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_2O_2 detoxification. But, to date, no haem-containing enzymatic defence against toxic H_2O_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_2O_2-dependent inactivation in the absence of electron donor by 480-fold. Spectral studies show that H_2O_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_2O_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_2O_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_2O_2), *Leishmania major*, promastigote, transmembrane domain.

INTRODUCTION

Under physiological conditions, APX (ascorbate peroxidase) catalyses the oxidation of ascorbate with H_2O_2 through the well-known peroxidative one-electron transfer mechanism [1]. It has been established that APX catalyses these reactions through the following steps:

$$\text{APX} + H_2O_2 \rightarrow \text{Compound (I)} + H_2O \quad (1)$$

$$\text{Compound (I)} + H_2A \rightarrow \text{Compound (II)} + HA^{\bullet} \quad (2)$$

$$\text{Compound (II)} + H_2A \rightarrow \text{APX} + HA^{\bullet} + H_2O \quad (3)$$

$$HA^{\bullet} + HA^{\bullet} \rightarrow H_2A + A \quad (4)$$

where H_2A 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_2A) by two one-electron transfer reactions with the formation of monodehydroascorbate radical ($HA^{\bullet}$), 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 π-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 (γ-*meso* position) and the other near the δ-*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 γ-*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^{30} 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

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_2O_2 is continuously produced as a by-product of photorespiration, fatty acid β-oxidation, photosynthesis and oxidative phosphorylation. The H_2O_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 ($NO^{\bullet}$), peroxynitrite ($ONOO^-$), hydroxyl radicals ($OH^{\bullet}$), H_2O_2 and superoxide radicals ($O_2^{\bullet -}$). However, *Leishmania* species use intracellular thiols [16], lipophosphoglycan [17], iron superoxide dismutase [18], HSP70 (heat-shock protein 70) [19], ovothiol 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_2O_2. Hence, the mechanism by which it withstands the toxic effects of H_2O_2 is still unclear. To date, no haem containing enzymatic defence against H_2O_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_2O_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_2O_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, 353 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′-AGGTAATGGCTGCGTAGCG (610 nt upstream) and the antisense primer2: 5′-CGTGTCCGAGGAGATACTAACG (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′-AAAGGATCCGGCACCTCGCGGCGAGCGAAAGGC, 5′-AAAAGGATCCACCGGCATCGCTGTCGGCACC and 5′-AAAAGGATCCGAGGAGCCGCCGTTCGACATC were used for the amplification of LmAPX, Δ12 LmAPX and Δ34 LmAPX respectively. The antisense primer 5′-AAAGGTACCTTAGCTCTCCGAAGCGGGTGCT 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 dibasic 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^{2+}-nitrilotriacetate 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 5.25; 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 $mM^{-1} \cdot cm^{-1}$.

Size-exclusion chromatography

Native forms of the Δ12 LmAPX and Δ34 LmAPX were analysed by gel-filtration chromatography using a Protein Pak SW 300 column (Nihon Waters, Japan, Tokyo) in an HPLC system (Waters), preequilibrated with 50 mM phosphate buffer (pH 6.5) and 250 mM NaCl. 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 (660 kDa), dimer BSA (132 kDa), ovalbumin (43 kDa) and RNAase (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_{obs}) of LmAPX were obtained at 424 nm by the mixing of enzyme (1.0 μM) in the presence of various concentrations of ascorbate with equimolar amounts of H_2O_2. Monophasic transient traces were fitted to a single exponential process to obtain pseudo-first-order rate constants.

