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Role of proximal methionine residues in *Leishmania major* peroxidase

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ABSTRACT

The active site architecture of *Leishmania major* peroxidase (LmP) is very similar with both cytochrome *c* peroxidase and ascorbate peroxidase. We utilized point mutagenesis to investigate if the conserved proximal methionine residues (Met248 and Met249) in LmP help in controlling catalysis. Steady-state kinetics of methionine mutants shows that ferrocytochrome *c* oxidation is <2% of wild type levels without affecting the second order rate constant of first phase of Compound I formation, while the activity toward a small molecule substrate, guaiacol or iodide, increases. Our diode array stopped-flow spectral studies show that the porphyrin π-cation radical of Compound I in mutant LmP is more stable than wild type enzyme. These results suggest that the electronegative sulfur atoms of the proximal pocket are critical factors for controlling the location of a stable Compound I radical in heme peroxidases and are important in the oxidation of ferrocytochrome *c*.

Introduction

Class I peroxidases, cytochrome *c* peroxidase (CCP), ascorbate peroxidase (APX),[1] and the gene duplicated bacterial catalase–peroxidase, are known to have origin in prokaryotes. They catalyze the peroxide-dependent oxidation of various electron donors to give two molecules of oxidized substrate and two molecules of water. The catalytically essential residues on the proximal and distal heme sides of Class I peroxidases are highly conserved. Although all enzymes are structurally similar yet the nature of physiological substrates and Compound I are fundamentally different [1–4]. This Compound I is the oxoferryl moiety (Fe(IV)=O) with a porphyrin π-cation radical or a protein-based radical species. The electronic structure of the relevant Compound I species in catalase–peroxidase is most probably an oxoferryl form in combination with a unique adduct radical [5]. APX uses porphyrin-π cation radical as a Compound I for oxidation of micromolecules (ascorbate) whereas CCP utilizes proximal tryptophan protein radical for oxidation of macromolecules (cytochrome *c*) [6]. Crystal structures reveal that the structural differences between CCP and APX in proximal site include: (a) a K^+ atom is present in APX within 8 Å from proximal tryptophan; (b) three Met residues are present in CCP within 8 Å from proximal tryptophan [7–9]. Mutational studies suggest that the absence of the positively charged K^+ ion and the presence of three Met residues (M172, M230 and M231) within 8 Å of the proximal Trp residue stabilize the Trp radical in CCP. Similarly, the K^+ containing triple Met mutant shows 99% less cytochrome *c* oxidation activity compared to wild type [9]. Indeed, other groups also have shown that Met residues act as an important controlling factor in oxidizing equivalents of CCP Compound I [10–12]. Due to the lack of ascorbate oxidation capability in CCP, previous works have been unable to show any change of the ascorbate activity after replacing the three key Met residues and introducing K^+ ion into the proximal pocket of CCP [9].

We have reported a novel functional hybrid enzyme of APX and CCP from *Leishmania major* known as *Leishmania major* peroxidase (LmP) which oxidizes both ascorbate as well as cytochrome *c* [13–16]. It plays a protective role in ROS mediated apoptosis [17,18]. Recently we have shown by using LmP knock out cell line of *Leishmania major* that LmP controls parasite differentiation and survival within macrophages by regulating the ROS content of the cell [19]. LmP has two distinct electron transfer pathways to oxidize both macro- and micro-molecules employing both tryptophan and porphyrin-π cation radical, respectively, as evidenced by the W208F LmP mutant [13]. Recently X-ray crystal structure has established the protein architecture of LmP as a structurally hybrid enzyme of APX and CCP [20] and these three different proteins have conserved proximal (His-Trp-Asp) and distal (Arg-Trp-His) triads for efficient peroxidase catalysis [1,6,14,21]. Furthermore we found that tryptophan radical stabilizing proximal methionine residues are conserved in CCP (M230 and M231) and LmP (M248 and M249) but absent in APX. Like CCP, we propose that the electronegative sulfur atoms of both M248 and M249 residues might play a role for enhancing cytochrome *c* oxidation and simultaneously they might be responsible for decreasing ascorbate or guaiacol oxidation through enhanced intra-molecular electron transfer from the porphyrin cation radical to the W208. Hence M248A, M249A, M248A + M249A, M248L + M249L and

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[1] *Abbreviations used:* APX, ascorbate peroxidase; LmP, peroxidase from *Leishmania major*; CCP, cytochrome *c* peroxidase; HS, high spin; LS, low spin; WT, wild type.

