Chapter III

Identification, cloning and purification of LDMCO
3.1. Summary

*Leishmania donovani* causes a life threatening disease called visceral leishmaniasis. Like other organisms, it also depends on iron for survival, growth and other essential cellular activities. But iron uptake mechanism of *Leishmania spp.* in general is not established so far. Throughout the evolution, multicopper oxidases (MCOs) have a conserved and important role in iron uptake of many organisms by their capacity as a ferroxidase (Ferrous to Ferric). In this Chapter, we confirmed the presence of a MCO in LD. By taking the advantage of published *Leishmania major* genome database, we identified a putative MCO gene, cloned it and purified the protein to homogeneity by affinity chromatography from LD. The purified recombinant LDMCO was found to contain ferroxidase activity. We also raised antibody against LDMCO in rabbit and used the antisera to confirm the presence of MCO in LD by western analysis. The presence of ferroxidase activity is also confirmed in whole cell lysate of LD. The presence of a ferroxidase in LD opens the possibility for a MCO mediated iron internalization process, which will be determined in the later part of the study.
3.2. Introduction

Iron is the fourth most abundant metal in earth crust but it is not readily available for living world because at neutral pH and aerobic conditions it is generally present as ferric form. The ferric form is restricted for the use of organisms due to its high insolubility with an equilibrium concentration of $10^{-17}$M, whereas microbes can only grow optimally in an equilibrium concentration of $10^{-8}$M to $10^{-6}$M (Huston et al., 2002). Thus during evolution, organisms have developed several ways to overcome this problem. Bacteria and some eukaryotes synthesize siderophores that binds to ferric form and mobilize it for its high affinity uptake (Champomier-Vergès et al., 1996). In yeast, multicopper oxidases (MCO) are involved in high affinity iron uptake. These are copper containing enzymes that oxidize ferrous form into the ferric form and help it to get inside the cell by oxidase-permesae system (Stearman et al 1996). One of the best-known members of this class of enzymes is laccase, also known as p-diphenol: O$_2$ oxidoreductase found in plants. In S. cerevisiae, high affinity iron uptake is mediated by an oxidase Fet3p that converts ferrous form into ferric form and then ferric form is transported inside cell by an iron transporter Ftr1 (De Silva et al 1997 and Hassett et al., 1998). In mammals, ceruloplasmin, the homolog of Fet3p, has a central role in iron homeostasis (Harris et al 1999). Furthermore, basolateral surface of the intestine utilizes a membrane MCO hephaestin (Vulpe et al., 1999) for iron release. Another MCO, FLP is involved in iron homeostasis in green algae Chlamydomonas reinhardtii (Herbik et al., 2002). MCOs have been described to have a variety of different physiological functions for bacteria, including manganese oxidation, copper tolerance, and iron oxidation (Huston et al 2002; Cooksey, 1994). All these studies show that the multicopper oxidases (MCOs) are essential part of high affinity iron uptake system of an organism or a cell. Although MCOs are detected in all the organisms but their presence are not yet reported in parasites including protozoa.

Many protozoa are human pathogens and cause dreadful diseases. One of them is Leishmania donovani that causes a vital visceralizing infection known as visceral leishmaniasis or kala-azar that has a high fatality rate in human if not treated properly. Very less is known about the mechanism by which it utilizes iron. Iron plays an
important role in LD for its survival and proper growth (Soteriadou et al., 1995; unpublished data from our lab). In general, iron is a component of heme, iron–sulfur clusters and acts as a cofactor in many important enzymes in *leishmania spp.* Iron is also required for antioxidant enzyme iron superoxide dismutase that plays an important role in protection against the oxidative damage inside macrophage resulting from activation of the NADPH oxidase (Paramchuk et al., 1997). It also contributes to essential functions such as mitochondrial energy metabolism, electron transport, DNA and RNA synthesis and detoxification of toxic oxidants (Wooldridge and Williams, 1993). Uptake of iron for these essential enzymes for survival of pathogen like LD is essential (Wilson and Britigan, 1998). There are some studies to understand the mechanism of iron uptake for *Leishmania spp.* Some studies proposed the presence of a glycoprotein that can bind transferrin in *Leishmania infantum* membrane (Soteriadou, 1995). *Leishmania chagasi* was shown that it could acquire iron from lactoferrin and transferrin (Wilson et al., 1994). But the exact mechanisms of obtaining iron by *Leishmania* are not clear so far. All these studies were performed mainly in *in-vitro* promastigote form of *Leishmania.* In *vivo* LD faces iron scarcity with in its hosts. LD spend part of their life cycle as promastigotes in the midgut of sand fly (pH=7.5), the other part as amastigotes within the phagolysosomes (pH=5.0) of host macrophages (Hyde, 1990) where iron is not readily available. Moreover macrophage is known to limit the access of iron to the pathogen as a defense strategy against the pathogen (Weinberg, 1992), so amastigotes might face larger challenge to accumulate iron. At the low iron concentration environment oxidase-permease system is suitable for iron uptake because MCOs have a very low $K_m$ so that they work at their $V_{max}$ even when iron is available in very low concentration. There is so far no consideration of oxidase mediated iron uptake in *Leishmania* in the literature. So we hypothesized the presence of an oxidase that is involve in iron uptake in LD as a component of oxidase-permease system and that might be important for high affinity iron uptake and survival of this parasite in the host macrophage.
3.3. Results