Inactivation of LmAPX by H_2O_2

The rate of inactivation of LmAPX by H_2O_2 was measured by preincubation of LmAPX with different concentrations of H_2O_2 in 50 mM phosphate buffer (pH 7.5) in the absence of electron donors. After the addition of H_2O_2, at various time intervals the incubation mixture was transferred to a cuvette containing 1 ml of assay mixture of 144 nM enzyme, 50 mM phosphate buffer (pH 7.5), 20 mM guaiacol and 0.3 mM H_2O_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_2O_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 86.79 % 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 Thr^{209} 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^{192}, Trp^{208} and Asp^{253} in LmAPX are superimposed with the corresponding His^{163}, Trp^{179} and Asp^{208}. The key distal residues of LmAPX (His^{68}, Trp^{67} and Arg^{64}) are also found to be in identical position with respect to the distal site residues of APX (His^{42}, Trp^{41} and Arg^{38}). The most significant difference is that of the Phe^{201} residue in LmAPX, which substitutes Arg^{172} 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 δ-aminolevulinic acid, but total protein goes into inclusion bodies and cannot be purified in soluble form (results not shown). However, Δ12 LmAPX and Δ34 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 Δ34 LmAPX eluted at a position expected of monomeric enzyme (33 kDa), whereas the elution pattern of Δ12 LmAPX showed that 70 % of the protein were eluted as monomeric enzyme (35.5 kDa), 15 % eluted as a dimeric 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 Δ12 LmAPX may be due to hydrophobicity of the transmembrane domain. The Δ34 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 Δ12 LmAPX and Δ34 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 Δ34 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_2O_2 to the resting state of Δ34 LmAPX produces oxyferryl compound (II) [oxyferryl compound (II) is produced via compound (I), a very short-lived ferryl heam iron with porphyrin π 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_{408}/A_{280}) for Δ34 LmAPX and Δ12 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 Δ12 LmAPX

