**Leishmania major** encodes an unusual peroxidase that is a close homologue of plant ascorbate peroxidase: a novel role of the transmembrane domain

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Haem-containing enzymes (peroxidase and catalase) are widely distributed among prokaryotes and eukaryotes and play a vital role in H\(_2\)O\(_2\) detoxification. But, to date, no haem-containing enzymatic defence against toxic H\(_2\)O\(_2\) has been discovered in *Leishmania* species. We cloned, expressed and purified an unusual plant-like APX (ascorbate peroxidase) from *Leishmania major* (LmAPX) and characterized its catalytic parameters under steady-state conditions. Examination of its protein sequence indicated approx. 30–60% identity with other APXs. The N-terminal extension of LmAPX is characterized by a charged region followed by a stretch of 22 amino acids containing a transmembrane domain. To understand how the transmembrane domain influences the structure–function of LmAPX, we generated, purified and extensively characterized a variant that lacked the transmembrane domain. Eliminating the transmembrane domain had no impact on substrate-binding affinity but slowed down ascorbate oxidation and increased resistance to H\(_2\)O\(_2\)-dependent inactivation in the absence of electron donor by 480-fold. Spectral studies show that H\(_2\)O\(_2\) can quickly oxidize the native enzyme to compound (II), which subsequently is reduced back to the native enzyme by an electron donor. In contrast, ascorbate-free transmembrane-domain-containing enzyme did not react with H\(_2\)O\(_2\), as revealed by the absence of compound (II) formation. Our findings suggest that the single copy LmAPX gene may play an important role in detoxification of H\(_2\)O\(_2\) that is generated by endogenous processes and as a result of external influences such as the oxidative burst of infected host macrophages or during drug metabolism by *Leishmania*.

Key words: ascorbate peroxidase, guaiacol, hydrogen peroxide (H\(_2\)O\(_2\)), *Leishmania major*, promastigote, transmembrane domain.

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**INTRODUCTION**

Under physiological conditions, APX (ascorbate peroxidase) catalyses the oxidation of ascorbate with H\(_2\)O\(_2\) through the well-known peroxidative one-electron transfer mechanism [1]. It has been established that APX catalyses these reactions through the following steps:

\[
\text{APX} + \text{H}_2\text{O}_2 \rightarrow \text{Compound (I)} + \text{H}_2\text{O}
\]

\[
\text{Compound (I) + H}_2\text{A} \rightarrow \text{Compound (II) + HA}^*\]

\[
\text{Compound (II) + H}_2\text{A} \rightarrow \text{APX} + \text{HA}^* + \text{H}_2\text{O}
\]

\[
\text{HA}^* + \text{HA}^* \rightarrow \text{H}_2\text{A} + \text{A}
\]

where H\(_2\)A represents the reducing substrate (ascorbate) and A is dehydroascorbate. Compounds (I) and (II), being two and one-electron oxidation states above native ferriperoxidase respectively oxidize ascorbate (H\(_2\)A) by two one-electron transfer reactions with the formation of monodehydroascorbate radical (HA\(^*\)), a fairly reactive and unstable species, which is reduced back to ascorbate and dehydroascorbate (A). Presteady-state mechanistic information for oxidation of ascorbate by native and recombinant pea cytosolic APXs [2,3] and for tea APX [4] is available. EPR and UV–visible spectroscopic features of compound (I) are consistent with the formation of a porphyrin \(\pi\)-cation radical intermediate [as found in HRP (horseradish peroxidase)], and not a protein-based radical species as found in cytochrome \(c\) peroxidase [5,6]. In addition to the known activity of APXs towards ascorbate, it is well known that these enzymes are rather indiscriminate in their choice of substrate and are able to catalyse the oxidation of non-physiological (often aromatic) substrate, in some cases at rates comparable with that of ascorbate itself [7].

For several reasons, substrate recognition and binding in APX is more complex than it might first appear. Although NMR-derived distance constraints for binding of ascorbate to APX are consistent with the existence of two distinct binding sites, one close to the 6-propionate (\(\gamma\)-meso) position and the other near the \(\delta\)-meso position of the haem [8], site-directed mutagenesis together with chemical modification experiment are indicative of a single ascorbate interaction at the haem edge in the region of Arg\(^{172}\), Cys\(^{32}\) and the haem propionates (close to the \(\gamma\)-meso) position [9]. The refined atomic positions in the ascorbate-bound APX crystal structure show H-bonds between the 2-OH and 3-OH groups of the ascorbate and the protein (Arg\(^{172}\)), and between the 2-OH group of the ascorbate and the (deprotonated) haem 6-propionate [10]. This structure also shows that the side chain of Lys\(^{90}\) swings in from the solvent to provide an additional H-bond to the 6-OH group of the ascorbate for stabilization of the substrate binding [10]. Additionally, steady-state oxidation of ascorbate by some APXs does not obey the normal (hyperbolic) Michaelis–Menten kinetics, suggesting either allosteric effect, which seems unlikely [11], or more than one substrate-binding site [12], or the disproportion of monodehydroascorbate molecules to give back ascorbate and dehydroascorbate [13]. Interestingly, oxidation of

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Abbreviations used: APX, ascorbate peroxidase; HRP, horseradish peroxidase; LmAPX, *Leishmania major* APX.