3.3.1. *Leishmania donovani* contains ferroxidase activity

To verify the presence of any ferroxidase activity LD whole cell lysate was prepared and ferroxidase assay was performed. The whole cell lysate was found to contain ferroxidase activity (Fig III.1A). The activity was assayed by the ability of leishmanial whole cell lysate to convert ferrous into ferric as estimated by measuring absorbance as described in Materials and Methods and reconfirmed by *In membrane* and *In gel* ferroxidase assay (Fig III.1B and III.1C). To confirm the oxidation of ferrous iron is actually due to ferroxidase activity, a specific inhibitor of MCO, sodium azide was added and significant inhibition was detected (Fig III.1A). Similarly, heat inactivation abrogated the ability of leishmanial cell lysate to oxidize iron, reconfirming that oxidation was due to the presence of an enzyme (Fig. III.1A, B and C).

3.3.2. *Leishmania major* has a putative multicopper oxidase

The genome of *Leishmania major* has been published (Ivens AC et al., 2005), which provided us a chance to search for a putative gene for MCO. We searched leishmanial genome for homology with other characterized MCOs using copper binding signature motifs (Fig. III.2) and we identified a putative open reading frame of multicopper oxidase in chromosome 3, containing 1908 bp (Fig III.2). The ORF codes for a 635 amino acids long chain and the translated product contains signature motif for multicopper oxidase and has four putative copper binding sites (Fig. III.2).

3.3.3. Cloning and sequencing of LDMCO gene

*Leishmania major* and *Leishmania donovani* shows a high (~ 96 %) homology to each other nucleotide sequence in general, thus we designed long primer (~ 18 mer of complimentary region) from LM to clone LDMCO from LD genomic DNA. After PCR amplification a product of expected size of 1908 bp was found (Fig. III.3A). Restriction digestion mapping and sequence analysis confirmed the amplification of right product. Then the product was cloned in pET 28(a) + and pMAL-c2X *E coli* expression vectors and was verified by restriction (Fig. III. 3B) and sequencing. The product should express as 635 amino acid containing protein of apparent molecular weight of 72 kDa.
Figure III. 1. LD contain ferroxidase activity

(A) Shows that whole cell lysate (LWL) of LD can oxidize iron (ferrous to ferric) by spectrophotometric assay. The ferroxidase activity is inhibited by sodium azide (1µM, a known ferroxidase inhibitor and heating).

(B and C) Shows zymogram demonstrating the ferroxidase activity in LWL, In membrane and In gel assay respectively. In all cases heating at 95°C for 10 min causes lose of activity. 50µg protein was used for zymogram. Zymogram technique has been describe in details in Chapter II.
NC_004916. Leishmania major ...[gi:32189699]

Nucleotides-
5'atgcgtgcac cgctcgcgtt gctgctgctg accattgttg tgtgcctgca cacagcagcg ggtaatgctg ctgatgctgc cccttcaccc
gtcatacttc ccagaggcag aagcgacacg aaggtcacac tctacttgcg caccggacgg gtctccatcc cgctcgagtg gatcgatggg
aaaggggtct tcttcgagta cacaggacgc ttttatgaag tgggtaacag cggaccgatg cttcccggcc ccacgctgaa ggtcaaccct
ggcggtagaa ttgtcttgac gctagtgaac gacctgggaa aggaagggat ggtcaacatg acaagtggga tgaacacgct ccacggtcct
aacatcacga acgtgcactt ccacggcatg cacagcgatc ctaaaatgga caatcctttc aaagttgcac tcccaggcga gacactggtg
tacaagatta atgttccgcg cgatcacgag ccaggccttc actggtacca tgcgcactct cacggtgccg tctactatca cgtgatgggt
gggatgttcg gggccatcga tgtgggggaa ggcgactttga cgaagacccc aagggccccc ttctatggt ggggtgctca ggtctctagc
gtcacactgt atgcggtaaa acgtgcggag agtaggcggag gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac gtaaagtcgc gtaggcggtac
3'

