Biochimica et Biophysica Acta 1834 (2013) 2057–2063

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Effect of distal His mutation on the peroxynitrite reactivity of *Leishmania major* peroxidase

Rina Saha, Moumita Bose, Sumit Sen Santara, Jayasree Roy, Rajesh K. Yadav, Subrata Adak *

Division of Structural Biology and Bio-informatics, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata, 700 032, India

ARTICLE INFO

Article history:
Received 9 April 2013
Received in revised form 21 June 2013
Accepted 24 June 2013
Available online 2 July 2013

Keywords:
Leishmania
Heme protein
Peroxidase
Peroxynitrite
Steady-state catalysis
Rapid kinetics and mutation

ABSTRACT

The conserved distal histidine in peroxidases has been considered to play a major role as a general acid-base catalyst for heterolytic cleavage of an O – O bond in H_2O_2. However, heme peroxidases react with peroxynitrite to form transient intermediates but the role of the distal histidine in this reaction is still unknown. In order to investigate catalytic roles of the histidine at the distal cavity, two *Leishmania major* peroxidase (LmP) mutants (H68E, H68V) were prepared. The rate of transition from ferric H68V to Compound ES by H_2O_2 is decreased by approximately five orders of magnitude relative to wild type, which is consistent with electron donor oxidation data where the H68V is ~1000 fold less active than wild type. In the reaction with peroxynitrite, the formation rate of intermediates in the mutants is not significantly lower than that for the wild type, indicating that the His68 has no major role in homolytic cleavage of an O – O bond in peroxynitrite. EPR spectroscopic data suggest that the transient intermediates formed by the reaction of LmP with H_2O_2 exhibits an intense and stable signal similar to CCP Compound ES whereas in case of the reaction with peroxynitrite, this signal disappears, indicating that the transient intermediate is Compound II. Rapid kinetics data suggest that the distal His68 mutants display higher decay rates of Compound II than wild type. Thus, His68 mutations minimize Compound II formation (inactive species in peroxynitrite scavenging cycles) by increasing decay rates during the steady state and results in higher peroxynitrite degrading activity.

1. Introduction

NO rapidly reacts with the superoxide anion (O_2^-) to produce peroxynitrite ($ONOO^-$) that is a potent oxidizing and nitrating agent [1]. Numerous biological compounds present in physiological systems have been shown to be altered by $ONOO^-$ [2]. DNA, protein and membrane lipids are the sensitive biotargets for $ONOO^-$mediated oxidative damage [3–5]. $ONOO^-$ has been shown to be a critical factor in several inflammatory disorders, including atherosclerosis, rheumatoid arthritis, myocardial dysfunction and autoimmune diabetes [6–10]. Thus, it is essential to find out the scavenger of $ONOO^-$ within the cell. CO_2 rapidly scavenges $ONOO^-$ in biological system but its reaction product ($ONOOCO_2^-$) is a stronger nitrating agent than peroxynitrous acid [11,12]. However, several reports suggest that the selenocysteine-containing glutathione peroxidase and peroxiredoxin catalyzes the detoxification of $ONOO^-$ [13,14].

Several groups of researchers have shown that $ONOO^-$ rapidly reacts with heme containing proteins including hemoglobin [15], myoglobin [16], peroxidases [17], pseudoperoxidase [18], nitric oxide synthase [19], catalase [17], cytochrome *c* [20], cytochrome P450 [21], and cytochrome *c* oxidase [22]. The synthetic ferric iron porphyrins have been shown to catalyze the isomerization of $ONOO^-$ to nitrate in vitro [23,24] and to be cytoprotective against $ONOO^-$ in vivo [25]. In addition, kinetic studies of the reaction of $ONOO^-$ with metmyoglobin and methemoglobin suggest that the ferric iron forms of these proteins catalyze the isomerization of $ONOO^-$ to nitrate [15,16,26]. Kinetic evidence suggests that metmyoglobin reacts with $ONOO^-$ to form ferryl myoglobin and NO_2 [27] via initial formation of a caged radical intermediate [Fe^{IV} = O•NO_2] and this caged pair reacts mainly via internal return to form metMb and NO_3^- in an oxygen rebound scenario. Unlike the synthetic ferric iron porphyrins, metmyoglobin and methemoglobin, $ONOO^-$ reacts with various peroxidases and converts native enzymes to the quasi-stable and catalytically inactive form of Compound II [17]. Thus, it is quite clear that the nature of the intermediate has been an important point of debate in the synthetic ferric iron porphyrins and heme proteins work, which remains to be solved unambiguously.