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the aromatic substrate guaiacol, which is thought to bind close to the δ-meso position [2], shows normal Michaelis kinetics.

In plants, H$_2$O$_2$ is continuously produced as a by-product of photosynthesis, fatty acid β-oxidation, photosynthesis and oxidative phosphorylation. The H$_2$O$_2$-induced oxidative damage is minimized by the concerted action of powerful antioxidant enzymes. Most important among them are catalases, which are localized to peroxisomes, glyoxysomes and mitochondria and APX, which is located in both the chloroplasts and the cytoplasm [14, 15].

Parasitic protozoa of the order Kinetoplastida are the causative agents of several medically important tropical diseases including visceral (Leishmania donovani) and cutaneous (L. major) leishmaniasis. During an infective cycle of Leishmania in the vertebrate host, the parasite must survive in the rigorous oxidizing environment of the macrophage. In order to survive under such oxidative burst conditions, they must evade the toxic effects of nitric oxide (NO*), peroxynitrite (ONOO*), hydroxyl radicals (OH*), H$_2$O$_2$ and superoxide radicals (O$_2$*- ). However, Leishmania species use intracellular thiols [16], lipophosphoglycan [17], iron superoxide dismutase [18], HSP70 (heat-shock protein 70) [19], ovot catalase A, trypanothione [20] and peroxidoxins [21] to overcome a variety of reactive oxygen and nitrogen species [22] during their life cycle. Unlike most eukaryotes, Leishmania lacks catalase and selenium-containing glutathione peroxidases, enzymes capable of rapidly metabolizing high levels of H$_2$O$_2$. Hence, the mechanism by which it withstands the toxic effects of H$_2$O$_2$ is still unclear. To date, no haem containing enzymatic defence against H$_2$O$_2$ has been identified in Leishmania. Partial genome sequencing of L. major confirms the presence of a subset containing the open reading frame that putatively codes for a protein homologous to unusual plant-like ascorbate-dependent haemoperoxidase. To understand better the structure–function aspects of the peroxidase protein, we cloned, expressed and characterized an APX-like protein from L. major, LmAPX. Our study reveals the physical and catalytic features of LmAPX, which shows marked susceptibility to H$_2$O$_2$ in ascorbate-depleted medium. Evidence has been presented to show that a 22 amino acid hydrophobic region present at the N-terminus of LmAPX plays an important role in controlling H$_2$O$_2$ susceptibility and ascorbate oxidation.

**EXPERIMENTAL**

**Materials**

L. major and L. donovani were procured from the Leishmania strain bank of our Institute. All reagents and materials were purchased from Sigma or sources reported previously [23–25].

**Detection of peroxidase activity in Leishmania cell lysate**

Promastigotes of L. major and L. donovani were cultured in DMEL (Dulbecco’s modified Eagle’s liquid) media and blood/agar media respectively at 22°C. Promastigotes, grown up to stationary phase, were harvested by centrifugation at 6000 g for 10 min, and the pellets were resuspended in 10 ml of 50 mM Tris/HCl buffer (pH 7.5) containing 0.1 mM ascorbate, 1 mM PMSF and 1 mM protease inhibitor I and II (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). The resuspended cells were disrupted by sonication and the lysate was centrifuged at 15000 g for 30 min. The supernatant was designated as the crude extract.

Peroxidase-mediated oxidation of guaiacol, iodide and ascorbate was measured by following the change in absorbance at 470, 535 and 290 nm respectively as described previously [26–28].

**Genomic DNA isolation from L. major**

Genomic DNA was isolated from L. major logarithmic promastigotes by using Qiagen genomic DNA isolation kit at room temperature (27°C) [24, 25].

**Cloning of LmAPX from genomic DNA**

The sense primer1: 5′-AGGTAATTGGCTCGTAGC (610 nt upstream) and the antisense primer2: 5′-CCTGTCCGGAGGATACCTACAC (458 nt downstream of putative APX gene) were used to amplify the desired portion (1980 bp) from L. major genomic DNA by PCR. The amplified product (1980 bp) was gel-purified by using the Qiagen kit. The coding region of full-length LmAPX, Δ12 LmAPX (12 amino acids deleted from the N-terminal sequence of LmAPX) and Δ34 LmAPX (34 amino acids deleted from the N-terminus sequence of LmAPX) were amplified by using the amplified product (1980 bp) as the template. The following sense primers: 5′-AAAAAGATCCGCCATCTGGGC-GCGGCGGAAAGGC, 5′-AAAAAGTCCACCGGATCTGGTCG-TGGCGACC and 5′-AAAAAGGATCCAGGAGGGCCGGCTTTC-GACATC were used for the amplification of LmAPX, Δ12 LmAPX and Δ34 LmAPX respectively. The antisense primer 5′-AAAAATGACCTTACCTCCTGGACGCGTGC was used in each case. Each of the amplified products was cloned into the BamHI and KpnI sites of the prokaryotic expression vector pTrcHisA (Invitrogen) and DNA was sequenced by using an automated DNA sequencer.

