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Role of Some Metal Ions on Steady–state Kinetics of Engineered Wild–type and Manganese (II) Binding Site Mutants of Recombinant *Phlebia radiata* Manganese Peroxidase 3 (rPr-MnP3)

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Abstract This study investigated the steady-state kinetics of engineered wild-type and manganese (II) binding site mutants of recombinant *Phlebia radiata* manganese peroxidase 3(rPr-MnP3). The effect (activation or inhibition) of some metal ions (Co^{2+}, Zn^{2+} Cu^{2+} and Na^{+}) on the activity of rPr-MnP3 enzymes was also studied. The results obtained showed that the rPr-MnP3 mutants in which the metal binding functionality has been largely lost have been created. Na^{+} (mono-valent ion) and Co^{2+}showed similar characteristics by exhibiting stimulatory effects on the activity of wild-type rPr-MnP3. However, Cu^{2+} and Zn^{2+} had mixed inhibitory effects on wild-type and mutants (E40H, E44H, E40H/E44H). It was observed that Cu^{2+} was by far the strongest inhibitor of engineered rPr-MnP3 enzymes while Co^{2+} exhibited a non-competitive inhibitory effect on the double mutant (E40H/E44H) and D186H activities. In addition, Zn^{2+} and Cu^{2+}also had non-competitive inhibitory effect on D186H mutant enzyme activity. The results obtained further showed that the competitive inhibitory effect of Cu^{2+}observed in other rPr-MnP3 enzymes is largely removed in D186H mutant enzyme. Generally, histidine substitution retained a strong selectivity for Cu^{2+} as competitive inhibitor. Zn^{2+} being generally non-competitive suggest involvement of sites other than the Mn (II) binding site. This study showed that rPr-MnP3 enzymes function with alternate ligands in the Mn^{2+} binding site and does not have absolute obligate requirement for all carboxylate ligand set.

Keywords: *peroxidase, Phlebiaradiata, steady-state, wild-type, mutants, metal ions, inhibitors*

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1. Introduction

A white-rot causing corticoid basidiomycete *Phlebia radiata* functions as an effective degrader of natural and synthetic lignins, lignin modelled compounds and various xenobiotics in the environment [1-7]. According to Hatakka and Hammel [6], white-rot basidiomycetous fungi are the principal organisms for the degradation of wood lignocellulose with a remarkable ability to effectively degrade and/or mineralize the recalcitrant lignin polymer. The unique ability of white rot fungi to degrade lignin is largely attributable to the main lignin modifying enzymes produced by white rot fungi [7]. *Phlebiaradiata*, a white-rot wood-decaying fungus, expresses and secretes multiple extracellular lignin modifying enzymes (LME), class II type of heme–containing peroxidases [8]including three isoenzymes of manganese peroxidase (MnP, EC 1.11.1.13) and three lignin peroxidases (LiP, EC 1.11.1.14), as well as two multicopper laccases (EC 1.10.3.2) [7,9,10,11,12]. Manganese peroxidase (MnP), a heme–containing enzyme belonging to the plant peroxidase like protein superfamily was first discovered in the white-rot fungus *Phanerochaete chrysosporium* [13,14,15]. This extracellular oxidoreductase class II fungal peroxidase has also been found in most of the wood lignin and litter-decaying basidiomycetes [7,12,16].

The manganese peroxidase (MnP), which is an effective degrader of a variety of environmental aromatic pollutants [14,17], has received considerable attention with respect to its ability to degrade lignin over the past years [18,19,20]. MnP occurs as a series of isoenzymes encoded by a large number of genes giving rise to the three different types of fungal MnP enzymes that have been structurally characterised [12]. These isoenzymes include: (i) long-tail type of MnPs found in *Phanerochaete chrysosporium* and in *Phlebia radiata* (Pr-MnP2 enzyme) [21,22], (ii) short-tail type of MnPs which are frequently detected as gene models in fungal genomes, but first isolated and described in *Phlebia radiata* (rPr-MnP3)

[10,22] and (iii) the versatile peroxidase (VP, 1.11.1.17) which is structurally similar to the short-tail type MnP, but enzymatically displaying hybrid activity for both Mn^{2+} ions and veratryl alcohol [23]. Evidence from the crystal structure of MnP from *Phanerochaete chrysosporium* showed that the manganese binding site in MnP is situated near the internal propionate of the heme [24,25,26]. The Mn (II) ion is coordinated to one oxygen atom of the carboxylic group of this propionate. The Mn^{2+} coordination sphere is further completed by 3 other oxygen from 3 different carboxylate groups-the side chains of acidic residues Glu35, Glu39 and Asp 179 and by two water molecules, thereby giving a six-coordinate octahedron.

