 1998. They used promastigote culture filtrate proteins (CFP) plus *Corynebacterium parvum* to inject the susceptible strain of mouse, Balb/c and found that it confers resistance to infection with *Leishmania major*. To further find out which component was responsible for conferring resistance they screened an amastigote cDNA expression library, where they found a clone encoding a 22.1 kDa protein having similarity to the eukaryotic TSA-thiol specific-antioxidant protein. This was later on found to be a similar peroxidase, which had been described by LEVICK et al., 1998. Also found were two clones encoding the previously identified promastigote surface antigen 2 (PSA-2) protein from *L. major*, a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein encoded by members of a highly polymorphic gene family (MURRAY et al., 1991). The recombinant peroxidase also elicited in vitro proliferative responses from peripheral blood mononuclear cells of human leishmaniases patients and significant antibody titers against the peroxidase were detected in sera of both cutaneous- leishmaniases and visceral- leishmaniases patients. These results show that the peroxidase protein could definitely be used as at one of the component of a leishmaniases vaccine.

The similarity between most of the *L. donovani* and *L. major* genes and gene function is extended by studies done by BEBARS et al., 2000 where they immunized mice with heat killed *L. donovani* promastigotes and challenged equal numbers of mice with *L. donovani* and *L. major*. What they found was interesting, though not unexpected. The results showed high-crossreactivity paralleled by a high cross protection between *L. donovani* and *L. major*.

This is not surprising given the fact that out of the 69% of the *L. major* genes remaining unclassified, more than 40 % are potentially *Leishmania*-(or kinetoplastid-) specific (MYLER 2000 Aug).
4.4. **Kinetic mechanism of LdTXNPx**

The kinetic pattern observed with LdTXNPx was essentially the same as *Crithidia* TXNPx and *T. cruzi* TXNPx (GUERRERO et al, 2000; NOGOCEKE et al, 1997; MONTEMARTINI et al, 1998b, 1999) in that oxidation of the reduced enzyme by the hydroperoxide and its regeneration by reduced TXN takes place in two independent steps, which means that at no given time there are more than two bimolecular complexes. This is known as the ping-pong mechanism of enzyme reaction. Replotting the ordinate intercepts of the primary plot, which are the reciprocal apparent maximum velocities, against the reciprocal concentrations of the *Crithidia* TXN2H6 yields the limiting maximum velocity and the Michaelis constants.

LdTXNPx displays ping-pong kinetics (essentially parallel lines in reciprocal primary Dalziel plots) with defined maximum velocity and \( K_m \) values unlike its other relatives, namely the *Crithidia fasciculata* TXNPx (NOGOCEKE et al, 1997) and glutathione peroxidases (URSINI et al, 1995; FLOHÉ et al, 1989) where infinite maximum velocity and no real Michaelis constants are observed. *Trypanosoma cruzi* TXNPx displays kinetics similar to the Leishmanial relative (GUERRERO et al, 2000). This simply means that in the enzymes displaying infinite maximum velocity, formation of enzyme-substrate complexes is slower than the consecutive decay of complexes, while the opposite assumption must be made for the enzymes that display defined maximum velocity.

The kinetic pattern of LdH6TXNPx can be described by the Dalziel equation for multi-substrate kinetics (Dalziel, 1957).

\[
[E_0]/v = \phi_0 + \phi_1/[A] + \phi_2/[B] + \phi_{1,2}/[A][B]
\]

where \([E_0]\) is the total enzyme concentration, \(A\) is the substrate, \(t\)-BOOH and \(B\) is *Crithidia* TXN2H6. The empirical coefficients \(\phi_1\) and \(\phi_2\) are simply the reciprocal values of the net forward rate constants characterizing the reactions of the reduced enzyme with hydroperoxide, \(k_1'\), and of the oxidized enzyme with reduced tryparedoxin, \(k_2'\).
The non-convergence of the slopes on the plot of $1/[t$-BOOH] against $E/v$ for diverse concentrations of TXN suggests that the last term of the above equation is zero, bringing us to the equation for two-substrate ping-pong kinetic reactions