**Expression and purification of proteins**

pTrcHisA vector alone, the recombinant pTrcHisA/LmAPX, pTrcHisA/Δ12 LmAPX and pTrcHisA/Δ34 LmAPX vectors were used to transform Escherichia coli BL21D3 cells. Transformed cells were grown overnight in 50 ml Luria–Bertani broth containing 100 µg ml$^{-1}$ ampicillin at 37°C in a shaker. The overnight grown cultures were then inoculated into 500 ml Terrific broth (12 g of tryptone, 24 g of yeast extract, 9.4 g of di-basic potassium phosphate, 2.2 g of monobasic potassium phosphate and 4 ml of glycerol/litre of medium). When the culture reached an absorbance of 0.8 at 600 nm, 0.5 mM isopropyl β-D-thiogalactoside and 0.4 mM δ-aminolevulinic acid were added, and the bacteria were further grown for 18 h at 22°C. Cells were harvested by centrifugation at 6000 g for 10 min, and the pellets were resuspended in 10 ml of 50 mM phosphate buffer (pH 7.5) containing 0.1 mM ascorbate, 150 mM NaCl, 1 mM PMSF, 1 mM protease inhibitors I and II (Roche Molecular Biochemicals) and 1 mg/ml lysozyme. The resuspended solution was kept for 1 h at 4°C and then the cells were broken by sonication. The lysate was centrifuged at 15000 g for 30 min. The supernatant, designated as the crude extract, was loaded on to an Ni$^{3+}$-nitrioltriacetate column. After loading the crude extract, the column was washed with washing buffer (50 mM phosphate buffer, pH 7.5, containing 0.1 mM ascorbate and 1 mM PMSF; 10 column volumes) and then washed further by 50 mM phosphate buffer (pH 7.5; 10 column volumes). The pure enzyme was eluted with either 50 mM phosphate or acetate buffer (pH 4.0) and then dialysed three times against 0.1 mM ascorbate and 50 mM phosphate buffer (pH 7.5) to adjust neutral pH. It is worth mentioning here that the enzymes eluted with either phosphate or acetate behaved identically as far as the optical spectra and kinetic parameters are concerned. In the case of ascorbate-free LmAPX preparation, whole purification was carried out in the absence of ascorbate.
**Protein concentration determination**

The haem was identified and quantified by the pyridine–haemochrome method [29]. The molar absorption coefficient of LmAPX at 408 nm was 101 mM$^{-1}$·cm$^{-1}$.

**Size-exclusion chromatography**

Native forms of the $\Delta 12$ LmAPX and $\Delta 34$ LmAPX were analysed by gel-filtration chromatography using a Protein Pak SW 300 column (Waters, Japan). The column was run at room temperature with a flow rate of 0.5 ml/min. The absorbance was monitored at 280 nm. The column was calibrated using thyroglobulin, albumin (660 kDa), dimer BSA (132 kDa), ovalbumin (43 kDa) and RNase (12.5 kDa).

**Binding and kinetic measurement of LmAPX**

All spectral studies were performed on a Shimadzu UV-1601 spectrophotometer using quartz cells of 1 cm light path. The difference spectrum of enzyme–ligand versus enzyme was obtained as described previously [26,27].

Compound (II) spectrum of LmAPX is stable and could be detected by a conventional spectrophotometer [compound (I) in LmAPX is a short-lived species]. Pseudo-first-order rate constants for compound (II) reduction ($k_{\text{obs}}$) of LmAPX were obtained at 424 nm by the mixing of enzyme (1.0 $\mu$M) in the presence of various concentrations of ascorbate with equimolar amounts of H$_2$O$_2$. Monophasic transient traces were fitted to a single exponential process to obtain pseudo-first-order rate constants.

**Inactivation of LmAPX by H$_2$O$_2$**

The rate of inactivation of LmAPX by H$_2$O$_2$ was measured by preincubation of LmAPX with different concentrations of H$_2$O$_2$ in 50 mM phosphate buffer (pH 7.5) in the absence of electron donors. After the addition of H$_2$O$_2$, at various time intervals the absorbance was monitored at 408 nm using cuvettes containing 1 ml of 144 nM enzyme, 50 mM phosphate buffer (pH 7.5), 20 mM guaiacol and 0.3 mM H$_2$O$_2$. During the substrate protection study against inactivation, a high concentration of electron donor was added to the preincubation mixture containing the enzyme before the addition of H$_2$O$_2$.