The coordination between Mn^{2+} and its ligands to form octahedral complexes is typical of Mn^{2+} complexes [27,28,29]. The presence of specific ligands [28] is an indication of conservation of the topology required for Mn (II) coordination and for a similar role in catalysis [24, 30]. The MnP is a heme–containing glycoprotein that uses manganese as the reducing substrate, which is its unique feature among other peroxidase. Themain physiological function of MnP is the oxidation of Mn^{2+} to Mn^{3+}ions [14,31,32].The MnP oxidizes the one-electron donor Mn^{2+} to Mn^{3+} as shown schematically in the set of chemical equations below:

$$MnP+H_2O_2 \rightarrow MnPI+H_2O$$

$$MnPI+Mn^{2+} \rightarrow MnPII+Mn^{3+}$$

$$MnPII+Mn^{2+} \rightarrow MnP+Mn^{3+}+H_2O$$

Mn^{3+} produced enzymatically is believed to form a diffusible oxidant complex with dicarboxylic acid chelators such as oxalate which is also secreted by the fungus [32,33,34,35]. The Mn^{3+}organic acid complex then oxidizes phenolic substrates, including lignin substructure model compounds [36] and aromatic pollutants [14,17,37,38], as well as possible mediator molecules [37,38]. The conformation and function of proteins depend significantly on many factors such as amino acid sequence and interacting cofactors [39,40]. It is widely known that metals in protein structure play significant role in influencing catalysis, transportation and storage [41,42].

Transition metals cations such as Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Co^{2+} have the strongest coordination interactions, and thus occur in metalloproteins [43]. Depending on their environment, transition metals provide various redox potentials as well as coordination sidesand that is the reason why enzymesemploy metal ions or organic ligands carrying metal ions such as heme groups. It has been shown that a variety of bulk and trace metals are integral to the actions of one or more enzyme systems; or at the least have known activating effects [31]. However, the physiological significance of many of these observations may be questioned as one enzyme may be activated by a specific set of metal ions, but not by any other metals.Further, ions of a given horizontal group of the periodic table (Mn^{2+}, Co^{2+}, $Cu^{2+}$and Zn^{2+}) unexpectedly differ in their ability to replace each other in activation or inhibition of a metalloenzyme. As one moves from left to right in a given period of the Periodic Table, the chemical properties of the elements slowly change. The variations in mass, ionicradius and ion potential (which to some extent determines ability to form coordination complexes in this group of transition metals) are small [44]. This observed difference in specificity is quite significant such that antagonistic pairs of ions are best sought within a given horizontal group.

Transition metals cations such as Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} are members of the transition series which are closely related [45] and according to Irving-Williamsorder, almost every ligand the stability of its complexes increases in the order: Mn^{2+}< Fe^{2+}< Co^{2+}< Ni^{2+}< Cu^{2+}> Zn^{2+} [46]. Although theyhave equal charge, these metal ions have only slight differences in atomic weight, ionic radius and potential, and mobility. These metal ions are capable of forming coordination complexes with a variety of organic functional groups, which maybe ionic covalent type. Although $Mn^{2+}$is regarded as the physiological activating ion for MnP [17,47], the extent of physiological activation by the other ions is not clearly understood [31,48]. Therefore, the physiological control of metalloenzymes activity may be affected by availability of metal ion and trace metal antagonisms [41]. Manganese have been widely studied with regard to its role as an important cofactor of several electron-transfer metalloproteins [49,50], however, there is paucity of information in terms of the mechanism by which manganese binds to MnP during catalytic turnover. Therefore, it is essential to effectively understand characterisation of recombinant *Phlebia radiata* manganese peroxidase (rPr-MnP3) function, and the rate of rPr-MnP3 activation or inhibition may depend on the configuration and stability of coordination complexes of the metallic ion with substrate or protein.

Enzyme inhibition or activation is usually extensively analysed due to its great interest both in the study of enzyme mechanisms [43] and in pharmacological studies [44]. Despite considerable progress with regard to the study on the binding site for Mn (II) and the catalytic mechanism of MnP, the precise nature of the effect of other divalent metal ions is still unclear. To further investigate these interactions, $NaSO_4$was used as control in this present study, in addition to Zn^{2+}, Cu^{2+}, and Co^{2+} as effectors. To the best of our knowledge, there has been no study conducted on the possibility of replacement of Mn (II) by alternative divalent metal ions at the Mn (II) binding site. In this present study, the influence of divalent metal ions on the steady-state kinetics of rPr-MnP3 have been investigated. In addition, the Mn (II) ligands for the engineered recombinant *Phlebia radiata* MnP3 enzymes Glu40, Glu44 and Asp186 (Figures 1A & B)were evaluated. Furthermore, this studyreports the first comprehensive study of the reactions of a novel MnP (engineered rPr-MnP3) with some divalent metal ions (Cu^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+}). The differences in the steady state enzyme kinetics of rPr-MnP3 enzymes in the presence of divalent metal ions have been discussed as an attempt to compare the metal ions sensitivity.