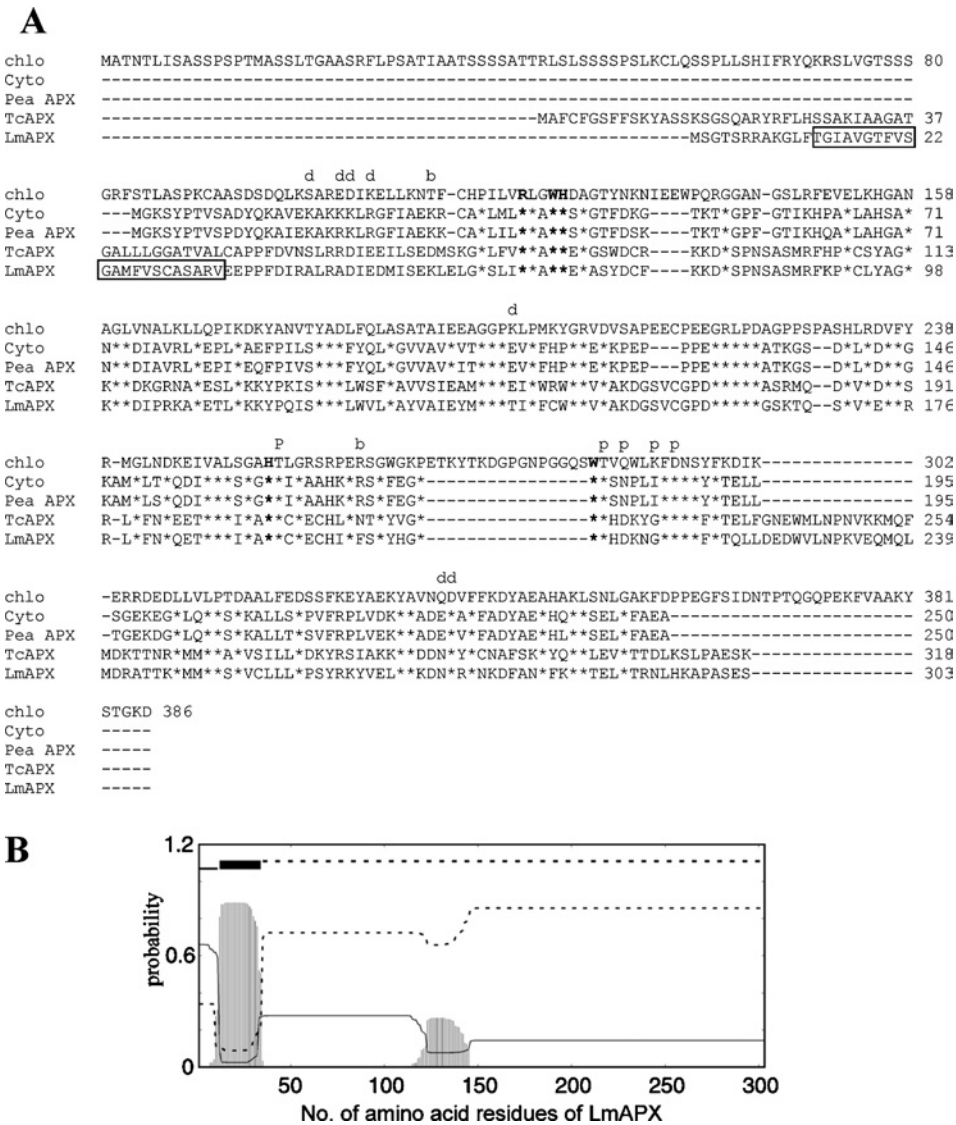

Figure 1 Sequence alignment of APXs from different sources

(**A**) The sequence of LmAPX was aligned with *T. cruzi* APX (TcAPX; CAD30023); tabacco stromal APX (chlo; BAA78553), pea APX (AAA33645) and soya-bean 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. (**B**) TMHMM (transmembrane hidden Markov model) posterior probabilities for LmAPX sequence (35) are shown. (TMHMM is a program for the prediction of transmembrane helices in proteins. The TMHMM is very well suited for the prediction of transmembrane helices because it can incorporate hydrophobicity, change 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 the 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 Arg^{172}. The full-kinetic profile of both mutants could not be generated and the direct determination of the kinetic parameters (K_m, k_{cat}) was not possible. Hence specific activities are calculated at 0.5 mM ascorbate concentration (a similar condition was used to measure the specific activity of pea cytosolic APX [3]). Table 1 showed that the transmembrane domain containing LmAPX has 5-fold higher activity compared with Δ34 LmAPX. 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 TcAPX proteins each exhibit a lower ascorbate oxidation compared with pea APX supports the idea that the absence of the Arg^{172} 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 K_m values of both Δ12 LmAPX and Δ34 LmAPX for guaiacol, iodide and H_2O_2 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