**RESULTS**

**Primary structure analysis**

The 0.9 kb genomic DNA fragment, coding for a 303 amino acid long LmAPX possesses 62.73% identity and 68.79% similarity with Trypanosoma cruzi APX. 35% identity and 60.72% similarity with pea cytosolic APX. 34% identity and 61.39% similarity with soya bean cytosolic APX, and 31.35% identity and 64.35% similarity with chloroplast APX [30–33]. Similarity of the primary sequences of LmAPX with that of chloroplast, T. cruzi, pea and soya-bean cytosolic APXs indicate that LmAPX is related to the class I group of haemoperoxidases. However, the sequence identities between LmAPX with other classes of superfamily are found to be less than 18% (results not shown). The charged residues in the dimer interface of the pea APX are not similar to LmAPX. Figure 1(A) also provides a sequence alignment of the proximal cation-binding loop in various APXs. LmAPX has the side chain Thr209 residue instead of aspartic acid, indicating that the proximal cation-binding loop is very similar to the K$^+$-binding site of APX [34]. The other feature that distinguishes LmAPX from the plant enzymes is the presence of a sequence insertion, of unknown function, near the C-terminus containing charged amino acids. A notable feature that differentiates LmAPX from the cytosolic APX is its N-terminal extended portion. TargetP V1.0 prediction [35] result indicates that in LmAPX, the extended region of the N-terminal sequence codes for a positive charged region (12 amino acids) which is followed by a stretch of 22 amino acids containing a hydrophobic region that has the potential to form a transmembrane domain (Figure 1B). Sequence analysis predicts that the overall structural elements of LmAPX are quite similar to the cytosolic APX. Furthermore, Swiss-Model protein modelling also predicts that the entire LmAPX sequence is highly compatible with structures of the distal as well as proximal site of haem in cytosolic APX protein (Figure 2). All of the key residues on the proximal site of the haem are conserved between LmAPX and APX: His$^{195}$, Trp$^{208}$ and Asp$^{253}$ in LmAPX are superimposed with the corresponding His$^{183}$, Trp$^{197}$ and Asp$^{208}$. The key distal residues of LmAPX (His$^{48}$, Trp$^{57}$ and Arg$^{68}$) are also found to be in identical position with respect to the distal site residues of APX (His$^{42}$, Trp$^{51}$ and Arg$^{68}$). The most significant difference is that of the Phe$^{201}$ residue in LmAPX, which substitutes Arg$^{12}$ of the APX, the crucial residue for the ascorbate binding as well as oxidation. It is worth mentioning that in contrast with plant APX, the parasite-specific T. cruzi APX, which is more close to LmAPX, also lacks this arginine residue at the ascorbate-binding site [30].

**Physical and spectral characteristics of LmAPX**

Recombinant N-terminal histidine-tagged LmAPX protein is overexpressed in E. coli cells by induction with isopropyl β-D-thiogalactoside and 8-amino-levulinic acid, but total protein goes into inclusion bodies and cannot be purified in soluble form (results not shown). However, $\Delta 12$ LmAPX and $\Delta 34$ LmAPX are both expressed as active and soluble forms. To investigate the native state molecular mass of both the variants of LmAPX, purified proteins were subjected to gel filtration using HPLC. Results shown in Figure 3(A) indicate that $\Delta 34$ LmAPX eluted at a position expected of monomeric enzyme (33 kDa), whereas the elution pattern of $\Delta 12$ LmAPX showed that 70% of the protein were eluted as dimeric protein (35.5 kDa), 15% eluted as a monomeric state (71 kDa) and the rest 15% of the protein eluted at a wide range of molecular mass (oligomerization of the protein). This result suggests that the tendency of oligomerization of the $\Delta 12$ LmAPX may be due to hydrophobicity of the transmembrane domain. The $\Delta 34$ LmAPX is a monomer instead of a dimer as observed with cytosolic APX probably because the charged residues in the dimer interface of the pea APX are absent from LmAPX. The $\Delta 12$ LmAPX and $\Delta 34$ LmAPX migrated on denaturing SDS-polyacrylamide gel at a molecular mass of 35.5 and 33 kDa respectively, identical with their calculated molecular mass (Figure 3A, inset). The UV–visible spectra of ascorbate-free $\Delta 34$ LmAPX shows the presence of a Soret peak at 408 nm with secondary peaks at approx. 500 and 640 nm (Figure 3B). Addition of a 5 molar excess of H$_2$O$_2$ to the resting state of $\Delta 34$ LmAPX produces oxyferryl compound (II) [oxyferryl compound (II) is produced via compound (I), a very short-lived ferryl haem iron with porphyrin ð cation radical [6]] absorbing at 420 nm at the Soret region with visible peaks at 532 and 560 nm. The calculated purity number Rz ($A_{\alpha\alpha}$/$A_{\alpha\beta}$) for $\Delta 34$ LmAPX and $\Delta 12$ LmAPX were 0.98 (Figure 3B) and 0.9 (results not shown) respectively.

To characterize further the effects of transmembrane domain on LmAPX catalysis, turnover of other common electron donors were investigated. Table 1 shows the steady-state data of ascorbate, guaiacol and iodide oxidation of both $\Delta 12$ LmAPX.
The sequence of LmAPX was aligned with T. cruzi APX (ToAPX: CAD30032), tobacco stromal APX (chlo; BA47853), pea APX (AAAT3645) and soybean cytosolic APX (Cyto; T07056). The residues identical with LmAPX sequence are denoted by an asterisk. The amino acid residues of the proximal and distal sites of haem implicated in the redox activity of APXs are denoted by boldface letters. The boxed region in LmAPX represented the transmembrane domain. The residues involved in electrostatic interactions of the dimer formation, ascorbate binding and K⁺-binding site in plant APX are represented by d, b and p respectively.