Heme peroxidases rapidly react with H_2O_2 to produce Compound I or Compound ES that contains Fe^{IV} = O and a cation radical [28–31]. The initial step is believed to be the formation of peroxidase–peroxo complexes [32] that undergo very rapid heterolytic O – O bond cleavage with the help of a widely conserved distal histidine [33–35]. Similarly, the reaction of the Fe^{III} state of heme peroxidase with $ONOO^-$ produces transient intermediates [17] but nothing is known regarding the role of the active site residues of peroxidase in $ONOO^-$ dependent transient

Abbreviations: LmP, *Leishmania major* peroxidase; HRP, horseradish peroxidase; CCP, cytochrome c peroxidase; $OONO^-$, peroxynitrite; Compound I, Fe(IV) = O heme with a porphyrin radical; Compound ES, Fe(IV) = O heme with an amino acid radical

* Corresponding author. Tel.: +91 33 2473 6793; fax: +91 33 2473 5197.
E-mail address: adaks@iicb.res.in (S. Adak).

1570-9639/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbapap.2013.06.018

intermediates formation. However, pseudoperoxidase from *Leishmania major* (LmPP) lacking the distal histidine displays lower peroxidase activity and higher $ONOO^-$ decomposition activity than the V90H mutant of LmPP [18].

In the present work, mutagenesis is used to simplify the mechanism of heterolytic and homolytic O–O bond cleavage in H_2O_2 and $ONOO^-$, respectively, by creating a *L. major* peroxidase mutant that lacks the distal His residue. The mutations of distal His-68 to Val and Glu display 3–5 orders of magnitude decreased rate of the heterolytic O–O bond cleavage in H_2O_2 but do not significantly change the rate of the homolytic O–O bond cleavage in $ONOO^-$. Our present experiments have indicated that the rate of $ONOO^-$ decomposition is increased by distal His mutation. A detailed characterization of the $ONOO^-$ decomposition reaction of the wild type LmP, His68Val and His68Glu mutant is reported here.

2. Materials and methods

2.1. Materials

$ONOO^-$ was obtained from Calbiochem, La Jolla, CA, 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 concentration of $ONOO^-$ was measured spectrophotometrically before each experiment by measuring the absorbance at 302 nm ($\varepsilon_{302} = 1705\ M^{-1}\ cm^{-1}$). Horse cytochrome c, potassium iodide, guaiacol and imidazole were obtained from Sigma-Aldrich. The sources of other reagents were described previously [18,36–38].

2.2. Molecular biology

Site-directed mutagenesis of Δ34LmP DNA in the pTrcHisA expression plasmid (coding for amino acids 34–303 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 H68V, 5′-CGCCTCGCATGG**GTG**GAGGCCGCCTCG-3′; antisense H68V, 5′-CGAGGCGGCCTC**CAC**CCATGCGAGGCG-3′; and sense H68E, 5′-CGCCTCGCATGG**GAG**GAGGCCGCCTCG-3′; antisense H68E, 5′-CGAGGCGGCCTC**CTC**CCATGCGAGGCG-3′. The mutations were confirmed at the molecular biology core facility of the Indian Institute of Chemical Biology.

2.3. Expression and purification of wild-type and mutant LmP

Wild-type and mutant enzymes were overexpressed in *Escherichia coli* BL21 D3 and purified by using Ni^{2+}-nitrilotriacetate affinity chromatography as reported earlier [39]. Concentrations of wild type, H68V and H68E enzymes were determined from the 406 nm absorbance of the heme, using an extinction coefficient of 101, 104 and 110 $mM^{-1}\ cm^{-1}$, respectively.

2.4. Peroxidase activity measurement

H_2O_2-driven ascorbate, guaiacol and cytochrome c oxidation by LmP (wild type and mutant) were assayed as described earlier [39]. In short, 0.1 μM enzymes were incubated with 0.5 mM ascorbate or 20 mM guaiacol at 50 mM phosphate buffer pH 7.5 in a final volume of 1.0 ml. In case of cytochrome c oxidation, 10 nM enzyme was incubated with 50 μM ferrous horse cytochrome c at 50 mM phosphate buffer pH 7.5. Reactions were initiated by adding 0.3 mM H_2O_2. The rate of ascorbate, guaiacol or cytochrome c oxidation was determined from the change of absorbance at 290 nm, 470 nm, or 550 nm respectively. The concentrations of oxidized ascorbate, guaiacol and cytochrome c were determined from $\varepsilon_{290} = 2.8 \times 10^3\ M^{-1}\ cm^{-1}$, $\varepsilon_{470} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$ and $\varepsilon_{550} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$, respectively.

