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Original Contribution

# *Leishmania major* ascorbate peroxidase overexpression protects cells against reactive oxygen species-mediated cardiolipin oxidation

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ABSTRACT

Heme peroxidases are a class of multifunctional redox-active proteins found in all organisms. We recently cloned, expressed, and characterized an ascorbate peroxidase from *Leishmania major* (LmAPX) that was capable of detoxifying hydrogen peroxide. Localization studies using green fluorescent protein fusions revealed that LmAPX was localized within the mitochondria by its N-terminal signal sequence. Subcellular fractionation analysis of the cell homogenate by the Percoll density-gradient method and subsequent Western blot analysis with anti-LmAPX antibody further confirmed the mitochondrial localization of mature LmAPX. Submitochondrial fractionation analysis showed that the mature enzyme (~3.6 kDa shorter than the theoretical value of the whole gene) was present in the intermembrane space side of the inner membrane. Moreover, expression of the LmAPX gene was increased by treatment with exogenous $H_2O_2$, indicating that LmAPX was induced by oxidative stress. To investigate the biological role of LmAPX we generated *Leishmania* cells overexpressing LmAPX in the mitochondria. Flow-cytometric analysis, thin-layer chromatography, and $IC_{50}$ measurements suggested that overexpression of LmAPX caused depletion of the mitochondrial ROS burden and conferred a protection against mitochondrial cardiolipin oxidation and increased tolerance to $H_2O_2$. These results suggest that the single-copy LmAPX gene plays a protective role against oxidative damage.


The *Leishmania* genus are unicellular DNA-containing parasites that multiply in macrophages of a human host and are responsible for various forms of leishmaniasis, which is a devastating disease with significant morbidity and mortality, especially in the human populations of developing countries. On infection of the macrophage, *Leishmania* has to resist its microbiostatic and microbicidal mechanisms for successful colonization [1]. To face these challenges, the *Leishmania* species use intracellular thiols [2], lipophosphoglycan [3], iron superoxide dismutase [4], HSP70 [5], ovothiol A [6], trypanothione, and peroxidoxins [7,8] to overcome a variety of reactive oxygen and nitrogen species [1] during their life cycle. However, unlike most eukaryotes, *Leishmania* lack catalase and selenium-containing glutathione peroxidases, enzymes capable of rapidly metabolizing high levels of $H_2O_2$. Hence, the mechanism by which it withstands the toxic effects of $H_2O_2$ is still obscure. In 1985, a group of workers reported that the *Leishmania* amastigotes can scavenge a large amount of $H_2O_2$ [2]. The removal of $H_2O_2$ in amastigotes is markedly inhibited by aminotriazole or sodium azide, which are well-known inhibitors of heme-containing enzymes such as catalase or peroxidase [2]. With this background knowledge, recently, we discovered the unusual plant-like peroxidase from *Leishmania major* (LmAPX) [9]. When the amino acid sequence of LmAPX is compared with other Class I, Class II, or Class III superfamilies as well as guaiacol peroxidase, the sequence identity of LmAPX is higher with Class I (~35%) compared to other classes (<18%). LmAPX is a functional hybrid between cytochrome c peroxidase (CCP) and ascorbate peroxidase (APX). This hybrid shows catalytic properties common to these two peroxidases, in being able to oxidize cytochrome c and ascorbate as substrates [10,11].

Most proteins in a eukaryotic cell are encoded in the nuclear genome and synthesized in the cytosol, and many need to be further sorted to one or other subcellular compartments by an N-terminal targeting sequence [12,13]. The hidden Markov model and SignalP server prediction result suggests that the N terminal of LmAPX has a signal peptide and probable transmembrane helix with cleavage site between residues 32 and 33. However, this type of prediction is not always correct and hence demands confirmation with unambiguous experimental data.

Although the plant APX has been extensively characterized and shown to be responsive to several environmental stresses [14–18], almost nothing is known about the cellular features of LmAPX, which include its precise intracellular localization, physiological function, and regulation. It is therefore important to understand what the exact function of LmAPX is, where this enzyme is localized, and how its synthesis is controlled at the molecular level. In this article, we provide evidence for the first time that LmAPX is the heme-containing peroxidase of the parasite and resides in the intermembrane space of

*Abbreviations:* LmAPX, *Leishmania* major ascorbate peroxidase; APX, ascorbate peroxidase; CCP, cytochrome c peroxidase; ROS, reactive oxygen species.

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the mitochondrial inner membrane in *Leishmania* with a form ~3.6 kDa shorter than the full-length gene. It is also important for $H_2O_2$ detoxification and protection of cardiolipin oxidation under oxidative stress and is up-regulated by increasing concentrations of exogenous $H_2O_2$ at 37°C.

## Materials and methods

### *Materials*

2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA), dihydrorhodamine 123, MitoSOX Red, 10-N-nonyl acridine orange (NAO), and Mitotracker Deep Red-CMX-ROS were procured from Molecular Probes (Eugene, OR, USA). Digitonin and protease inhibitor cocktail were obtained from Roche. Anti α-tubulin antibody was purchased from Upstate Cell Signaling. Cytidine diphosphate diacylglycerol, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidic acid were purchased from Avanti Polar Lipids (Alabaster, AL, USA). $^{32}P_i$ was purchased from the Board of Radiation and Isotope Technology, India. Thin-layer chromatography plates (Cat. No. 4855-820) were purchased from Whatman (Clifton, NJ, USA). Chloroform, methanol, and ethanol were from Merck (Germany) and triethylamine was from Rankem (India). Cardiolipin and all other chemicals were purchased from Sigma (St Louis, MO, USA) or sources previously reported [9,10,19,20]. The conversion of the fluorescent probe DCFDA to DCFH (reduced 2′,7′-dichlorofluorescein) was done by dissolving in ethanol and hydrolyzing with 10 mM NaOH for 60 min in the dark and then it was diluted to 10 μM for work [21].

