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Mutation of Val90 to His in the pseudoperoxidase from *Leishmania major* enhances peroxidase activity

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ABSTRACT

Pseudoperoxidase from *Leishmania major* (LmPP) catalyzes the breakdown of peroxynitrite though it can hardly react with H_2O_2. Our modeling structure predicts that a conserved His to Val switch near the distal heme pocket of LmPP may determine the profile of its H_2O_2 activity. To test this hypothesis, we have generated complementary mutations in the LmPP (V90H) and studied the formation of Compounds I and II. The rate of transition from high spin ferric state of V90H to Compound I by H_2O_2 is increased by approximately three orders relative to wild-type LmPP, which is consistent with electron donor oxidation data where the V90H mutant enzyme is ~30 fold more active than wild type. Thus, our data indicate that a lower rate for heterolytic cleavage of the O–O bond of H_2O_2 in wild type LmPP is caused by the His/Val switch in heme distal site. In the catalysis of peroxynitrite scavenging, V90H LmPP has lower catalytic activity compared to the wild type enzyme. In contrast to peroxynitrite scavenging, the second order rate constant of peroxynitrite binding step in mutant enzyme does not change significantly compared to the wild-type. Spectral data suggest that the distal Val90 residue in LmPP prevents the ferryl species formation in the presence of peroxynitrite. The lower peroxynitrite scavenging activity of the mutant reflects increased peroxidase activity rather than isomerase activity.

1. Introduction

Peroxidases play extremely diverse functions in biology [1,2]. Heme peroxidase reduces one molecule of H_2O_2 to form two molecules of water through the oxidation of reducing substrate, including aromatic compounds and iodide [3,4]. The first step of the catalytic cycle forms Compound I intermediate, which is an oxyferryl porphyrin π-cation radical (Fe^{IV}=O $\pi^{\cdot+}$) in horseradish peroxidase (HRP) [5] or oxyferryl tryptophan radical (Fe^{IV}=O Trp$\cdot^{+}$) in cytochrome c peroxidase (CCP) [6]. Generally, Compound I in HRP performs one-electron oxidation of small organic molecules (electron donors), producing the second intermediate, compound II (Fe^{IV}=O). Compound II is further reduced back to the resting ferric state by another one-electron reduction with the reducing substrate [1,3,4,7]. However, the oxidation of iodide is mediated by one two-electron transfer reaction to Compound I only [4].

The most versatile function and spectroscopic uniqueness of peroxidases has mainly been recognized to the structural components of the heme environment that designate the heme active site, specifically amino acids located in the proximal and distal pockets of heme [8]. High-resolution crystal structures and site-specific mutagenesis studies of peroxidases [9–14] indicate that the catalytic machinery required to activate H_2O_2 is highly conserved. Mostly, the key catalytic residues in the distal site of the heme peroxidases are a histidine (His42 in HRP) and an adjacent arginine (Arg38 in HRP). The histidine is thought to facilitate formation of the initial iron-peroxide complex by deprotonating the peroxide and subsequently to promote cleavage of the oxygen–oxygen bond by protonating the distal oxygen [15]. The arginine is proposed to facilitate O–O bond scission by electrostatic stabilization of dioxygen bond cleavage [15]. Ortiz de Montellano's group has proposed in earlier studies that the ferryl species of Compounds I and II are partially or fully shielded from direct interaction with substrates [16,17]. The catalytic histidine residue (His42) may be part of the proposed barrier that restricts access of substrates to the ferryl species, and a reduction in the size of this residue greatly increases the peroxygenase activity of HRP [14].