Translation-
"MRAPLALLLLTIVVCLHTAAGNAADAAPSPVILPRGRSDTKVTLYLRTGRVSIPLEWIDGKVFFEY
TGRFYEVNSGPMLPGLPTKLVNPGGRIVLTVNDLGKEGMVNMTSGMNTLHGPNITNHFHGMH
SDPKDMNFKVALGETLYVKNIRPHDFGLWYHAHSHGAVYHVMcGMFGAEVGGDFT
KTPRPHFHWSQILMVWHLRINLNSCRDCGPMSSLSADAMSLLFDPDRIVDREGNEYEMPALLFL
VNGQHRRPTVRVKHRPMRLRIGFAAGSCYNIMSPLKQCAFHTMAIDAGLRTVEVEVGWLYTT
ATRRSLVVDCAEGTPYVSHGDPDSALFYLKSRGRQKGRAVAFVPYTMKPSGQLQJLGQNTF
YERIESQRSTLDPEPETYYVVLQGPNCSLOSNSTCHYEFFQGINGRRIEGYHGFVVLQAVTVARYV
DPTDKRPHPLHFHNHFQFSEFPRGNNHETNAMYMVGISHSERDTIPILDVTTIRWKAATYV
GEVYVYHCHALQHEDRGMMSLYLHYPKSKGAVQVQEQQSTGAFLSWPHRSHAYSLLVF
ASAAAAAWGFRHRCACCSPAAQSAFDRASYAGTPGEHPLERRA"
Figure III. 3. Cloning, expression and purification of LDMCO

(A). Agarose gel analysis of PCR product performed using genomic DNA of L.D. Lane 1: Arrows shows right size product of LDMCO which is 1908 bp. Lane 2: Shows Marker.


(C). SDS-PAGE was performed and results marked as- Lane 1: Uninduced bacterial cell lysates containing pMALc2X-LDMCO, lane 2: same with IPTG-induced cell lysate, lane 3: binding after maltose beads, lane 4: eluted MBP-LDMCO, lane 5: repurified MBP-LDMCO.

(D). SDS-PAGE with - Lane 1: Uninduced bacterial cell lysate containing pET28(a)-LDMCO, lane 2: same with induced cell lysate, lane 3: after binding with Ni-NTA beads and lane 4: eluted purified His-tagged LDMCO.
3.3.4. Expression and purification of LDMCO

Expression and purification of LD recombinant LDMCO are shown in Fig. III. 3 (C-F) using *E. coli* BL21 transformed with vector pET 28(a) + -HIS-LDMCO and *E. coli* DH5α transformed with vector pMALc2X-MBP-LDMCO and both were induced with IPTG yielded a 72-kDa protein and 112 kDa protein respectively as predicted from the cDNA (Fig III.3. C-D). Both the HIS-LDMCO and MBP-LDMCO were purified by affinity chromatography using specific columns. Then, anti HIS-MCO antibody was generated in rabbit and quality of purification was checked by both coomassie staining and western blot analysis by using anti-HIS-MCO antibody (Fig III.3E, F and G).

3.3.5. Sequence analysis of Putative LDMCO

The 1.9 kb ORF encoded the putative oxidase was cloned from LD and confirmed by sequence analysis. The ORF implicates polypeptide of 635 amino acids with a predicted molecular mass of 72 kDa. The isoelectric point was predicted to be at a pH value of 8.4. In amino acid sequence comparisons, the product of the putative ORF from LD showed the highest overall identity to the multicopper oxidase Fet3p from *S. cerevisiae* (Fig. III.6). Motif searches revealed four amino acid sequences that correspond to copper-binding sequences of the family of multicopper oxidases at amino acid positions 116 to 140, 155 to 181, 460 to 485 and 525 to 551 (Fig. III. 4, 5 and 6). Prosite scan confirm the presence of MCO type 2 signature motif between 531 to 542 amino acids (Fig. III.5). Blast search showed that other characterized members of this family are laccases, ceruloplasmin, Fet3p and ascorbate oxidase. We found homologies between LDMCO and each of the members of the multicopper oxidase group. The highest identities between LDMCO and other MCOs exist around the copper-binding sequences. The human homologue to LDMCO, ceruloplasmin, is also a multicopper oxidase and shows sequence homology to around all four potential copper-binding regions. The putative transmembrane domains 580 to 596 amino acid and the potential secretary signal sequences were found between 1 to 21 amino acids respectively (Fig. III.5). Homologous genes containing the copper-binding domains that characterize MCOs were also identified in BLAST searches of the genomes of several other organisms including trypanosomes including *T. brucei* and *T. cruzi* (Fig. III.6).
Figure III. 3. Contd.