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M248C + M249C mutants were created to test this proposal. We found that cytochrome *c* turnover was decreased and guaiacol or iodide reactivity was enhanced in M248A, M249A, M248A + M249A, M248L + M249L and M248C + M249C mutant enzymes. Detailed spectroscopic, kinetic, and structural characterization of the methionine mutants have been described.

Materials and methods

Materials

All reagents and materials were obtained from Sigma or sources previously reported [14,15,22,23].

Construction of site-directed mutants

Site directed mutagenesis was done using the Quick Change polymerase chain reaction *in vitro* mutagenesis kit from Stratagene. Integrated DNA Technologies synthesized oligonucleotides used to construct site-directed mutants in LmP. Mutations and corresponding oligonucleotides are listed below, with mutagenic codons in bold. Sense M248A: 5′-GCGACGACAAAGCTG**GCG**ATGCTTCCCAGT GAC-3′; antisense M248A: 5′-GTCACTGGGAAGCAT**CGC**CAGCTTTGT CGTCGC-3′; sense M249A: 5′-GCGACGACAAAGCTGATG**GCG**CTTCC CAGTGAC-3′; antisense M249A: 5′-GTCACTGGGAAG**CGC**CATCAGCT TTGTCGTCGC-3′; sense M248A + M249A: 5′-GCGACGACAAAGCTG **GCGGCG**CTTCCCAGTGAC-3′; antisense M248A + M249A: 5′-GTCAC TGGGAAG**CGCCGC**CAGCTTTGTCGTCGC-3′; sense M248L + M249L: 5′-GCGACGACAAAGCTG**CTGCTG**CTTCCCAGTGAC-3′; antisense M248L + M249L: 5′-GTCACTGGGAAG**CAGCAG**CAGCTTTGTCGTCGC -3′; sense M248C + M249C: 5′-GCGACGACAAAGCTG**TGCTGC**CTTCC CAGTGAC-3′; antisense M248C + M249C: 5′-GTCACTGGGAAG**GC AGCA**CAGCTTTGTCGTCGC-3′. DNA sequencing at the Indian Institute of Chemical Biology core facility confirmed incorporated mutant. DNA containing the desired mutations was transformed into *Escherichia coli* for protein expression.

Expression and purification of wild-type and site-directed LmP mutants

Wild-type LmP (34 amino acids deleted from N-terminal sequence of LmP gene) and mutants containing a His_6 tag attached to their respective N terminus were overexpressed in *E. coli* strain BL21 (DE3) using a pTrcHisA vector and were purified by using Ni^{2+}–NTA resin as described previously [14,22]. UV–visible spectra were recorded in a Shimadzu-2550 spectrophotometer using 50 mM phosphate buffer pH 7.5. The heme was quantified by the pyridine haemochrome method [24] and concentrations of wild type, M248A, M249A, M248A + M249A, M248L + M249L and M248C + M249C mutant enzymes were determined from Soret peak of the heme using an extinction coefficient of 101, 95, 104, 93, 105 and 98 $mM^{-1}\,cm^{-1}$, respectively. The Rz values (A408/