(TMHHMM is a program for the prediction of transmembrane helices in proteins. The TMHHMM is very well suited for the prediction of transmembrane helices because it can incorporate hydrophobicity, charge bias, helix lengths and grammatical constraints into one model for which algorithms of parameter estimation and prediction already exist.) The posterior probabilities for transmembrane helix, inside or outside are displayed. The prediction showed that the 12–34 region of LmAPX represented the transmembrane domain. The dotted and solid lines represented the outside and inside of the membrane respectively.

and Δ34 LmAPX. In the case of ascorbate oxidation, both the truncated forms of LmAPX displayed non-Michaelis–Menten kinetics under steady-state condition. A linear dependence on substrate concentration was observed and saturation was not detected at any accessible concentration suggesting that the binding of ascorbate is weak probably due to the absence of Arg172. The full-kinetic profile of both mutants could not be generated and the direct determination of the kinetic parameters (Km, kcat) was not possible. Hence specific activities are calculated at 0.5 mM ascorbate concentration (similar condition was used to measure the specific activity of pea cytosolic APX [3]). The rate of ascorbate oxidation of Δ12 LmAPX was approx. 15-fold lower as compared with the pea cytosolic APX [2,3,9] but similar to the T. cruzi APX [30]. The fact that variant LmAPX and ToAPX proteins each exhibit a lower ascorbate oxidation compared with pea APX [2,3,9] supports the idea that the absence of the Arg172 side chain in parasite-specific APX may disrupt their ability to utilize ascorbate as a source of electron. As opposed to ascorbate oxidation both proteins exhibited Michaelis–Menten-type kinetics in the presence of guaiacol and iodide. From the Lineweaver–Burk plot, the calculated KmA values of both Δ12 LmAPX and Δ34 LmAPX for guaiacol, iodide and H₂O₂ are very similar to each other (Table 1) indicating that the substrate affinity of the recombinant LmAPX is unaltered after removing the transmembrane domain. When Δ12 LmAPX and Δ34 LmAPX were purified from the overexpression system in the absence of ascorbate, the Δ12
Figure 2  Ribbon structural model showing the position of Lys55 and Phe201 in LmAPX relative to the ascorbate binding Lys30 and Arg172 in APX.

The green and red colour residues represent residues of APX and LmAPX respectively. The model is based on published crystal structures for soya-bean cytosolic APX-ascorbate complex [10]. The distal site residues His68, Arg64 and Trp67 and proximal site residues His192, Asp253 and Trp208 of LmAPX are superimposed with the corresponding distal and proximal site residues of APX.

Figure 3  The size-exclusion chromatography, SDS-polyacrylamide gel electrophoresis and light absorbance spectra recorded after purification of LmAPX.

(A) Size-exclusion chromatography of purified Δ12 LmAPX and Δ34 LmAPX by HPLC. Solid and dotted lines depict the elution profile of Δ12 LmAPX and Δ34 LmAPX respectively. The inset shows that proteins were visualized with Coomassie Blue stain. Lane 1, purified Δ34 LmAPX; lane 2, molecular mass standards; and lane 3, purified Δ12 LmAPX. (B) UV–visible spectra of ascorbate-free Δ34 LmAPX before (········) and after the addition of 7 μM H₂O₂ (——).
The catalytic activities were determined at 25°C as described in the Experimental section. The values represent the means ± S.E.M. for three measurements each.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ascorbate* Specific activity (units/mg)</th>
<th>H₂O₂† Kₘ (µM)</th>
<th>Guaiacol† Kₘ (µM)</th>
<th>Iodide† Kₘ (µM)</th>
</tr>
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<tbody>
<tr>
<td>Ascorbate-bound</td>
<td></td>
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<tr>
<td>Δ12 LmAPX</td>
<td>15 ± 0.9</td>
<td>25 ± 4</td>
<td>6.25 ± 1</td>
<td>174 ± 10</td>
</tr>
<tr>
<td>Δ34 LmAPX</td>
<td>3.2 ± 0.1</td>
<td>27 ± 3</td>
<td>6.66 ± 1</td>
<td>143 ± 10</td>
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<tr>
<td>Ascorbate-free</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ12 LmAPX</td>
<td>ND‡</td>
<td>200 ± 25</td>
<td>10.1 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Δ34 LmAPX</td>
<td>3.0 ± 0.1</td>
<td>25 ± 2</td>
<td>8.2 ± 1</td>
<td>111 ± 11</td>
</tr>
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</table>

* Specific activities are reported at 0.5 mM ascorbate concentration (measurable concentration), which was used to measure the specific activities of pea APX [3].
† Fit with Michaelis–Menten equation.
‡ ND, not detectable.

LmAPX enzyme showed low activity and high Kₘ value for H₂O₂, whereas Δ34 LmAPX was found to be highly active (Table 1) indicating that ascorbate is essential during the purification of Δ12 LmAPX for its stabilization. This result is consistent with the previous report where it has been shown that chloroplast APX (containing a transmembrane domain) is a labile enzyme in an ascorbate-depleted medium when compared with cytosolic APX [14].