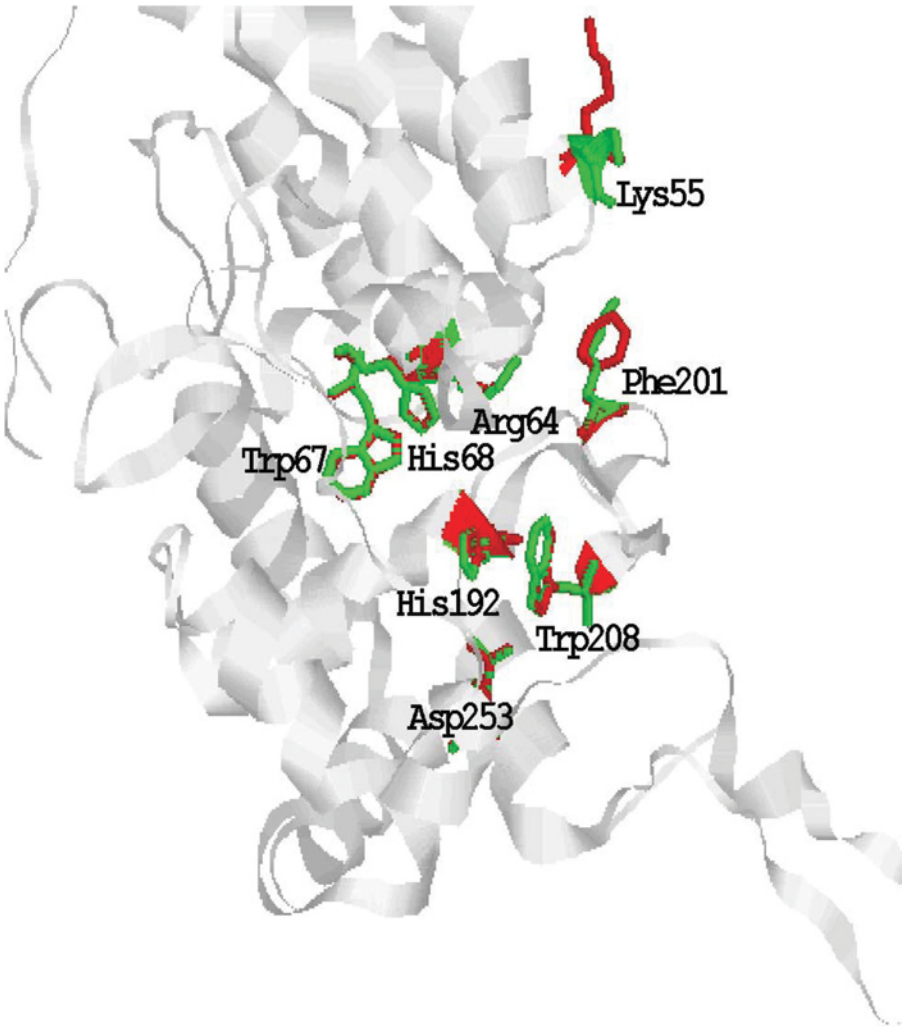

Figure 2 Ribbon structural model showing the position of Lys^{55} and Phe^{201} in LmAPX relative to the ascorbate binding Lys^{30} and Arg^{172} in APX

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 His^{68}, Arg^{64} and Trp^{67} and proximal site residues His^{192}, Asp^{253} and Trp^{208} of LmAPX are superimposed with the corresponding distal and proximal site residues of APX.

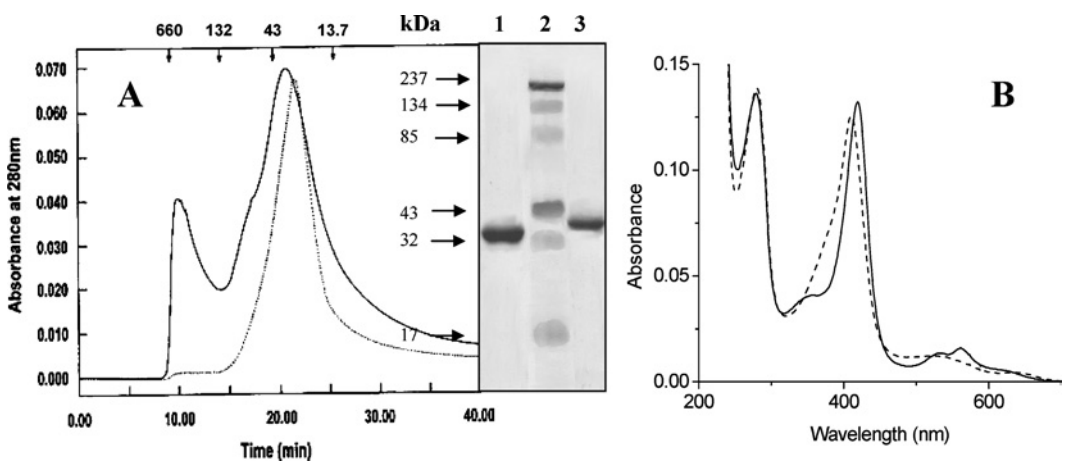

Figure 3 The size-exclusion chromatography, SDS-polyacrylamide gel electrophoresis and light absorbance spectra recorded after purification of LmAPX

(**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 H_2O_2 (——).

Table 1 Comparative analysis of steady-state oxidation of Δ12 LmAPX and Δ34 LmAPX

Both enzymes were purified from an overexpression system in the presence or absence of ascorbate. The turnover number (k_{cat}) is expressed as mol of product formed · (mol of protein)$^{-1}$ · min^{-1}. 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.