$$\frac{[E_0]}{v} = \phi_0 + \phi_1[A] + \phi_2[B]$$

This is interpreted as the absence of a ternary complex of the enzyme with both substrates during the reaction. This equation describes an enzyme-substitution mechanism involving two substrates, whereby enzyme-substitution, in case of peroxidase, means oxidation or reduction of the enzyme, respectively. The complex catalytic mechanism of the peroxiredoxins that contain two interacting reaction centers has been discussed in detail by MONTEMARTINI et al, 1999 and CHAE et al, 1994. $\phi_0$, assumes a defined value defining typical Michaelis-Menten-type saturation kinetics which is observed only when an intramolecular reaction of an enzyme substrate complex occurs with the overall lowest rate constant. The $\phi_1$ values for the three enzymes- *C. fasciculata* CfTXNPx (NOGOCEKE et al, 1997), *T. cruzi* TcTXNPx (GUERRERO et al, 2000) and *L. donovani* LdTXNPx do not differ substantially implying that the oxidative part of the catalytic cycle may proceed with largely identical rate constants. Therefore, the reductive part of the cycle i.e. the enzyme/TXN complex may be the rate-limiting step, which may be due to the suboptimal fit of the heterologous His-tagged substrate.

### 4.5. Molecular Mechanism of catalysis

The first peroxidoxin to be reported, yeast TSA or thioredoxin peroxidase exists as a homodimer of 25 kDa subunits, linked by two disulphide bonds between the conserved Cys47 and Cys170. It was also revealed through site-directed mutagenesis that Cys47SH oxidizes to form cysteine sulphenic acid, which then reacts with the second cysteine in the conserved region to form an intermolecular disulphide bond (CHAE et al, 1994), the sulphhydryl groups being regenerated by the transfer of reducing equivalents from NADPH by thioredoxin reductase to thioredoxin and finally to the disulphide of TSA (KWON et al, 1994; CHAE et al, 1994). Therefore,
Cys47 and not Cys170 is the site of oxidation by a putative substrate. Additional evidence for the involvement of the two conserved cysteines in the VCP motif came from studies by NOGOCEKE et al, 1997 where they observed that two molecules of N-ethyl maleimide react with TXNPx when previously reduced by TXN plus trypanothione.

CHAE et al in 1994 suggested the following mechanism for thioredoxin peroxidase of yeast-the hydroperoxide reacts with the cysteine of the N-terminal conserved region and forms a sulphenic acid derivative; which then forms a disulphide bridge with the second conserved cysteine in the adjacent subunit in the homo-oligomeric protein after which the reduced enzyme is regenerated by Tryparedoxin. A similar mechanism exists in the chemically unrelated selenium-containing peroxidases (FLOHÉ et al, 1973; URSINI et al, 1995), where a selenocysteine residue is activated by proton-donating amino acid residues to react with hydroperoxides, and the resulting selenenic derivative is reduced stepwise by glutathione. Interestingly, the kinetic patterns of glutathione peroxidase and both the Crithidia fasciculata and Leishmania donovani peroxidase show similar fashions with ping-pong mechanism with infinite maximum velocities and Michaelis constants.

X-ray crystallization would have given the exact structure of the active site of this enzyme and probably the residues involved in catalysis. We tried mutating both the conserved cysteines, because mutating an additional cysteine (Cys83 in HBP23 and Cys91 in hORF6) allowed the hORF6 and HBP23 proteins to be crystallized (CHOI et al, 1998; HIROTSU et al, 1999). But all our attempts were unsuccessful as the protein tended to precipitate when it was concentrated. Aggregation of the protein has been observed in previous cases too, but replacing the particular cysteines solved the problem, because maybe that the inter protein disulfides promoted oligomerisation. So, we opted for a PCR-based site-directed mutagenesis approach and heterologously expressed as N-terminally Histidine-tagged proteins in E.coli. This approach we know, may not give us the most accurate picture but it was the best option under the circumstances. However, CHOI et al, 1998 and HIROTSU et al, 1999 have reported crystal structures of the oxidized forms of a 1-Cys peroxiredoxin called human open reading frame 6(hORF6) and a 2-Cys-peroxiredoxin heme binding protein (HBP23) respectively. The structure of Crithidia fasciculata tryparedoxin peroxidase in its
recombinant reduced form has been determined using multi-wavelength anomalous dispersion methods applied to a selenomethionyl derivative. The results show that it belongs to a distinct subgroup of the thioredoxin superfamily and that there are distinct quaternary structure alterations induced by the change in the oxidation state; from dimer in the oxidized state to decamer in the reduced form. The strong sequence homology between different peroxiredoxins suggests that the residues contributing to protein reactivity may be the same.