H₂O₂-dependent inactivation of LmAPX

Chloroplast APX is known to be distinct from cytosolic APX with respect to H₂O₂ susceptibility in the absence of an electron donor [36]. To ascertain whether Δ12 LmAPX (like chloroplast APX) and Δ34 LmAPX (like cytosolic APX) displayed similar properties, both the enzymes were preincubated with different concentrations of H₂O₂ that resulted in concentration and time-dependent irreversible inactivation of the enzyme following pseudo-first-order kinetics (Figures 4A and 4B). When kₘ values obtained from the slope of each line were plotted against H₂O₂ concentration, a straight line (Figures 4A and 4B inset) was obtained from which a second-order rate constant was calculated to be 6000 M⁻¹·min⁻¹ for Δ12 LmAPX and 16.7 M⁻¹·min⁻¹ for Δ34 LmAPX at 30°C. The result shows that Δ12 LmAPX is 480-fold more susceptible to H₂O₂-dependent inactivation as compared with Δ34 LmAPX. H₂O₂-dependent inactivation of both enzymes were protected by a high concentration of ascorbate or aromatic donor (guaiacol) indicating that electron donors scavenged the preincubating H₂O₂ by reducing the compound (I) and (II) back to the native state.

Spectral properties of Δ12 LmAPX and Δ34 LmAPX with H₂O₂

Figure 5(A) shows the change in resting state of ascorbate-free Δ12 LmAPX spectrum when 20 molar excess of H₂O₂ was added at 25°C. In contrast with other peroxidases, the initial spectrum was unaltered in the region of Soret and visible peaks of enzyme at 408, approx. 500 and 640 nm. This result indicated that ascorbate-free Δ12 LmAPX could not react with H₂O₂. Thus our spectral observation is consistent with the kinetic result where ascorbate-free Δ12 LmAPX was found to be catalytically inactive (Table 1). Figure 5(B) shows the change in the resting state of ascorbate-bound Δ12 LmAPX spectrum when 5-fold molar equivalent of H₂O₂ was added. The initial spectrum showed a higher absorbance and red-shifted to 420 nm at the Soret region with simultaneous appearance of a double hump at 532 and 560 nm in the visible region [1,37,38]. This initial spectrum is reminiscent of compound (II) of the other peroxidase. This compound (II) species returns to the ferric state of the enzyme within 30 s. These results suggest that ascorbate-bound Δ12 LmAPX is in active form, which is able to react with H₂O₂ to form compound (II) [via compound (I)] which subsequently reacts...
with ascorbate (electron donor) to form the native enzyme. The rate of compound (II) reduction by ascorbate for \( \Delta 12 \) LmAPX is very slow compared with pea APX [2,3] and follows monophasic kinetics (Figure 5C). The compound (II) reduction is linearly dependent on ascorbate concentration (Figure 5C). The second-order rate constant derived from this linear dependence was \( 6.01 \pm 0.03 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1} \). In contrast with ascorbate-free \( \Delta 12 \) LmAPX, the ascorbate-free \( \Delta 34 \) LmAPX enzyme was found to be in an active state and exhibited peaks at 408, approx. 500 and 640 nm. When five molar excess of \( \text{H}_2\text{O}_2 \) was added to the ascorbate-free \( \Delta 34 \), the native enzyme immediately formed \( \alpha/\beta \) bands at 532 and 560 nm with shifting of Soret band from 408 to 420 nm, and simultaneous decrease of absorbance at 640 nm. The ascorbate-free \( \Delta 34 \) LmAPX-oxidized species [compound (II)] is found to be stable for several hours. The red shift in the Soret band and double hump at the visible region of this species is very similar to the well-known peroxidase compound (II). Upon addition of 10 \( \mu \text{M} \) ascorbate to the compound (II) of ascorbate-free \( \Delta 34 \) LmAPX, 34 amino acids deleted from N-terminus sequence of LmAPX; the enzyme intermediate returned back to a native state within 60 s (Figure 5D, broken line), indicating that the ascorbate was oxidized by the LmAPX–\( \text{H}_2\text{O}_2 \) intermediate.
As binding of ascorbate is a prerequisite for oxidation [9,10], the interaction of ascorbate with both Δ12 and Δ34 LmAPX were studied by optical difference spectroscopy [26,27] in the presence or absence of guaiacol. The binding of ascorbate gave a characteristic difference spectrum of the LmAPX–ascorbate complex versus LmAPX, having a maximum at 433 nm and a minimum at 413 nm (Figure 6A for Δ12 LmAPX and Figure 6B for Δ34 LmAPX). The apparent equilibrium dissociation constant, $K_d$, for the LmAPX–ascorbate complex as calculated from the plot of $1/\Delta \text{A}$ versus $1/[\text{ascorbate}]$ (Figure 6C for Δ12 LmAPX and Figure 6D for Δ34 LmAPX) was $33 \mu M$. When binding was studied in the presence of guaiacol, ascorbate also interacted with the LmAPX–guaiacol complex, showing a similar characteristic difference spectra at the Soret region; however, the nature of the binding was found to be competitive. This is further substantiated by the finding (Table 2) that the binding of ascorbate to both forms of LmAPX ($K_d = 33 \mu M$) is significantly increased ($K_d = 100 \mu M$ for Δ12 and $K_d = 400 \mu M$ for Δ34 LmAPX) in the presence of guaiacol. This indicates that ascorbate interacts at a site close to the guaiacol (aromatic donor) binding site.