2. Materials and Methods

2.1. Materials

The complete MnP3 gene of *Phlebia radiata* strain 79 (ATCC 64658) was generously provided by Dr.Taina Lundell, Department of Food and Environmental Sciences, Division of Microbiology, University of Helsinki, Finland. The Gene Bank accession number for the cDNA encoding

peroxidase Pr-MnP3 is AJ566200. The Pr-MnP3 cDNA was present in vector pCR2.1.TOPO. The *Escherichia coli* expression vector pFLAG1 was obtained from International Biotechnologies Inc, UK. Peroxidases, wild-type, E40H, E44H, E40H/E44H, DI86H and 186N (RZ = 5.6, 2.1, 4.5, 5.2, 4.4 and 5.0, respectively) were produced, activated and purified based on the procedure described by Ufot and Akpanabiatu [51]. All chemicals used in this study were obtained from Sigma–Aldrich, UK and Fisher Scientific, UK. Restriction enzymes were supplied by NEBiolabs, UK. All spectroscopic measurements in this study were carried out using ultraviolet spectrophotometer (UV-2401 PC, Shimadzu Scientific Instruments, Addison, IL).

2.2. Steady-state Kinetic Analysis of rPr-MnP3 Activity with Manganese (II)

Mn (II) is assumed to be the *in vivo* substrate for manganese peroxidase. A set of apparent steady-state kinetic constants for Mn (II) to Mn (III) oxidation for each of the engineered *Phlebiaradiata* MnP3 enzymes was obtained by measuring the initial rates of assays at 238 nm for varying $MnSO_4$ concentrations: 0.02 - 1.0 mM for wild-type, D186H and D186N mutants and 1 – 35 mM for E40H, E44H, and E40H/E44H mutants. The concentration of $MnSO_4$ was increased for E40H, E44H and E40H/E44H enzymes since the concentration used for the wild-type, D186H and D186N could not produce detectable activity. Enzyme concentrations of 0.8nM and 0.2µM were used for wild-type and mutants, respectively. The assay buffer was 100 mM Na tartrate, pH 5.0 and 8.0 for wild-type and mutants, respectively. All assays were performed with a fixed 0.1mM hydrogen peroxide at 25°C using ultraviolet spectrophotometer (UV-2401 PC, Shimadzu Scientific Instruments, Addison, IL). In this study, the product being detected was actually a Mn (III) tartrate complex with ε_{238} = 6.5 mM^{-1} cm^{-1} [32]. To ensure quality control, best laboratory practices were adopted [52,53] and all measurements were triplicate determinations.

2.3. Steady-state Kinetic Analysis of rPr-MnP3 Activity with ABTS

ABTS[2,2'-azino-bis(3-ethylbenzthiozoline-6-sulphonic acid)] is a commonly used peroxidase substrate with very high levels of activity shown by plant peroxidases [41]. ABTS assays for wild-type rPr-MnP3 were carried out in 100 mM Na tartrate buffer, pH 3.0, with a fixed H_2O_2 concentration of 0.1mM, 0.01µM enzyme and ABTS final concentration of 3.5 mM for the wild-type enzyme while ABTS concentration range1 – 6.5 mM was used for the mutants variants. Formation of the radical product as a function of time was measured, at 25°C, by monitoring the increase in absorbance at 414 nm. The initial rates were determined using ε_{414} = 36.8 $mM^{-1}cm^{-1}$ [41] and allABTS assays measurements were triplicate determinations.

2.4. Sensitivity of Mn (II) and ABTS Oxidation by Engineered *Phlebia radiata* Wild-type and Mutant MnP3 Enzymes to Some Metal Ions

To study the effects (activation or inhibition) of some divalent metal ions [Co (II), Zn (II) or Cu (II)] and a monovalent metal ion [Na (I)] on the activity of engineered wild-type and mutants *Phlebia radiata* MnP3 enzymes using Mn (II) and ABTS as substrates, the respective assay mixtures were constituted as described above and metal ions were added at a fixed concentration: 0.8 mM Zn (II), 0.8mM Co (II) or 0.2 mM Cu (II), as well as 0.8 mM Na (I).

For all assays, initial rate for each assay was determined in triplicate for each substrate concentration and then converted to turnover numbers. Data analysis, plotting and manipulations were carried out using statistical software – SigmaStat®, Version 3.5 (Systat Software Inc., USA) and graphing software package – SigmaPlot®, Version 12.5 (Systat Software Inc., USA). From the K_m (Michaelis-Menten constant) and k_{cat} obtained, specificity constants (k_{cat}/K_m) were calculated.