To get a picture of what the active site comprised of in this peroxidase, ten mutants were constructed. The residues shown to be conserved by multiple sequence alignment of various peroxidases were taken as a starting point. The two conserved Cysteines were taken and exchanged for serine just to confirm their essentiality. The Tryptophan shown in Crithidial peroxidase to be a part of the catalytic triad was exchanged for the bulky Phenylalanine and also the positively charged Arginine. The Tryptophan in the second conserved motif was exchanged for Lysine. Arginine at position 158 was exchanged for an Aspartate. Threonine49 was exchanged for Serine, because it was conserved or replaced by Serine naturally in the peroxiredoxins. It was also exchanged for Valine to see the extent of its involvement in catalysis. Tyrosine at position 44 was mutated to a Phenylalanine and also to a Histidine. It was made sure that the ten different mutants had an identical conformation to rule out any variations due to difference in the conformation. Keeping the chromatographic behavior of all the mutants same in terms of elution from the columns with imidazol and salt did this. The mutants showed variations in the amount of protein expressed; the reason is unknown. But previous authors have observed this when creating mutants; MONTEMARTINI et al, 1999 when mutating TXNPx and STEINERT et al, 2000 when mutating TXN2.

Out of the ten variants designed based on a consensus sequence of LdTXNPx and peroxiredoxin-type peroxidases and heterologously expressed in E.coli, the ones that were already studied in the Crithidia fasciculata Tryparedoxin peroxidase reinforced the results already presented by MONTEMARTINI et al in 1999. C52S was completely inactive suggesting that the N-terminal conserved Cys was indeed the redox-active constituent of the N-proximal reaction center of LdTXNPx as suggested in TXNPx. However, MONTEMARTINI et al, 1999 had previously suggested that it
probably forms a catalytic triad with the Arginine128 and Tryptophan at position 87. In our studies, replacement of Tryptophan with positively charged Arginine and the bulky yet inert Phenylalanine suggested that the bulky Tryptophan played only a steric role in the triad. Actually, this has been already suggested by the remote location [over 11 Å (ALPHEY et al, 2000)] of the imino nitrogen of Tryptophan at position 87 from the sulphydryl group of Cys52 to form a direct hydrogen bond even if the residues are rotated to a more suitable position. Instead, the Threonine at position 49 seemed to be the more suitable third partner in the catalytic triad. This Threonine is either conserved or is replaced by Serine in a large variety of peroxiredoxins. Both residues enabled catalysis, and Serine was found to be more catalytic than Threonine. An apolar Valine destroyed the activity of the enzyme. So, the Tryptophan at position 87 plays only a steric role in keeping the active site in a certain conformation so that Cys52 sulphenic acid is able to react with reduced Cys173 and is yet protected from oxidation to sulfenic or sulfonic acid which would be deleterious for catalysis (CLAIBORNE et al, 1999). ALPHEY et al, 2000 have suggested that probably Tryptophan at this position affects the side-chains of the conserved Phenylalanine at position 48 and influence the decamer assembly since Phenylalanine 48 forms a number of important interactions at the dimer-dimer interface, which in turn may preserve Tryparedoxin peroxidase in its reduced active conformation, impeding autooxidation by an activated Cys173 of the second adjacent subunit. The model did not rule out the possibility of involvement of the Tyrosine at position 44 because neither an apolar Phenylalanine, nor a basic Histidine was able to conserve the activity of the enzyme. This is puzzling, since it implies some involvement of this residue in catalysis, which remains a mystery to be solved. As for the second conserved Cysteine at position 173, it suggested an activation of the cysteine by Arginine at position 158 and a structural role for the Tryptophan at 177. MONTEMARTINI et al had previously proved this in 1999 by constructing C173S, R128E and W177E and W177H.