	Ascorbate*	H_2O_2†	Guaiacol†		Iodide†	
Enzyme	Specific activity (units/mg)	K_m (μM)	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
Ascorbate-bound						
Δ12 LmAPX	15 ± 0.9	25 ± 4	6.25 ± 1	174 ± 10	2.5 ± 0.04	192 ± 20
Δ34 LmAPX	3.2 ± 0.1	27 ± 3	6.66 ± 1	143 ± 10	2.5 ± 0.02	274 ± 20
Ascorbate-free						
Δ12 LmAPX	ND‡	203 ± 25	10.1 ± 1	15 ± 1	3.1 ± 0.04	11 ± 2
Δ34 LmAPX	3.0 ± 0.1	25 ± 2	8.2 ± 1	111 ± 11	3.0 ± 0.3	225 ± 15

* 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 K_m value for H_2O_2, 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].

H_2O_2-dependent inactivation of LmAPX

Chloroplast APX is known to be distinct from cytosolic APX with respect to H_2O_2 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 H_2O_2 that resulted in concentration and time-dependent irreversible inactivation of the enzyme following pseudo-first-order kinetics (Figures 4A and 4B). When k_{obs} values obtained from the slope of each line were plotted against H_2O_2 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 H_2O_2-dependent inactivation as compared with Δ34 LmAPX. H_2O_2-dependent inactivation of both enzymes were protected by a high concentration of ascorbate or aromatic donor (guaiacol) indicating that electron donors scavenged the preincubating H_2O_2 by reducing the compound (I) and (II) back to the native state.

Spectral properties of Δ12 LmAPX and Δ34 LmAPX with H_2O_2

Figure 5(A) shows the change in resting state of ascorbate-free Δ12 LmAPX spectrum when 20 molar excess of H_2O_2 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 H_2O_2. 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 H_2O_2 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

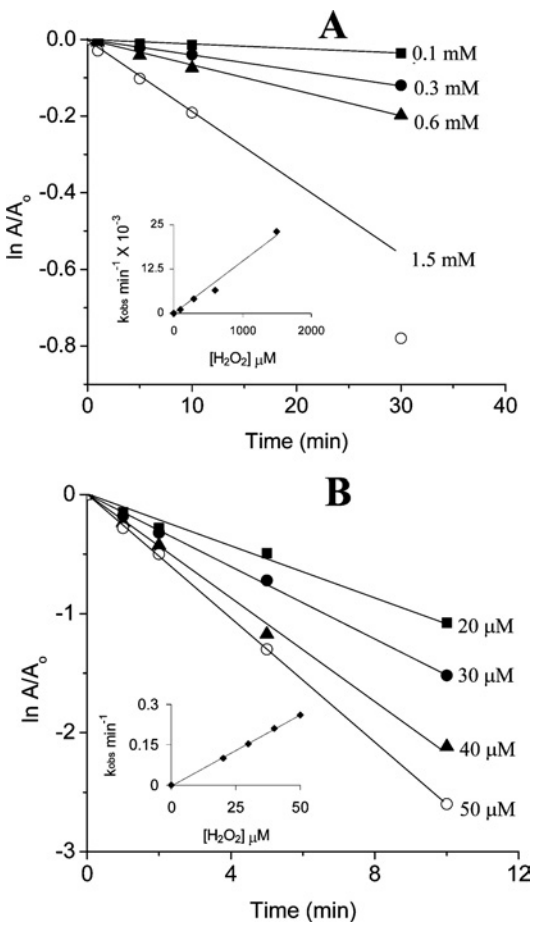

Figure 4 Kinetics of the inactivation of LmAPX by H_2O_2

(**A**, **B**) Time-dependent inactivation of Δ34 LmAPX and Δ12 LmAPX respectively. Enzyme (20 μM) was preincubated with different concentrations of H_2O_2 in 50 mM phosphate buffer (pH 7.5). Aliquots of 5 μl were assayed at the specified times for residual activity with guaiacol in 50 mM phosphate buffer (pH 7.5). Inset: k_{obs} versus H_2O_2 concentration, used for the determination of second-order rate constant of inactivation.