2.5. Rapid scanning experiments by stopped-flow spectrophotometry

The time-resolved spectra were collected from 340 to 700 nm on Hi-Tech stopped-flow instrument (KinetAsyst), using a rapid scanning diode array device (Hi-Tech MG-6560) designed to record 300 complete spectra within 450 ms in each mixing. Rapid scanning experiments involved mixing solutions containing high spin enzyme in 50 mM phosphate buffer, pH 7.5 with varying concentrations of $ONOO^-$ or H_2O_2 solutions at 25 °C. Kinetics of the formation of the transient intermediates was measured at 420 nm and signal-to-noise ratios were improved by averaging 10 individual traces. Average traces were fit to single exponential functions using software provided by the instrument manufacturer. Second order rate constants for the transient intermediate formation of LmP were obtained by the plotting of k_{obs} versus varying concentrations of $ONOO^-$ or H_2O_2.

The decay rates of $ONOO^-$ in the presence of wild type and mutants were studied with the stopped-flow instrument. The mixing time of the instrument is about 0.75 ms. The kinetic traces were collected at 340 nm instead of 302 nm for the decay rate of $ONOO^-$ [18]. The molar extinction coefficient of $ONOO^-$ is 868 at 340 nm and 1705 at 302 nm. For the detection of transient intermediates, the protein solutions in the presence of 100 mM phosphate buffer pH 6.0 or 7.5 were mixed with varying concentrations of $ONOO^-$. The kinetic traces were collected at 421 nm for the formation rate of transient intermediates. The results of the fits of the traces (averages of at least 10 single traces) from at least three experiments were averaged to obtain each observed rate constant, given with the corresponding standard deviation. $ONOO^-$ solutions were prepared by diluting the stock solution instantly before use with 0.005 M NaOH to get the required concentration.

2.6. Electron paramagnetic resonance measurements

X-band EPR spectra were measured on a Jeol (JESFA 200) apparatus. LmP (0.4 mM) was dissolved in 50 mM phosphate buffer (pH 7.5) with H_2O_2 (0.4 mM) or $ONOO^-$ (0.8 mM) for the measurement of EPR. EPR samples were prepared by combining 0.4 mM enzyme with 0.4 mM H_2O_2 or 0.8 mM $ONOO^-$. Those samples were immediately transferred to an EPR tube and flash frozen in liquid nitrogen within 3 min.

3. Results

3.1. Mutant LmP purification and spectroscopic characterization

LmP mutants were purified in absence of electron donor and typically yielded about 10 mg per liter of culture that was comparable to our yield of wild-type LmP expressed in the same system. The H68V and H68E LmP used in our experiment were purified to homogeneity and had the same molecular mass as wild-type LmP (data not shown). The UV–visible spectra of both His68 mutants showed the presence of a Soret peak at 406 nm with visible peaks at ~500 and 640 nm. Spectroscopic data revealed that both His68 mutants contained heme in a predominantly high spin state, which closely resembles that of wild type (Fig. 1A–C). The H_2O_2 mediated spectral changes were studied by rapid scan UV/vis spectroscopy between 340 and 700 nm at pH 7.0 and 25 °C to obtain a spectrum of the intermediate over the entire wavelength range. As shown in Fig. 1A–B, the Soret bands of wild type and H68E mutant LmP shifted from 406 nm to 420 nm. The final spectrum in H68E mutant was reminiscent of Compound ES of the wild type LmP due to α and β band in visible region at 560 and 532 nm [36]. These results are consistent with previous CCP and HRP results [40,41]. In contrast with the wild type LmP, H_2O_2 is unable to shift the Soret band from 406 nm to 420 nm in the H68V mutant upon addition of H_2O_2 at 50 mM phosphate buffer pH 7.0 (Fig. 1C). Although H68E and H68V mutants react with 20 equivalent of H_2O_2 with increased absorbance at 421 nm, the change in absorbance at 421 nm during the reaction with H_2O_2 is 88% for H68E and 9% for H68V compared to wild