The kinetic behavior of the mutants seemed to corroborate the specific activities of the mutants. The Michaelis constants show an increased affinity of the T49V mutant with both t-BOOH and tryparedoxin.
In conclusion, then, the present investigation modifies and complements our understanding of the molecular mechanism of LdTXNPx as follows: i) the highly conserved Tryptophan residues 87 and 177 are not involved in catalysis but appear required to stabilize the architecture of the reaction centers; ii) both conserved Cysteines are under electrostatic influence of nearby Arginine residues, which favors the dissociation of the SH groups; iii) C52 is further activated by hydrogen bonding from T49, which is conserved in all known TXNPx species but may be replaced by serine as seen in some other peroxiredoxins; Y44 appears to contribute to LdTXNPx catalysis in a way that remains to be elucidated.

The proposed mechanism of action is consistent with that deduced for the homologous thioredoxin peroxidase of yeast (CHAE et al, 1994) and is supported by the molecular modeling based on the structure of the human peroxiredoxin (CHOI et al, 1998). As suggested by MONTEMARTINI et al, 1999 for the Crithidia TXNPx, a similar catalytic principle can be applied to the Leishmania donovani LdTXNPx. The conserved Cysteine in the N-proximal VCP motif is activated by an Arginine and is readily oxidized by a hydroperoxide to yield a sulphenic acid derivative. This highly reactive derivative first reacts with the similarly activated cysteine in the C-terminal VCP motif of an adjacent subunit. The exposed intersubunit disulphide bridge allows reduction by Tryparedoxin.

4.6. Overview and impact on future research

The main properties of an ideal target for rational drug design (FAIRLAMB, 1996) are the essentiality of the target for the survival of the parasite in the mammalian host that should be thoroughly verified by molecular genetic approaches and should be absent from the mammalian host to permit selective inhibition. Also, the target should be open to detailed study at all levels. As described in the introduction section 1.6, various targets have been identified. Almost none of the targets described so far satisfy all the requirements, so the search for newer and more effective targets remains open. Also, because parasitism is associated with the loss rather than the acquisition of new metabolic pathways, it becomes a Herculean task to find a target that is considerably different from its host.
The *Leishmania*-host cell interaction is still a subject of interest (RITTIG et al, 2000). The promastigotes become located in phagolysosomes where they transform to and survive as 'aflagellated' amastigotes that hide their shortened flagellum within the flagellar pocket. Multiplication of these amastigotes is presumed to cause the eventual burst of the host cell, thereby releasing the infectious parasites.

One of the interesting vaccine candidate recently described is the sandfly saliva (KAMHAWI, 2000). The sandfly saliva contains a rich array of pharmacologically active compounds, which prevent the haemostatic mechanisms of the host. Also, they serve as immunosuppressants and have an exacerbative effect on *Leishmania* infectivity for their mammalian hosts. It has been supposed that the sandfly saliva may be used as a part of an anti-*Leishmania* vaccine for the mammalian host. More studies have to be done in this sector.

The weak antioxidant system has long been one of the more promising targets due to its apparent substantial difference from the hosts. Out of all the defense systems, Trypanothione seems to be suited for the reduction of hydroperoxide with formation of oxidized cyclic trypanothione disulfide, because its SH groups are more reactive than that of glutathione at physiological pH (MOUTIEZ et al, 1994). Trypanothione and the components which is it synthesized from (glutathione and spermidine) and the enzymes responsible for the synthesis (glutathionylspermidine synthetase, trypanothione synthetase, gamma-glutamylcysteine synthetase and ornithine decarboxylase) have still very much kept the researchers interested in them as antitrypanosomal and antileishmanial drug targets (AUGUSTYNS et al, 2001). This study provides us with a much better understanding of the antioxidant defense system of *Leishmania*. It also strengthens our view that this thiol-dependent antioxidant system is unique to trypanosomatids and substantially different from its mammalian hosts. With one more component of the human-pathogen group of *Leishmania* being found i.e. the terminal peroxidase in the cascade, the only component of the trypanothione-mediated hydroperoxide metabolism to be identified and characterized is the trypouredoxin. Trypouredoxin has been found to be significantly different from the human homologue, thioredoxin, as simply changing the active site motifs of a trypouredoxin does not change it into a thioredoxin (STEINERT et al, 2000).
This study certainly provides us one more molecular target for the development of the much-needed antiparasitic drug, and to start modern high-throughput screening for potential irreversible inhibitors, which is broadened by the presence of similar peroxidases throughout the various organisms. As *Leishmania* lack catalase and glutathione peroxidase, but have superoxide dismutase; the trypanothione system seems to be the only mechanism to detoxify hydrogen peroxides. In fact, some researchers are exploiting this feature in antiparasitic drug development. It seems to obtain a specific inhibitor of the parasite SOD; the combination of SOD inhibitors with suitable nitric oxide donors should generate peroxynitrite, which is toxic against these parasites (KRAUTH-SIEGEL et al, 1999). These studies are still in infancy and it remains to be seen whether it has potential side effects for humans who would disqualify this candidate too as many others before.