3. Results and Discussion

Genes encoding different MnP isozymes of *Phlebia radiata* have been isolated [22] and structurally, Pr-MnP3 protein resembles versatile peroxidase (VP) and lignin peroxidase (LiP), having a shorter C-terminal tail (Figure 1A). The production of recombinant proteins and their variants by site–directed mutagenesis is a pre-requisite for structure function studies. The recombinant *Phlebia radiata* manganese peroxidase 3 (rPr-MnP3) was engineered, expressed, refolded, purified and preliminary characterized [51,54]. The polymerase chain reaction (PCR)based whole plasmid amplification method was used to create site–directed mutations in which Glu 40, Glu 44 (were converted to His) and D186 (converted to His and Asn) the potential Mn (II) binding ligands in rPr-MnP3 [51,54]. This was done to probe the precise geometry of the Mn ligands within the binding site of rPr-MnP3 and also the flexibility of the Mn (II)binding site, with regard to the incorporation of other metal ions. The involvement of these residues in maintaining the catalytic activity of the enzyme and the structural integrity of the active site will be later discussed.

3.1. Steady-state Kinetic Analysis of Mn (II) and ABTS Oxidation by Wild-type and Mutants MnP3 from *Phlebiaradiata*

The steady-state kinetic parameters for Mn (II) oxidation by *Phlebiaradiata* MnP3 enzymes were determined as a function of Mn (II) and ABTS concentration. The residual kinetic parameters were calculated based on the control without adding any metal compound (set as 100%). Figure 2 (A & B) showed the results of the initial rates of Mn (II) and ABTS oxidation at different substrates concentrations. The plots depict the usual Michaelis-Menten kinetics and the kinetic parameters for Mn (II) and ABTS oxidation are as presented in Table 1. The apparent K_m and k_{cat} values of wild-type rPr-MnP3 for Mn (II) were found to be 0.17 ± 0.01mM and 175 ± 3.0 s^{-1}, respectively. The rPr-MnP3 variants had K_m values for Mn^{2+}, 65-fold (E40H), 50-fold (E44H), 117-fold (E40H/E44H), 139-fold (D186H) and 178 - fold (D186N) higher than that for the wild-type rPr-MnP3 (Table 1). The k_{cat}values for all mutants enzymes (E40H, E44H, E40H/E44H, D186H and D186N) were ~

93 % lower compared to that of the wild-type rPr-MnP3. Subsequently, the corresponding catalytic efficiencies for the mutant enzymes for Mn (II) oxidation are much lower compared to the wild-type rPr-MnP3.

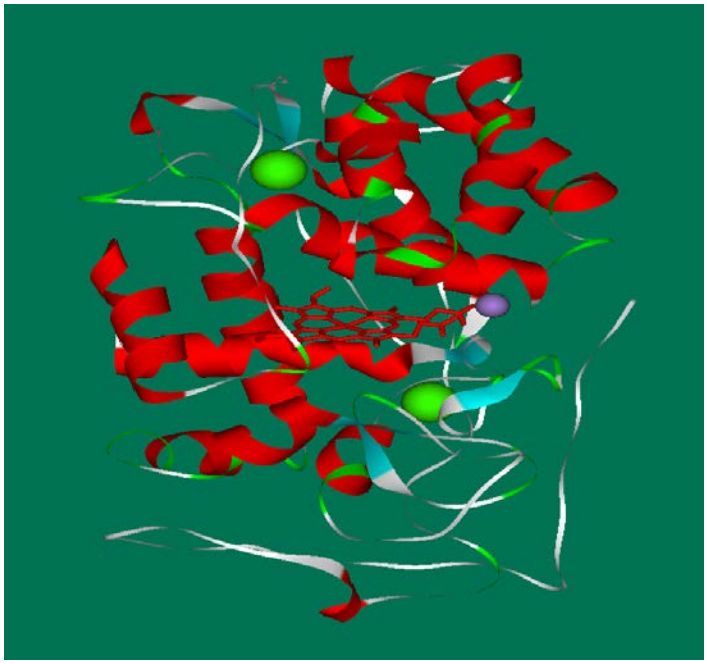

Figure 1A. Ribbon Model showing the overall fold of engineered MnP3 from *Phlebia radiata*. α-Helices and heme are shown in red, β-strands in white, distal and proximal calcium in green, manganese in pink. Image generated using Web Lab Viewer (Raswin) and the heme, Mn, and Ca obtained from crystal structure of versatile peroxidase from *Pleurotuseryngii*deposited in the protein Data Bank, entry code 3FJWA