**DISCUSSION**

Intracellular pathogen *Leishmania* possesses a strong antioxidant defence against the oxidants released by the macrophage under oxidative burst condition, for its survival and replication. In most pathogenic organisms, haemoproteins, e.g. catalase or peroxidase, play a major role in detoxification of $H_2O_2$, an oxidant. But to date, this type of enzymatic machinery has not been reported in *Leishmania*. This study for the first time describes the properties of an unusual plant-like haem-containing APX (LmAPX) from *L. major*. The sequence homology of LmAPX suggests that it belongs to a broad family of peroxidase evolutionarily related to class I peroxidase [39]. The sequence alignment studies detail the marked similarities among LmAPX with other APXs in the proximal/distal sides of the haem. In fact, His95,Trp79 and Arg46 on the distal haem side are found to be absolutely conserved. These distal histidine and arginine residues have been implied to work in concert in an acid/base catalysed cleavage of the peroxide $O-O$ bond [40,41]. Swiss-Model protein modelling also predicts that the amino acid residues at the proximal haem side of LmAPX, Asp115, proximal ligand His192 and Trp208 are identical with APX. Analysis of the crystal structure of a ascorbate and aromatic donor-bound APX, mutational and chemical modification studies have identified two distinct binding sites, the first of which contains a negative charged ascorbate-binding domain near the exposed $γ$-site of haem, whereas the second is thought to be a neutral aromatic donor-binding domain near the exposed 6-site of haem [29,42]. When the ascorbate-binding site of LmAPX is compared with that of APX, Lys55 in LmAPX was found to be identical with Lys30 of APX but Phe201 appears to be substituted for Arg207 that reportedly interacts with the 2′-OH and 3′-OH groups of ascorbate. Indeed, Arg207 is absolutely conserved among all other APXs examined so far except in TcAPX, which is the only known APX in the trypanosomatid family. Similar to LmAPX, TcAPX also lacks this residue where an asparagine residue (Asn216) occupies the homologous position. The specific activity of ascorbate and guaiacol oxidation in LmAPX is lower compared

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Spectrum of complex (nm)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta \epsilon_{\text{peak-mask}}$ (mM$^{-1}$·cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ12 LmAPX</td>
<td>413 433</td>
<td>33 ± 3</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>+ Guaiacol</td>
<td>409 433</td>
<td>100 ± 9</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Δ34 LmAPX</td>
<td>413 433</td>
<td>33 ± 4</td>
<td>3.33 ± 0.2</td>
</tr>
<tr>
<td>+ Guaiacol</td>
<td>409 433</td>
<td>400 ± 25</td>
<td>3.33 ± 0.2</td>
</tr>
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</table>

Table 2 Characterization of difference spectra and apparent dissociation constants ($K_d$) of LmAPX–ligand complexes

Measurement of apparent dissociation constants were made at 25 °C as described under the Experimental section. The concentration of LmAPX and guaiacol used were 10 μM and 20 mM respectively. The data were obtained from three experiments. The ligand was ascorbate in each case.
with the plant APX, probably due to slower rate of electron transfer from electron donor to compound (II). Compound (I) in ascorbate-bound LmAPX is short-lived and its spectrum decays too rapidly to capture by conventional spectrophotometer. Hence the reduction of compound (II) to ferric state is a rate-limiting step in the LmAPX catalysis. The rate of compound (II) reduction by ascorbate for both variants of LmAPX are monophasic and are slower than that obtained from the kobs-out of pea APX compound (II) reduction [3]. These results correlate well with what is seen in the steady-state kinetics data. Difference spectroscopic studies show that despite the absence of the crucial arginine residue at a position homologous to Arg172 of APX, the affinity (Kd) of LmAPX for ascorbate is still in the micromolar range, suggesting that other residues might be involved in ascorbate binding. It is possible that APXs from lower eukaryotes (TcAPX, LmAPX) probably utilizes some distinct ascorbate-binding mechanism.

The primary sequence of this peroxidase (LmAPX) was found to be comprised of a hydrophobic transmembrane motif at the N-terminus. It is well established that the general function of N-terminal hydrophobic transmembrane motif is to anchor the proteins to the membrane of different organelles including Golgi, ER, vacuoles, synaptic vesicles, mitochondria and peroxisomes [35]. Apart from this, the novel aspects that set apart Δ12 LmAPX from its transmembrane domain deleted counterpart (Δ34 LmAPX) are (i) Δ12 LmAPX has a higher tendency for oligomerization than Δ34 LmAPX at the native state; (ii) the enzyme Δ12 LmAPX in an ascorbate-free system is catalytically inactive, whereas ascorbate-free Δ34 LmAPX is catalytically active; (iii) ascorbate oxidation rates of Δ12 LmAPX are greater than Δ34 LmAPX at physiological pH in an ascorbate-supplemented system; and (iv) the second-order rate constant of Δ12 LmAPX inactivation by H2O2 is 480-fold higher compared with Δ34 LmAPX in the absence of electron donors. These properties of the N-terminal transmembrane domain of this enzyme provide a unique perspective on LmAPX structure–function.