Ultimately, however the sequencing of the *Leishmania* and other Trypanosomatid genomes will open up new frontiers to potential drug and vaccine targets and hopefully result in the production of cheaper drugs and effective vaccines, as the diseases associated with this group of organisms remain to this date a third world disease, although its association with AIDS has already started a few people noticing. After that differential genome analysis is a good method for identification of species-specific genes. Structures of the proteins can then also be predicted based on sequence and experimental data (KRAUTH-SIEGEL et al, 1999). Sequencing of the *Leishmania major* Friedlin genome is in full swing with Chromosome 1 and 3 been completely sequenced, and chromosome 4 virtually complete. It is predicted that the sequencing of the whole genome may be complete by the end of 2003(MYLER et al, 2000). Most of the 70% of the newly identified genes are kinetoplastid specific. The genes are organized into large (>100-300 kb) polycistronic clusters of adjacent genes on the same DNA strand, chromosome 1 containing divergent genes, and chromosome 3 containing two convergent clusters.

So, the progress in *Leishmania* and for that matter Trypanosomatid research is showing signs of being fruitful very soon in terms of an effective therapeutics and vaccine. HANDMAN et al, 2000 have described that a DNA vaccine against *Leishmania major* also serves as a therapeutic. They vaccinated both resistant,
C3H/He and susceptible mice, Balb/c with DNA encoding the parasite surface antigen/gp46/M2, which resulted in the reduction of lesions and promoted healing.
5. SUMMARY

The trypanothione-mediated hydroperoxide metabolism seems to be common to all trypanosomatids and has been shown in *Crithidia fasciculata* to comprise of three major enzymes acting in concert to transfer the electrons from NADPH to the final peroxide donor. Tryparedoxin peroxidase is the terminal peroxidase; Tryparedoxin and Trypanothione Reductase being the other two major enzymes to detoxify hydroperoxides in a complex redox cascade.

This work shows the presence of at least one of the key enzymes, Tryparedoxin peroxidase in *Leishmania donovani*, the causative agent of the fatal disease Kala Azar. This is the first ever evidence of the presence of the seemingly unique Trypanosomatid antioxidant system in *L. donovani*.

The open reading frame encoding the gene Tryparedoxin peroxidase was cloned, N-terminally His-tagged and expressed in pET 22 and purified on a His-bind resin. The protein was expressed to high levels. The peroxidase, which has been named LdTXNPx, to keep the nomenclature uniform for the peroxidases cloned in our lab, exists as a homodimer of about 21 kDa subunits linked by two disulphide bonds between Cys 52 and C173. The enzyme kinetics were studied and the enzyme was found to display ping-pong kinetics with a $k_{cat}$ of 10.0 s$^{-1}$.

Site-directed mutagenesis was done to determine the active-site structure of the enzyme or at least do some kind of study into the active-site structure. Since all attempts to crystallize the protein failed because of the precipitation of the protein at high concentrations, site-directed mutagenesis seemed the only way to look at the active site. Based on the sequence alignments with different related proteins and some intelligent derivations, some seven amino acids were chosen for the task (See Appendix, Figure 20). As both the VCP regions are highly conserved in most of the known and related peroxidases, the two cysteines were taken for mutagenesis so as to confirm their involvement in acting as the redox-active centers. They were exchanged for Serine so as to see the activity of the SH group, by replacing it with a hydroxyl group, without affecting all the other factors.
The model suggests that both the highly conserved Cysteines embedded in the characteristic VCP motifs, acts as the redox-active centers, the N-proximal cysteine being activated by the Arginine, R128. Threonine at position 49 was exchanged against Valine and Serine; basically to study the function of the –OH group. The activities of the mutant proteins indicate the definite involvement of T49 in the catalytic triad formed by Cysteine52 and Arginine128 (See Appendix, Fig 21). The Tryptophans, W87 and W177 seem to play only a steric role, and appear to stabilize the proximal and distal centers respectively. The mutation of Tyrosine44 showed some role of the amino acid as exchanging it to Phenylalanine or Histidine abolished all protein activity.
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7. APPENDIX