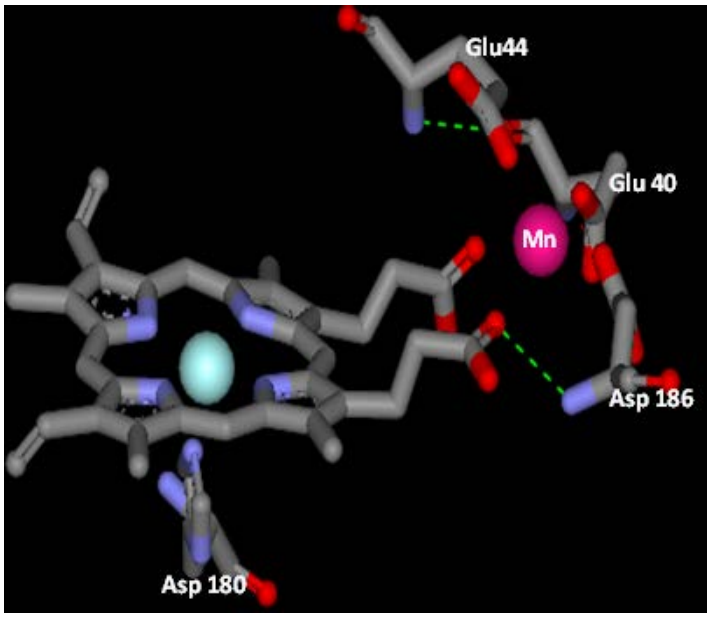

Figure 1B. A model of manganese (II) binding site, with its associated amino acids ligands, of engineered P. radiata MnP3. The Mn (II) (pink) at the edge of the heme propionate is surrounded by ligands, Glu40, Glu44 and Asp186.Image generated using WebLab Viewer (Accelrys) and the heme, Mn, and ligands obtained from crystal structure of versatile peroxidase from Pleurotuseryngii deposited in the protein Data Bank, entry code 3FJWA

Table 1. Steady-state Kinetic Parameters for Mn (II) and ABTS Oxidation by Wild-type and Mutant MnP3 Enzymes (E40H, E44H, E40H/E44H, D186H and D186N) from *Phlebia radiata*

Enzyme	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m ($mM^{-1}s^{-1}$)	
	Mn (II)	ABTS	Mn (II)	ABTS	Mn (II)	ABTS
Wild-type	0.17 ± .01	1.4 ± 0.1	175.0 ± 3.0	912 ± 41	1029.0	651.4
E40H	11 ± 0.4	0.5 ± 0.1	12.0 ± 0.2	369 ± 13	1.0	738.0
E44H	8.5 ± 0.5	0.3 ± 0.1	12.0 ± 0.3	491 ± 24	1.5	1637.0
E40H/E44H	20.0 ± 3.0	4.2 ± 0.4	1.7 ± 0.1	615 ± 28	0.08	146.4
D186H	23.0 ± 4.0	0.24 ± 0.03	0.8 ± 0.1	1007 ± 27	0.04	4196.0
D186N	30.0 ± 4.0	1.3 ± 0.1	8.0 ± 0.5	762 ± 28	0.3	586.0