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 H_2O_2 to form compound (II) [via compound (I)] which subsequently reacts

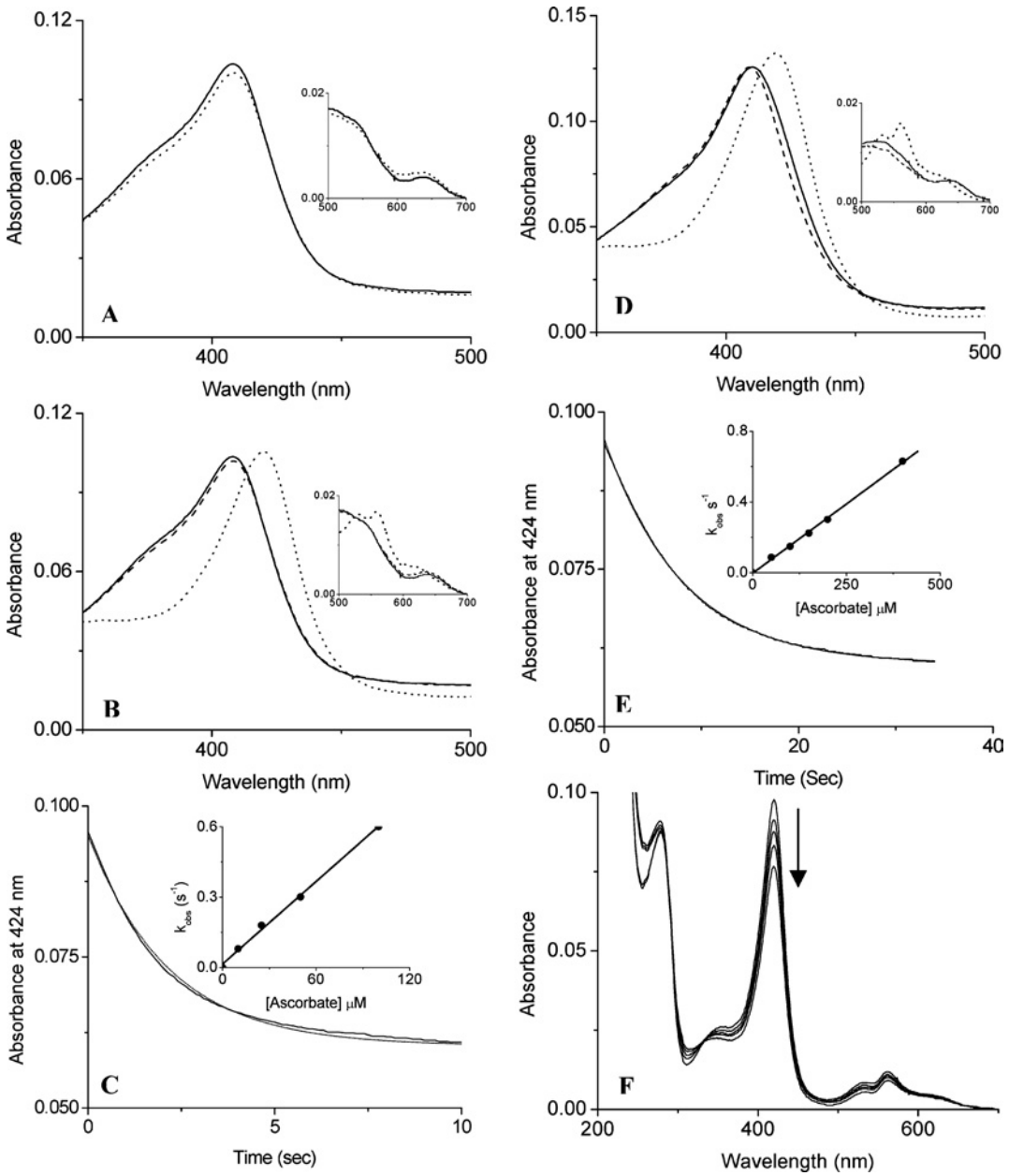

Figure 5 Spectral studies of LmAPX

(**A**) Shows the spectrum of ascorbate-free Δ12 LmAPX before (——) and after the addition of H_2O_2 (· · · · · · · · ·). (**B**) Scans of ascorbate-bound Δ12 LmAPX before (——) and after the addition of equimolar H_2O_2 at initial (· · · · · · · · ·) and after 30 s (- - - -). (**C**) Kinetic trace for Δ12 LmAPX compound (II) reduction by 50 mM ascorbate. The dotted line is a fit of the data to a single exponential function. (**D**) Scans of ascorbate-free Δ34 LmAPX before (——) and after the addition of 5-fold molar excess H_2O_2 (· · · · · · · · · ·), and after the addition of 10 μM ascorbate (- - - -). (**E**) Kinetic trace for Δ34 LmAPX compound (II) reduction by 50 mM ascorbate. The dotted line is a fit of the data to a single exponential function. (**F**) Spectrum of ascorbate-free Δ34 LmAPX in the presence of 0.5 mM H_2O_2 at different time intervals (30 s, 1 min, 2 min, 5 min and 10 min). Insets of (**A**, **B** and **D**) represent a zoom of the visible spectrum of their corresponding scans. Insets of (**C** and **E**) show plots of pseudo-first-order constants (k_{obs}) versus ascorbic acid concentration for the reduction of compound (II) derivatives of Δ12 LmAPX and Δ34 LmAPX respectively.