(E) Lane 1: Purified homogeneous MBP-LDMCO protein, lane 2: marker.
(F) Lane 1: Purified homogeneous His-LDMCO and lane 2: marker.
(G) Western blot analysis, Lane 1: MBP-LDMCO, lane 2: Leishmanial whole cell lysate and lane 3: His-LDMCO, using anti serum produced against HIS-LDMCO raised in rabbit.
Leishmania donovani

Nucleotides-

5' atgcgtgcac cgctcgcgtg gccatgttgt tgtgttcttc ccacgagcag aagggacag agggtcctgt gttggttgcag cgtgtcgtct tccctcgtgt cgtcgtcgtg cgtcgtcgtg 3'

Translation-

MRAPLALLLTLVCLHTAAAGNAADAAAPSVILLPRGRSDTKTVLRLTGTVSIPLEWIDGKVFE YTGRTVEVGSNGMLPPTLVNPGRRIVLTVNDLGKEGMVNTSNGMNTIHGPNTNTNVH FF HMGMHPDKNPFPVFALGKYNPFWHTPBLGHPHLWYHAHSGAVYHVHYNMGMFGAIDV

Figure III. 4. DNA and protein sequences of cloned multicopper oxidase from Leishmania donovani. It was confirmed by sequencing to be of 1908 bp long and ORF codes for 635 amino acid long chain with a signature motif of multicopper oxidase, which is a copper binding site.
Figure III. 5- (A) Shows amino acid sequence of LDMCO with putative copper binding domains. (B) Shows the predicted protein map for LDMCO. (C) Shows the domain information for LDMCO.
**Figure III.6-Amino acid sequence alignment for the four copper binding domains of LD with other MCOs.** The number across the top refers to the sequence position in LD protein. Dark gray shading indicates residues conserved in six or more of the sequences, while light grey shading indicates residues conserved in four or five of the eight sequences.
Figure III.7- Recombinant LDMCO contains ferroxidase activity. MBP-tagged recombinant LDMCO was purified and ferroxidase activity was checked by (A) spectroscopic assay and (B) *in gel* assay. Standard deviation was within 10%.
3.3.6. Ferroxidase activity of recombinant LDMCO
To finally confirm that cloned LD gene is actually containing ferroxidase activity, the ferroxidase assay was performed. Ferroxidase activity was detected in purified MBP-tagged protein. This was confirmed both by spectroscopic (Fig. III.7A) and in gel assay (Fig. III.7B).

3.4. Discussion
Multicopper oxidases (MCOs) are complex copper-containing enzymes and usually contain a variety of different physiological functions, including manganese oxidation, copper tolerance, and mainly iron oxidation (Huston, 2002; Cooksey, 1994). They oxidize ferrous form into the ferric and help it to get inside the cell by oxidase-permease system of organisms in diverse areas of evolution. MCOs are long known with some prescribed in vitro functions like iron oxidation. Studies with yeast (Askwith et al., 1994, 1996) defined its function as a ferroxidase in vivo and involved in high-affinity iron uptake. A similar function in iron uptake in iron deficiency was ascribed for mammalian homologue ceruloplasmin (Muckhopadhyay et al., 1998). Later MCOs are also found in bacteria (Kim et al., 2001) and green algae (Herbik et al., 2002) and implicated with respective iron homeostasis. So far, presence of a multicopper oxidase in any trypanosomatid parasite or parasite in general was not reported. By identifying and expressing a functional recombinant LDMCO ferroxidase this study thus describes the presence of a multicopper oxidase in parasite for the first time.

Like all organisms LD also require iron for its proper growth particularly inside the phagolysosome where it require iron superoxide dismutases for protection against the oxidative damage resulting from activation of the NADPH oxidase (Paramchuk et al., 1997). Macrophage is known to limit the access of iron for the availability of pathogens as a defense strategy (Weinberg, 1992). *Leishmania* initially localize in phagolysosomes and TF receptors do not traffic through lysosomes (Wilson and Britigan, 1998). So, LD survives in condition where it faces iron scarcity. Incidentally, not much is known regarding iron uptake mechanism of *Leishmania*. Initially, a couple of studies proposed the presence of a 70 kDa transferrin binding
protein in *Leishmania* spp. via which it can take iron (Voyiatzaki and Soteriadou, 1990; Voyiatzaki and Soteriadou, 1992) but until now neither transferrin receptor homologous gene was found in *Leishmania* nor the presence of transferrin receptor is not established. Later evidence was found that 70 kDa *Leishmania* surface protein is non-specific, which suggested that instead of transferrin receptor, protein bound iron is reduced by a NADPH requiring *Leishmania* reductase for internalisation by the parasite by yet unknown mechanism (Wilson *et al.*, 2002). There was so far no consideration of ferroxidase mediated iron uptake in *Leishmania* in the literature. Thus identification of an azide-inhibitable ferroxidase present in *Leishmania donovani* opened the possibility as a mediator of iron internalisation process in this parasite, which may be essential for high affinity iron uptake from iron sources particularly for their survival within the phagolysosomes of the infected macrophages. Attempt will be made to understand the role of LDMCO in parasite iron uptake capacity in both the promastigote and amastigote forms.