Recently, we have cloned, expressed and characterized the pseudoperoxidase from *Leishmania major* (LmPP) [18]. Co-localization studies by confocal microscopy and subsequent Western-blot analysis with anti-LmPP antibody have confirmed that the mature enzyme is present in the plasma membrane of amastigote stage of *Leishmania* [18]. Recently we have shown by using a LmPP knock out cell line of *L. major* that LmPP controls parasite survival within macrophages [18]. The activity of LmPP is higher against peroxynitrite than that in H_2O_2 suggesting that the biological function of this enzyme is to act as a scavenger of peroxynitrite [18]. Although LmPP catalyzes the oxidation of iodide, thiocyanate and guaiacol by H_2O_2, the rate of oxidation was 3.5 orders slower than HRP [18]. On the basis of homology modeling [19,20], the fundamental conformation of LmPP is found to be similar

Abbreviations: LmPP, *Leishmania major* pseudoperoxidase; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); HRP, horseradish peroxidase; CCP, cytochrome c peroxidase; $OONO^-$, peroxynitrite

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to that of peanut peroxidase [11] and the coordinates of the side chain of the heme peripheral Arg86, Phe89 and His206 residues of LmPP are identical to Arg38, Phe41 and His-169 of peanut peroxidase (Fig. 1). Moreover, a model structure of LmPP active site based on peanut peroxidase crystal is also proposed that it contains an amino acid switch (His90 to Val90), which might create lower reactivity with H_2O_2 compared with that in the plant enzymes. We hypothesized that this substitution (His90 to Val90) may explain at least partially why the oxidation rate of the electron donor is slower than that of the plant peroxidase. We therefore generated mutations in LmPP (V90H) and studied the Compound I formation and measured the activity of iodide or guaiacol oxidation by H_2O_2. Our findings suggest that the His/Val switch created structural differences that altered peroxidase activity in LmPP.

2. Materials and methods

2.1. Materials

Ni^{2+}-nitrilotriacetate resin, potassium iodide, guaiacol and imidazole were obtained from Sigma-Aldrich. The sources of other reagents were described previously [18,21–23]. Peroxynitrite (Calbiochem, La Jolla, CA) was purchased as an aqueous solution (180–200 mM) and stored frozen at −80 °C until use. The stock solution was diluted with 0.005 M NaOH, and the peroxynitrite concentration was determined spectrophotometrically before each experiment by measuring the absorbance at 302 nm ($\varepsilon_{302} = 1705\ M^{-1}\ cm^{-1}$).

2.2. Mutagenesis

Site-directed mutagenesis of LmPP DNA in the pET15B expression plasmid (coding for amino acids 48–346 plus a six-His tag at the N terminus) was performed using the QuikChange site-directed mutagenesis kit from Stratagene. The mutation codon (bold) was incorporated into the primers as follows: sense V90H, 5′-CATTCGAACCGCGTTT**CAC**TTG GCGGCGCGCCGTGC-3′; antisense V90H, 5′-GCACGGCGCGCCGCCAA**G TG**AAACGCGGTTCGAATG-3′. The mutations were confirmed at the molecular biology core facility of the Indian Institute of Chemical Biology.

2.3. Protein expression and purification

Wild-type and mutant enzymes were overexpressed in *Escherichia coli* BL21 D3 and purified using Ni^{2+}-nitrilotriacetate affinity chromatography as reported previously [18]. Concentrations of LmPP and V90H enzymes were determined from the 423 nm absorbance of the heme, using an extinction coefficient of 69 $mM^{-1}\ cm^{-1}$ and 85 $mM^{-1}\ cm^{-1}$, respectively.

2.4. Peroxidase assay

H_2O_2-dependent iodide and guaiacol oxidation by LmPP (wild type and mutant) were assayed as described earlier [18]. In short, 0.5 μM enzymes were incubated with 2.0 mM iodide or 20 mM guaiacol at 50 mM phosphate buffer pH 5.5 in a final volume of