with ascorbate (electron donor) to form the native enzyme. The rate of compound (II) reduction by ascorbate for Δ12 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 $6.01 \pm 0.03 \times 10^3$ $M^{-1} \cdot s^{-1}$. In contrast with ascorbate-free Δ12 LmAPX, the ascorbate-free Δ34 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 H_2O_2 was added to the ascorbate-free Δ34, the native enzyme immediately formed α/β 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 Δ34 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 μM ascorbate to the compound (II) of ascorbate-free Δ34 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–H_2O_2 intermediate.

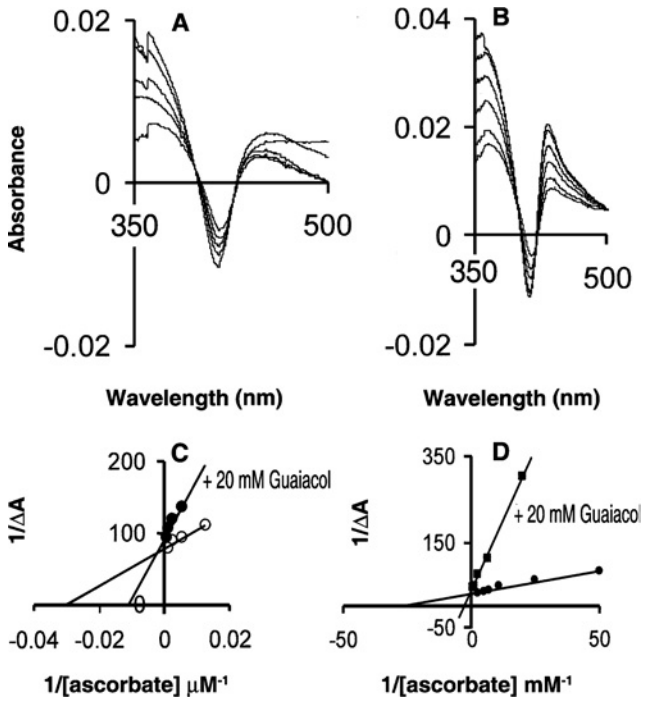

Figure 6 Difference spectra of LmAPX–ascorbate complexes

(**A**) Difference spectra of Δ12 LmAPX–ascorbate versus Δ12 LmAPX at pH 7.5. The concentration of ascorbate-free Δ12 LmAPX used was 6 μM, and the ascorbate concentrations were 80, 180, 380, 880 and 1880 mM. (**B**) Difference spectra of Δ34 LmAPX–ascorbate versus Δ34 LmAPX at pH 7.5. The concentration of ascorbate-free Δ34 LmAPX used was 10 μM, and the ascorbate concentrations used were 20, 40, 90, 140, 190 and 390 mM. (**C**, **D**) The measurement of K_d values of Δ12 LmAPX–ascorbate and Δ34 LmAPX–ascorbate respectively. The plot of 1/ΔA versus 1/[ascorbate] was used for calculating the K_d value of ascorbate in the presence or absence of 20 mM guaiacol.