7.1. DNA MARKERS:

1. DNA Molecular Weight Marker III, DIG-labeled
0.12 - 21.2 kb
_13 fragments_: 125, 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973, 5148, 21226 bp

2. DNA/Hind 111
23130, 9416, 6557, 4361, 2322, 2027, 564, 125 bp

3. 100 bp DNA ladder
The 100 bp DNA Ladder has a number of proprietary plasmids that are digested to completion with appropriate restriction enzymes to yield 12 bands suitable for use as molecular weight standards for agarose gel electrophoresis. The digested DNA includes fragments ranging from 100-1517 base pairs. The 500 and 1,000 base pair fragments have increased intensity to serve as reference points.

4. 1 kb DNA ladder
The 1 kb DNA Ladder has a number of proprietary plasmids that are digested to completion with appropriate restriction enzymes to yield 10 bands suitable for use as molecular weight standards for agarose gel electrophoresis. The digested DNA includes fragments ranging from 0.5-10.0 kilobases (kb). The 3.0 kb fragment has increased intensity to serve as a reference band.

7.2. Protein Markers

1. Prestained SDS-PAGE Standards, Low Range (Biorad)
These markers were used for Western Blots to identify the size of a protein. The molecular weights of these markers ranged from 14.4 kDa to 97.4 kDa. The constituents were 97.4 kDa Phosphorylase B (Rabbit Muscle), 66.2 kDa Bovine Serum Albumin (Bovine Plasma), 45 kDa Ovalbumin (Chicken egg white), 31 kDa Carbonic Anhydrase (Bovine erythrocytes), 21.5 kDa Soybean trypsin inhibitor (Soybean) and 14.4 kDa Lysozyme (Chicken egg white).

2. MultiMark™ Multi-colored Standard (Novex)
These multi-colored markers help to identify the correct size of the protein on Western Blots. The Molecular weights ranged from 4 kDa to 250 kDa. The proteins used as standard are: Insulin (4 kDa), Aprotinin (6 kDa), Lysozyme (17 kDa), Myoglobin-red (22 kDa), Myoglobin-Blue (30 kDa), Carbonic Anhydrase (42 kDa), Glutamic Dehydrogenase(60 kDa), Phosphorylase B(148 kDa) and Myosin(250 kDa).

3. 10 kDa Ladder (Gibco)
10 kDa protein markers were used virtually in all the protein gels stained with Coomassie or Silver staining. The markers show a band every 10 kDa higher starting from 10 kDa.

7.3. PRIMERS

1. Primers used for sequencing

M13 (-40) forward  5'-GTT TTC CCA GTC ACG AC-3'
M13 reverse         5'-CAG GAA ACA GCT ATG AC-3'
M13 (-20) forward   5'-GTA AAA CGA CGG CCA GT-3'

2. Primers used for sequencing the full-length gene. The restriction site is italicized and is shown in bold.

Forward Nde1 His: 5'-CAC CAT ATG CAT CAT CAT CAT CAT CAT CAT ATG TCC TGC GGT AAC GCC-3'
3. Primers used for site-directed mutagenesis. The exchanged codons are in bold and italics