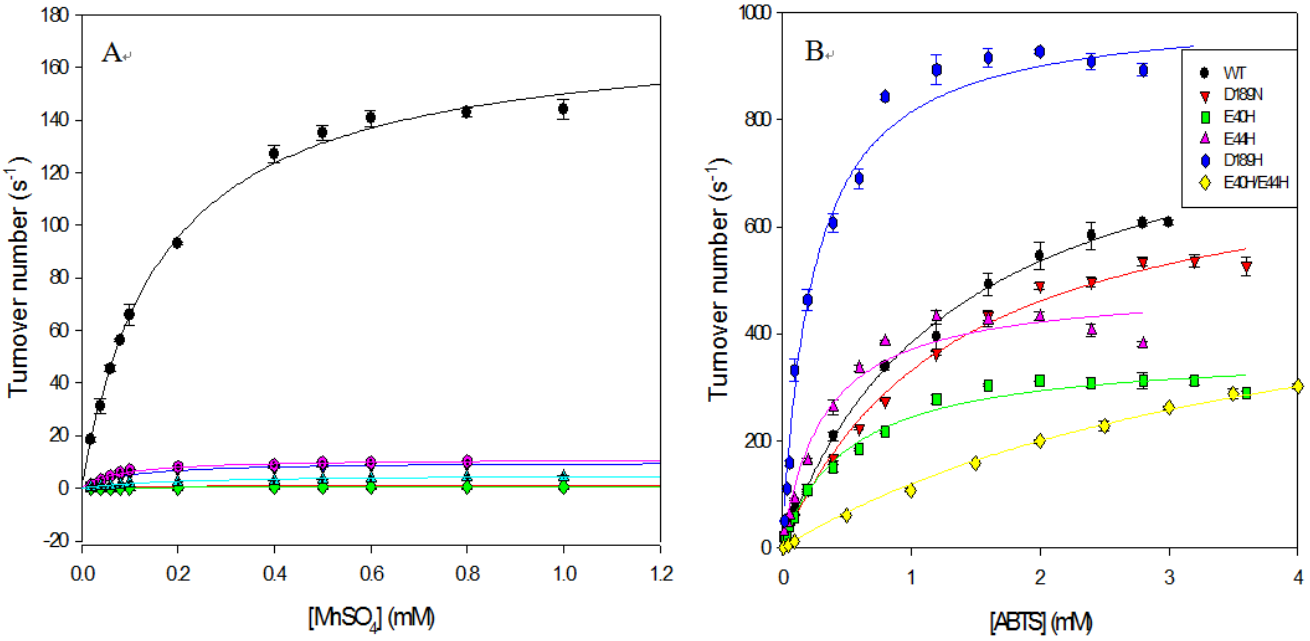

Figure 2. Steady-state Kinetic Parameters for (A) Mn (II) and (B) ABTS Oxidation by Wild-type and Mutant MnP3 Enzymes from *Phlebia radiata*.Each data point represents the mean of three independent determinations with standard errors indicated. Assays were carried out as described in materials and methods. For assays with Mn (II), a fixed concentration of 0.1 mM H_2O_2 was used and the data fitted to the Michaelis equation using Sigma Plot. For ABTS studies, a fixed concentration of 0.01 µM was used for all assays involving the wild-type enzyme while that of the mutant variants range from 0.1 – 6.5 mM. The kinetic parameters extracted from the plots are presented in Table 1

The apparent K_m value for ABTS of the wild-type Pr-MnP3 was found to be 1.4 ± 0.1mM, while the apparent k_{cat} value was 912 ± 41 s^{-1} (Table 2). The apparent K_m values for E40H, E44H and D186H were 2.7-fold, 4.3-fold and 5.8-fold lower, respectively while D186N was equivalent to that of the wild-type enzyme. In contrast, the apparent K_m value for ABTS of the double mutant (E40H/E44H) was approximately 3-fold higher than the wild-type. The resultsobtained also showed that the respective apparent k_{cat} values for ABTS oxidation of E40H, E44H, E40H/E44H and D186N mutant enzymes were 40%, 54%, 67% and 84% lower than the value of the wild-type enzyme. However, the k_{cat} of D186H mutant enzyme was approximately equivalent to that of the wild-type Pr-MnP3. The apparent K_m and k_{cat}values obtained were used to estimate the apparent catalytic efficiencies (k_{cat}/K_m), as a comparative measure of enzyme effectiveness. Catalytic efficiency of E40H and D186N were found to be approximately equivalent to that of the wild-type. In addition, catalytic efficiency of E44H and D186H were found to approximately 2-fold and 6-fold, respectively higher and approximately 4-fold lower for E40H/E44H rPr-MnP3 double mutant.

Table 2. Kinetic Parameters for Mn (II) and ABTS Oxidation *Phlebia radiata* Wild-type MnP3 in the Presence and Absence of Metal Ions

Metals Added	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m ($mM^{-1}s^{-1}$)	
	Mn (II)	ABTS	Mn (II)	ABTS	Mn (II)	ABTS
$MnSO_4$	0.17 ± 0.01	1.39 ± 0.14	175.0 ± 3.0	912 ± 41	1029.0	656
0.8mM Na_2SO_4	0.16 ± 0.01	1.12 ± 0.14	212 ± 5	1067 ± 55	1325	952
0.8mM $ZnSO_4$	0.28 ± 0.02	1.12 ± 0.14	228 ± 7	1260 ± 153	814	1125
0.2 mM $CuSO_4$	0.22 ± 0.04	1.40 ± 0.34	135 ± 9	1419 ± 153	613	1013
0.8 mM $CoSO_4$	0.14 ± 0.12	1.40 ± 0.22	262 ± 9	1365 ± 96	1871	975

The kinetics of enzymes and proteins with catalytic activity is key to understanding the mechanism of their catalysis. The crystal structure [28], homology modelling of *Phanerochaete chyrsosporium* MnP [55] and rPr-MnP3 model (as shown in Figure 1B) indicate that there is a cation binding site on the surface of MnP consisting of carboxylates of three acidic amino acid ligands (Asp179, Glu35, and Glu 39 for *Phanerochaete chyrsosporium* MnP [28] and Glu 40, Glu44 and Asp186 for rPr-MnP3), and one of the heme propionates. Manganese peroxidase 3 from *Phlebia radiata*, has no X-ray crystallographic data yet available. In this study, the relative importance of the ligands forming the Mn (II) binding site has been demonstrated by the kinetic characterization of mutants of key residues. This study focuses on the steady-state kinetics of engineered recombinant *Phlebia radiata* manganese peroxidase 3 wild-type and mutant enzymes. However, from our model (Figure 1B) the Mn (II) binding site was modeled comparable to the homologous *Phanerochaete chrysosporium* structure. Furthermore, rPr-MnP3 was used to study the mechanistic diversity of metal cations in the activity of MnP enzymes. In this study, characterization of these mutants would increase our understanding of the unique specificity of this enzyme and steady-state kinetic characterisation of the wild-type and mutant rPr-MnP3 suggests that this cation site is the productive Mn (II) binding site. In a previous study, Ufot and Akpanabiatu [51] showed that the manganese binding site mutants of rPr-MnP are essentially identical to the wild-type enzyme with respect to chromatographic properties, suggesting that these mutations did not result in gross conformational changes in the enzyme.