A similar monophasic behaviour was observed for Δ34 LmAPX with a second-order rate constant of $1.57 \pm 0.08 \times 10^3$ $M^{-1} \cdot s^{-1}$ (Figure 5E). This rate constant is approx. 4-fold lower than that of Δ12 LmAPX, which is consistent with our ascorbate oxidation data. The ascorbate-free Δ34 LmAPX was further studied to find out why the enzyme was inactivated in the presence of high concentrations of H_2O_2. Figure 5(F) showed that the Soret haem absorbance of the compound (II) was gradually decreasing with increasing intervals of time for high concentrations of H_2O_2 with the visible peak unaltered during decreasing Soret absorbance at 420 nm. This modified enzyme could not be reduced back to the native state by adding electron donors (results not shown), indicating that compound (II) is converted into the inactive species in the presence of a high concentration of H_2O_2. Thus our spectral observation is in line with the kinetic result where Δ12 LmAPX and Δ34 LmAPX were found to be susceptible to H_2O_2-dependent irreversible inactivation.

Ascorbate-binding study

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/ΔA versus 1/[ascorbate] (Figure 6C for Δ12 LmAPX and Figure 6D for Δ34 LmAPX) was 33 μM. 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 ($K_d = 33$ μM) is significantly increased ($K_d = 100$ μM for Δ12 and $K_d = 400$ μM for Δ34 LmAPX) in the presence of guaiacol. This indicates that ascorbate interacts at a site close to the guaiacol (aromatic donor) binding site.

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.

	Spectrum of complex (nm)			
Enzyme	Minimum	Maximum	K_d (μM)	$\Delta\varepsilon_{peak\text{-}trough}$ ($mM^{-1} \cdot cm^{-1}$)
Δ12 LmAPX	413	433	33 ± 3	2.4 ± 0.2
+ Guaiacol	409	433	100 ± 9	2.2 ± 0.2
Δ34 LmAPX	413	433	33 ± 4	3.33 ± 0.2
+ Guaiacol	409	433	400 ± 25	3.33 ± 0.2

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, His^{68}, Trp^{67} and Arg^{64} 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, Asp^{253}, proximal ligand His^{192} and Trp^{208} 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 δ-site of haem [2,9,42]. When the ascorbate-binding site of LmAPX is compared with that of APX, Lys^{55} in LmAPX was found to be identical with Lys^{30} of APX but Phe^{201} appears to be substituted for Arg^{172} that reportedly interacts with the 2′-OH and 3′-OH groups of ascorbate. Indeed, Arg^{172} 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 (Asn^{216}) occupies the homologous position. The specific activity of ascorbate and guaiacol oxidation in LmAPX is lower compared

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 $k_{obs\text{-}fast}$ 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 Arg^{172} of APX, the affinity (K_d) 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 H_2O_2 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 recombinant 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 H_2O_2. This is probably because the H_2O_2 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 H_2O_2 in the absence of electron donors by kinetic analysis of their inactivation reactions, compound (III) and P670 species formation. It was found that H_2O_2 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 H_2O_2 was required for inactivation [44], whereas APX was extremely sensitive to inactivation. Although the Δ12 LmAPX is more sensitive to H_2O_2-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 H_2O_2 [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 H_2O_2-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 K_d of ascorbate binding, which is consistent with a previously reported observation [8]. Therefore the spectrally derived ascorbate K_d 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 (K_d) 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 H_2O_2 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 scavenge a large amount of H_2O_2 [16]. The removal of H_2O_2 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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