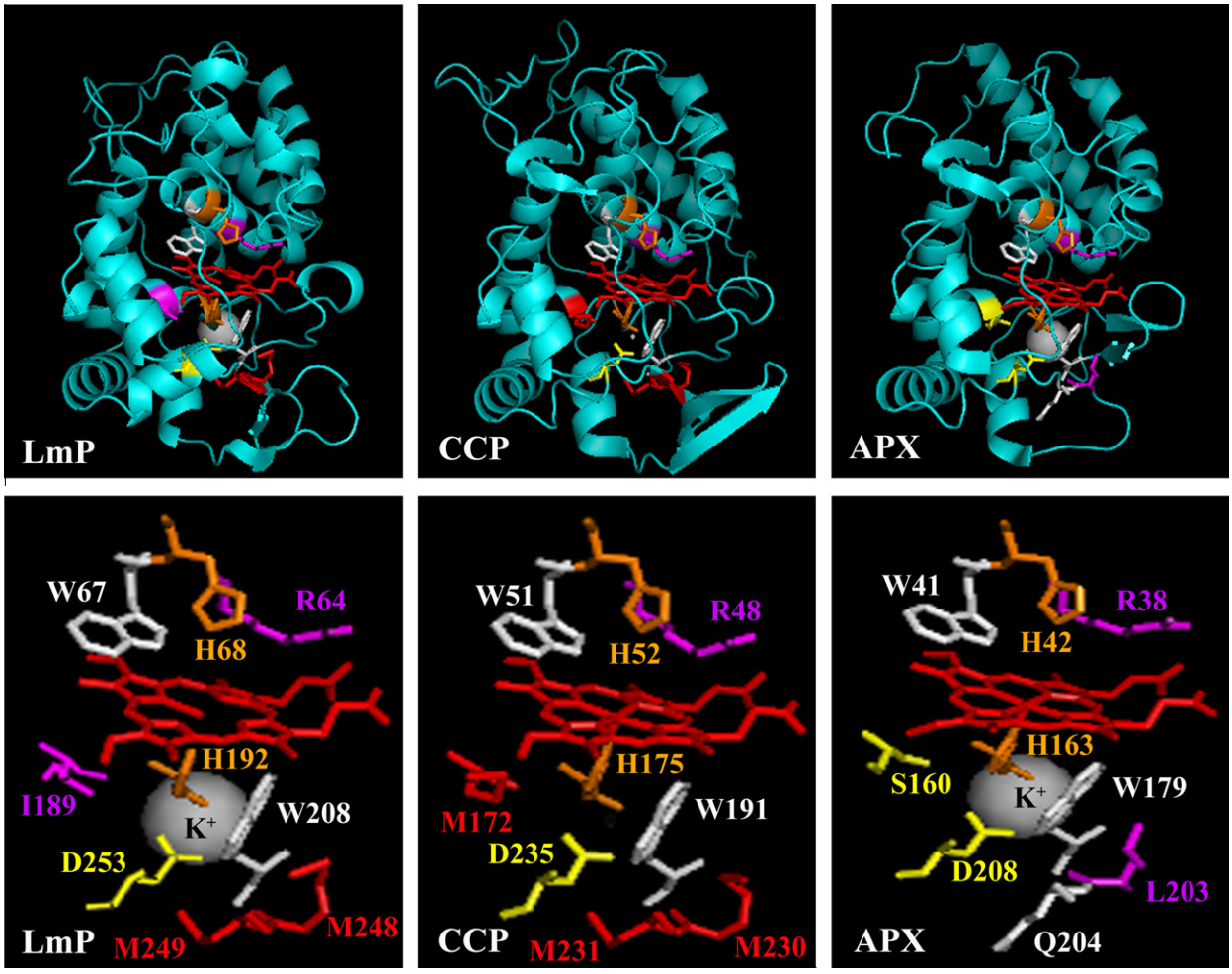

Fig. 1. The comparative study of LmP crystal structure (LmP) with both yeast CCP (CCP) and pea APX (APX). Upper panel showed cartoon structure with distal site, proximal site, electron rich methionine residue and potassium ion, where the heme of three enzymes was superimposed. The PDB coordinates were taken from published yeast CCP (PDB entry code: 2CCP), and pea APX (PDB entry code: 1APX). Structures were illustrated by using PyMOL [42] software. Lower panel is the same as upper panel except cartoon.