Further insight into this unexpected difference between Δ12 and Δ34 LmAPX was gained from the formation of the enzyme intermediates when the peroxidase reaction cycles were analysed spectrometrically. The reason for the lack of measurable peroxidase activity in ascorbate-free Δ12 LmAPX could be that the recombiant protein, despite its solubility, is incorrectly folded. However, a similar lack of peroxidase activity was observed in the native ascorbate-free chloroplast APX, which is purified from plants [36]. In general, several active enzymes are very unstable in the absence of substrate because the active site of substrate binding was found to be improperly folded. But the ascorbate-free Δ12 LmAPX enzyme can bind ascorbate with similar affinity as compared with Δ34 LmAPX, which rules out the possibility of incorrect folding at the ascorbate-binding site in ascorbate-free Δ12 LmAPX during purification. The spectral and kinetic evidence strongly support the view that the ascorbate-free Δ12 LmAPX enzyme is catalytically inactive due to its incapability of reaction with H2O2. This is probably because the H2O2 entry channel of ascorbate-free Δ12 LmAPX was hindered by the transmembrane domain.

Using previously established methods [43,44], we made an effort to examine the reactions of LmAPX with H2O2 in the absence of electron donors by kinetic analysis of their inactivation reactions, compound (III) and P670 species formation. It was found that H2O2 acted as a mechanism-based (suicide) inactivator in APX [44]; although, important differences were noticed between these enzymes. For HRP, it was established that a large stoichiometric excess of H2O2 was required for inactivation [44], whereas APX was extremely sensitive to inactivation. Although the Δ12 LmAPX is more sensitive to H2O2-dependent inactivation compared with Δ34 LmAPX, since both of them share identical active-site residues in catalysis, we suggest a similar overall mechanism involved in inactivation for both the enzymes. This inactivation process may be connected to the spontaneous reduction of compound (II) to an inactive species [compound (II)-like] followed by a decrease in haem Soret spectra. The compound P670-like species, which is formed in HRP-C under a high concentration of H2O2 [45], is not detected in the inactive state of both enzymes. No distinct spectral shift was observed for inactive species. A similar phenomenon occurs in both APX [44] and HRP-A2, where a P670-like species is not detected [46]. This was probably because the P670 species of LmAPX is inherently unstable and difficult to detect. The decreasing haem Soret spectra indicate that H2O2-dependent haem degradation is occurring in ascorbate-free-LmAPX.

In view of the fact that ascorbate leads to Soret spectral changes in LmAPX, it is logical to conclude that ascorbate binds near the exposed haem edge. However, the non-saturation kinetics of ascorbate peroxidation does not correlate with the calculated Kd of ascorbate binding, which is consistent with a previously reported observation [8]. Therefore the spectrally derived ascorbate Kd value of native LmAPX is different from that for kinetically active enzyme–substrate complexes under steady-state conditions. It has been predicted from ascorbate-dependent compound (II) reduction studies (biphasic rate constant at high ascorbate concentration) that APX has two competent ascorbate-binding sites for electron transfer including high-affinity (near the γ-haem edge) and low-affinity (δ-meso edge) binding sites [3]. Since we did not observe biphasic reduction of compound (II) with ascorbate, the possibility of multiple ascorbate-binding sites may be ruled out at least in our case. Interestingly, our binding studies indicate that ascorbate interacts at the haem edge as an electron donor since it competes with guaiacol. An alternative possibility might be that the binding of guaiacol to the native LmAPX perturbs the conformation of its ascorbate-binding site leading to lowered affinity of the site for the ascorbate (Kd) observed as apparent competition between ascorbate and guaiacol. The unambiguous identification of the actual ascorbate-binding site should, however, wait until the X-ray crystal structure of LmAPX is solved.

In various stages of its life cycle, the Leishmania species may come in contact with H2O2 as a result of direct stimulation of the macrophage respiratory burst [47,48]. This could occur in vivo during initial infection of promastigotes or in passage from one macrophage to another for amastigotes [47,48]. In the parasite, the reactive oxygen species are generated by endogenous processes and as a result of external influences such as host immune responses and drug metabolism [47,49]. In 1985, it was reported that the Leishmania amastigotes can scavange a large amount of H2O2 [16]. The removal of H2O2 by amastigotes was markedly inhibited by aminotriazole or sodium azide, which is an inhibitor of haem-containing enzymes, e.g. catalase or peroxidase [16]. Our preliminary results suggested that the Leishmania cell lysate has peroxidase activity (S. Adak, unpublished work). In the absence of catalase, the single copy APX gene may play a vital role in protecting this parasite against oxidative damage. This unusual LmAPX could thus be the fundamentals of a rational approach to the design and discovery of drugs against Leishmania infections.
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