The insignificant effects of these mutations on the spectroscopic properties of rPr-MnP3 and mutations of the manganese (II) binding ligands significantly altered the catalytic properties of wild-type rPr-MnP3 enzyme (Figure 2 A & B and Table 1). The image of the Mn (II) binding site is in total agreement with the activity behaviour after site-directed mutagenesis of rPr-MnP3 (Table 1). The rPr-MnP3 variants E40H, E44H, E40H/E44H, D186H and D186N completely lost the ability to oxidize Mn (II) at the substrate concentrations normally used to measure the activity of the wild-type rPr-MnP3 enzyme. It is known that Mn (II) oxidation could be detected with the use of only high substrate concentrations. The higher apparent K_m values obtained for the mutants enzymes indicate that the binding affinity of the mutant proteins for Mn^{2+} is significantly decreased with respect to the wild-type protein. Although rPr-MnP3 may possess other manganese (II) binding sites, the results obtained in this study strongly suggest that the Mn^{2+} binding site shown in Figure 1B [54] is the only productive catalytic site for Mn^{2+} oxidation, particularly since the double mutation (E40H/E44H) almost completely destroys rPr-MnP3 oxidation of Mn^{2+} (Table 1). The results obtained in this study are in agreement with the findings of few other studies [26,28,55].

Of the five mutants studied, two of them (E40H/E44H and D186N) and wild-type rPr-MnP3strictly obeyed standard Michaelis-Menten kinetics with ABTS oxidation (Figure 2B). However, the other three mutants (E40H, E44H, and D186H) in this present study showed some evidence of substrate inhibition effects [56]. The reduction in K_m of E40H, E44H and D186H suggests that the amino acid substitutions may have created enzymes with a higher affinity towards ABTS, resulting in higher effectiveness ratio with respect to ABTS for E40H, E44H and D186H. In contrast, the double mutant (E40H/E44H) with higher K_m for ABTS had a reduced affinity for ABTS resulting in reduced catalytic efficiency when compared to the wild-type enzyme. These observations suggest that the Mn^{2+} oxidation site in rPr-MnP3 is also the site for ABTS oxidation, although the effects of the mutations on the ABTS oxidation are less severe than for Mn (II) oxidation. Although it retained the ability to directly oxidize high redox-potential dyes, the substrate specificity of the rPr-MnP3 enzymes was in agreement with the findings of few other related studies [25,26,57].

3.2. Sensitivity of Mn (II) and ABTS Oxidation by Wild-type Pr-MnP3 Enzyme to Monovalent and Divalent Ions

The influence of divalent metal ions (Zn^{2+}, Co^{2+} and ,Cu^{2+}) on Mn (II) and ABTS oxidations by wild-type *Phlebia radiata* MnP3 enzyme are presented in Table 2 and Figure 3 (A & B). The monovalent sodium sulphate ion ($NaSO_4$) was also tested to see the possibility of behavioural similarity to the divalent metal ions.In this study, the Mn (II) oxidation revealed that the K_m increased in the presence of Zn^{2+} (65%) and Cu^{2+} (29%), and decreased in the presence of Co^{2+} (17.6%), and Na^+ (5.9 %). However, the turnover rate of the enzyme was observed to decrease in the presence of Cu^{2+}(~ 23%) and increase in the presence of Zn^{2+} (30%), Co^{2+} (~ 50%) and Na^+ (~ 21%). In addition, it was observed that Co^{2+} had ~80% increase in the specific activity of this enzyme compared to $MnSO_4$ oxidation alone. During ABTS oxidation, ~ 19% reduction in the K_m values of the enzyme was observed in the presence of $Zn^{2+}$and Na^+. The K_m values obtained for this enzyme in the presence of Co^{2+} and Cu^{2+} were the same as that obtained in ABTS only condition.In this present study, thek_{cat} increased in the presence of all metal ions thus: Na^+ (~17%), Zn^{2+} (~ 38%), Cu^{2+} (~ 56%) and Co^{2+} (~ 50%). An overall increase in specific activity was also observed for all metal ions; with the enzyme showing the highest efficiency in the presence of Zn^{2+} (~67%) when compared with ABTS only condition. Although significant progress has been made in identifying various trace metals (Cu, Co, Mn, Zn, Mg, Fe, etc.) required for the activity of a number of enzymes, these observations may be questioned as one enzyme may be activated by a specific set of metal ions while others are inhibited.