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Promoter</td>
<td>5'-TAA TAC GAC TCA CTA TAG GG-3'</td>
</tr>
<tr>
<td>T7 Terminator</td>
<td>5'-GCT AGT TAT TGC TCA GCG G-3'</td>
</tr>
<tr>
<td>C52S For</td>
<td>5'-GAC TTC ACC TTC GTG <strong>TCC</strong> CCG ACA GAG ATC ATC-3'</td>
</tr>
<tr>
<td>C52S Rev</td>
<td>5'-GAT GAT CTC TGT CGG <strong>GGA</strong> CAC GAA GGT GAA GTC-3'</td>
</tr>
<tr>
<td>T49V For</td>
<td>5'-CCC GCT CGA CTT CTG <strong>CTT</strong> CGT GTG CCC GAC-3'</td>
</tr>
<tr>
<td>T49V Rev</td>
<td>5'-GTC GGG CAC ACG AAG <strong>ACG</strong> AAG TCG AGC GGG-3'</td>
</tr>
<tr>
<td>T49S For</td>
<td>5'-CCC GCT CGA CTT CTC <strong>CTT</strong> CGT GTG CCC GAC-3'</td>
</tr>
<tr>
<td>T49S Rev</td>
<td>5'-GTC GGG CAC ACG <strong>AAG</strong> GAG AAG TCG AGC GGG-3'</td>
</tr>
<tr>
<td>W87F For</td>
<td>5'-CGC GCA CCT GCA G<strong>TT</strong> CAC GCT GCA GGA CCG C-3'</td>
</tr>
<tr>
<td>W87F Rev</td>
<td>5'-GCG GTC CTG CAG CTG <strong>GAA</strong> CTG CAG GTG CGC G-3'</td>
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<tr>
<td>W87R For</td>
<td>5'-CGC GCA CCT GCA <strong>GAG</strong> GAC GCT GCA GGA CCG C-3'</td>
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<tr>
<td>W87R Rev</td>
<td>5'-GCG GTC CTG CAG CTG <strong>CCT</strong> CTG CAG GTG CGC G-3'</td>
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<tr>
<td>Y44F For</td>
<td>5'-GTG CTC TTC TTC <strong>TTC</strong> CCG CTC GAC-3'</td>
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<tr>
<td>Y44F Rev</td>
<td>5'-GTC GAG CGG <strong>GAA</strong> GAA GAA GAG CA-3'</td>
</tr>
<tr>
<td>Y44H For</td>
<td>5'-GTG CTC TTC TTC <strong>CAC</strong> CCG CTC GA-3'</td>
</tr>
<tr>
<td>Y44H Rev</td>
<td>5'-GTC GAG CGG <strong>GTG</strong> GAA GAA GAG CA-3'</td>
</tr>
<tr>
<td>C173S For</td>
<td>5'-GAA GCA CGG CGA GGT <strong>GTC</strong> CCC CGC GAA CTG GAA G-3'</td>
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<tr>
<td>C173S Rev</td>
<td>5'-CTT CCA GTT CGC GGG <strong>GGA</strong> CAC CTC GCC GTG CTT C-3'</td>
</tr>
<tr>
<td>R128D For</td>
<td>5'-GAG GAG GTT CTG <strong>GAC</strong> CTG CTG GAG GC-3'</td>
</tr>
<tr>
<td>R128D Rev</td>
<td>5'-GCC TCC AGC AGG <strong>TCC</strong> AGA ACC TCC TC-3'</td>
</tr>
<tr>
<td>W177L For</td>
<td>5'-CCC CGC GAA <strong>CTT</strong> GAA GAA GGG CGC CCC C-3'</td>
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</table>
7.4. *E.coli* strains used in the study

*E.coli* SURE strain (Stratagene): e14⁻ (McrA⁻) Δ(mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 (Kan') uvrC [F⁺ proAB lacF²ZΔM15 Tn10 (Tet')]

*E.coli* TOP10F⁺ (Invitrogen): F' {lacI² Tn10(TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG

*E.coli* INVαF⁺ (Invitrogen): F' endA1 recA1 hsdR17 (rK', mK') supE44 thi-1 gyrA96 relA1 φ80lacZΔM15Δ(lacZYA-argF)U169

*E.coli* BL21(DE3) (Novagen): F' ompT hsdS₈(rB mB') gal dcm (DE3)

All these *E.coli* strains were obtained as Glycerol stocks from their respective manufacturers. A portion of the culture was scraped with a culture loop from the surface of the frozen culture and plated on an agar plate and allowed to grow overnight at 37°C before taking them for cultures.
Fig 20: Multiple sequence alignment of the parasitic peroxiredoxins having similarity to L. donovani peroxidase.
Fig 21: A model of the reaction center of reduced LdTXNPx showing the catalytic triad formed in the N-terminal domain (Adapted from FLOHE et al, 2000).