### *Parasite culture*

*L. major* (strain 5ASKH) promastigotes were cultured at 26°C in M199 medium supplemented with 40 mM Hepes (pH 7.4), 200 μM adenine, 1% penicillin–streptomycin (v/v), 50 μg/ml gentamycin, and 10% heat-inactivated fetal bovine serum. Transformed cells were maintained under the same conditions with additional supplementation of 200 μg/ml G418.

### *Cloning and construction of overexpression system in Leishmania*

Genomic DNA was isolated from *L. major* promastigotes using a Qiagen genomic DNA isolation kit as per the manufacturer's instructions. To amplify different versions of LmAPX, the following open reading frame primers were used: primer1, 5′-AAAA*CCCGGG*CGCATGTCCGGCACCTCGCGG-3′; primer2, 5′-AAAA*CCCGGG*CGCATGGAGGAGCCGCCGTTCGACATC-3′; primer3, 5′-AAAA*GGATCC*GCTCTCCGAAGCGGGTGCTTT-3′; and primer4, 5′-AAAA*GGATCC*TTAGCTCTCCGAAGCGGGTGCTTTG-3′. The segments in italic denote restriction sites: SmaI for primer1 and primer2 and BamHI for primer3 and primer4. Primer1 and primer2 were used as sense, and primer3 and primer4 were used as antisense for different purposes. The amplified product of primer1 and primer3 was cloned into the SmaI and BamHI sites of the pXG-B2863 vector to produce C-terminal-GFP-tagged full-length LmAPX protein. The amplified product of primer1 and primer4 (containing a stop codon) was cloned into same vector and the same sites to generate full-length LmAPX without GFP. Primer2 and primer3 were used to produce C-terminal-GFP-tagged Δ34 LmAPX protein.

### *Transformation*

Transformations of all the constructs were performed by electroporation [22] with a Bio-Rad Gene Pulsar apparatus using 450 V and 550 μF capacitance. Briefly, late log-phase promastigotes ($0.5–1.0\times10^7$) were harvested at 1200 g (4°C) for 10 min and washed twice in electroporation buffer (21 mM Hepes, 137 mM NaCl, 0.7 mM $NaH_2PO_4$, 6 mM glucose, pH 7.4). Cells were finally suspended at a density of $1\times10^8$/ml and 0.36 ml was taken into a 0.2-mm ice-chilled electroporation cuvette. Thirty micrograms of plasmid DNA dissolved in 40 μl of electroporation buffer was then added to the cuvette and incubated on ice for 10 min. The cells were incubated a further 10 min on ice and added to 10 ml of drug-free growth medium. After 24 h of revival 20 μg/ml G418 was added and the cells were kept at 26°C for another 10 days with mild shaking. The presence of transfected cells was monitored visually by microscope and the drug concentration was increased gradually with each passage. Finally all the transfected cells were maintained in 200 μg/ml G418.

### *Confocal microscopy*

Mitochondrial localization of C-terminal GFP-tagged chimeric LmAPX was visualized by TCS-SP Leica confocal microscope fitted with a krypton/argon laser [23]. Log-phase promastigotes ($1\times10^7$) were pelleted by centrifugation at 1200 g for 10 min at 4°C and washed twice with ice-cold phosphate-buffered saline (PBS). The cells were suspended in 1 ml PBS and 100 μl of the suspension was taken into a different microfuge tube. Five hundred nanomolar Mitotracker Deep Red CMX-ROS was added to each tube. Fifty microliters of the suspension was spotted on poly-L-lysine-coated slides and kept in the dark for 30 to 45 min. Nonadherent cells were removed by a gentle wash with ice-cold PBS and fixed with ice-cold 4% paraformaldehyde for 10 min. Slides were further washed and air-dried for 10 min. Differential visualization of the fluorophore was achieved using a 488 nm excitation filter and 522/535 nm emission filter for green fluorescence and a 633 nm excitation filter and 644/665 nm emission filter for red fluorescence.

### *Measurement of intracellular $H_2O_2$ consumption*

Intracellular $H_2O_2$ consumption was measured by fluorescence spectrophotometry with DCFDA as the probe [24]. Both wild-type and overexpressing cells ($1\times10^7$ cells/ml) were treated with 200 μM $H_2O_2$ for 30 min and washed twice at 1200 g (4°C) for 5 min with PBS. Cells were further incubated with 6 μM DCFDA for 30 min at 26°C in the dark with mild shaking. The intensity of fluorescence was recorded immediately in a fluorescence spectrophotometer at 502 nm for excitation and 529 nm for emission.

### *Measurement of intracellular peroxidase activity*

The dihydrorhodamine 123 (peroxidase substrate) was used to measure mitochondrial peroxidase activity in vivo. Cells were incubated with 6 μM dihydrorhodamine 123 (ex/em: 488/525) for 25 min at 30°C and washed twice in PBS and fluorescence was measured subsequently using a FACSCanto flow cytometer (Becton–Dickinson, San Jose, CA, USA).

### *Determination of $IC_{50}$*

The growth-inhibitory concentration of $H_2O_2$ was studied on both wild-type and overexpressing cells using Nunclon 24-well plates. Log-phase promastigotes of both wild-type and overexpressing cells were seeded at $3\times10^5$ cells/ml in 2 ml growth medium in the presence of increasing concentration of $H_2O_2$. Overexpressing cells were maintained in 200 μg/ml G418 throughout the experiment, and after 5 days of growth cell densities were determined using a hemocytometer.

### *Flow cytometry*

The cardiolipin-sensitive probe NAO was used to monitor changes in mitochondrial lipids *in vivo*. Briefly, after treatment