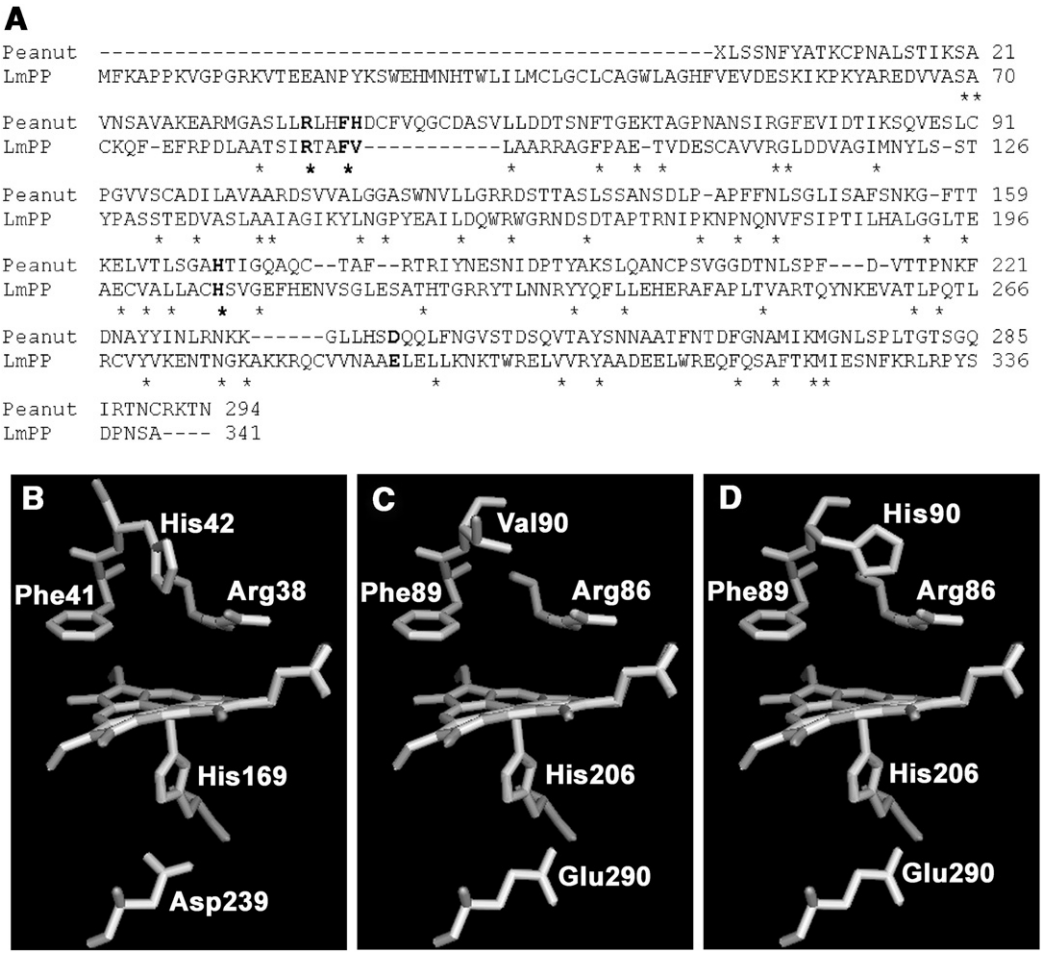

Fig. 1. Structural model is showing position of Val90 in LmPP relative to the His42 in peanut peroxidase. Panel A, the sequence of LmPP was aligned with peanut peroxidase. The residues that were identical with peanut peroxidase sequence were denoted by asterisk. Bold letters denoted active site residues. Panels B, C and D represent the crystal structure of peanut peroxidase, model structure of wild type LmPP and V90H mutant LmPP, respectively. The active site residues of distal site and proximal site of the heme are numbered. Based on the published X-ray crystallographic structures of peanut peroxidase (PDB entry code: 1sch), we constructed a three-dimensional model by homology modeling. Prediction of the three-dimensional structure of LmPP was done by knowledge-based homology modeling using the SWISS MODEL WORKSPACE [19] and PyMOL [35] software.