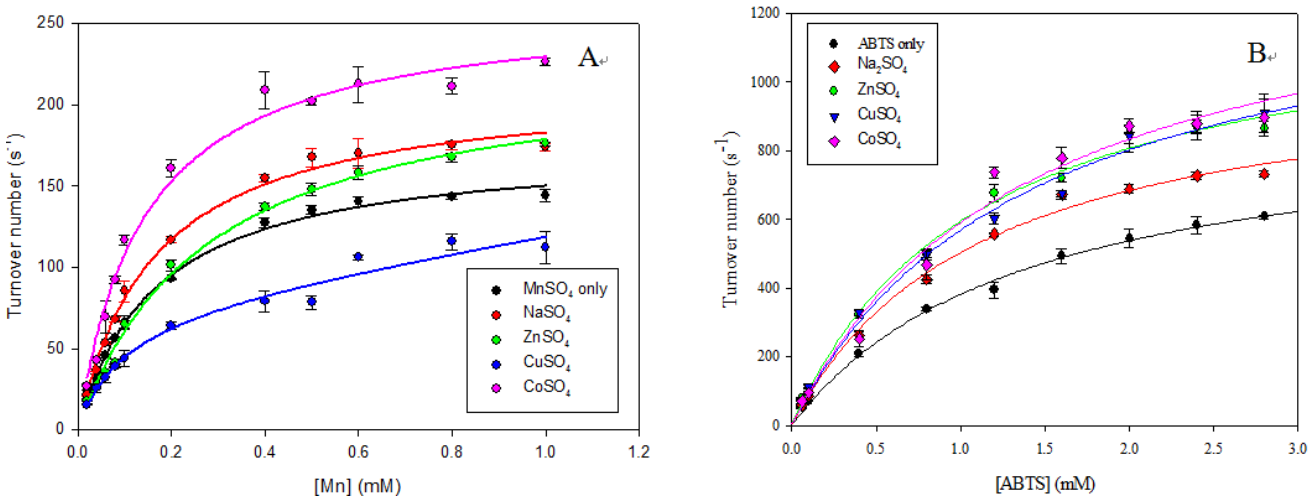

Figure 3. The Dependence of Turnover Number on (A) Mn (II) and (B) ABTS Concentration in the Presence of Monovalent and Divalent Ions. (—) $MnSO_4$ only, (—) 0.8 mM $NaSO_4$, (—) 0.8 mM $ZnSO_4$, (—) 0.2 mM $CuSO_4$, (—) 0.8 mM $CoSO_4$. Reaction mixtures contained 100 mM Na tartrate buffer, pH 5.0 at 25°C and fixed H_2O_2 concentration (0.1 mM). Varying concentrations of $MnSO_4$ and ABTS were used for figures A and B respectively as stated in the material and methods section. Apparent kinetic parameters were estimated from the best fit to the Michaelis-Menten equation

The steady-state kinetic results of Mn (II) oxidation by wild-type and mutant rPr-MnP3 enzymes in the presence of other metal ions revealed that inhibition by metal ions was not in itself, in some cases, a highly stringent process. For the wild-type rPr-MnP3, it was observed that both Na^+ and Co^{2+}exhibited similar behaviour and had stimulatory effects on enzyme activity. The stimulatory effect on activity of rPr-MnP observed in this present study is not in agreement with the findings of Whitwam et al. [57] where 0.1 mM of Co^{2+} inhibited MnP by 67%. In addition, Cu^{2+} and Zn^{2+} affected both K_m and k_{cat} with mixed inhibitory effects. Although the Cu^{2+} was by far the strongest competitive inhibitor, the degree of inhibition or stimulation was different for each metal ion relative to enzyme. The dual effects of metal ions on enzyme activity could also be attributed to a differential affinity of the metal ions for various states of the enzyme [58]. The results of thispresent study showed that sodium, the mono-valent ion (Na) occasionally influenced the activity of the *Phlebia radiata* enzymes in the same manner as the divalent metal ions. The apparent K_m and k_{cat} values for ABTS oxidation in the absence of any metal ion was found to be higher compared to Mn (II) oxidation. The findings of this study showed a decrease in the K_m of enzyme in the presence $ZnSO_4$ during ABTS oxidation. Although both Na^+ and $ZnSO_4$ had a stimulatory effect on apparent k_{cat}, Zn^{2+} exhibited a more significant stimulatory effect when compared to other conditions. It was observed that $CuSO_4$ and $CoSO_4$ had little or no effect on K_m but their apparent k_{cat}values were higher than that of Mn^{2+}.

3.3. Effects of Metal Ions on Mn (II) Oxidation by Mutant Pr-MnP3 Enzymes (E40H, E44H, and [E40H/E44H])

The result of the effect of some metal ions on Mn (II) oxidation by mutant *Phlebia radiata* MnP3 enzymes (E40H, E44H, and E40H/E44H) are presented in Figures 4 (A – C) and Table 3. In this study, there was an increase in the K_m of E40H (Cu^{2+}: 47.0 ± 7.0, Co^{2+}: 14.3 ± 1.0, Zn^{2+}: 14.0 ± 1.0 and Na^+: 13.2 ± 0.8) compared to Mn^{2+} with the K_m value of 11.0 ± 0.4. In addition, the results obtained showed an increasing trend in the K_m values of E44H mutant (Cu^{2+}: 24.0 ± 3.0, Zn^{2+}: 15.0 ± 1.4 and Na^+: 10. ± 1.0) except for Co^{2+} with a decrease in the K_m value (1.0 ± 0.4). The K_m value for the double mutant(E40H/E44H) was observed to have increased significantly only in the presence of Cu^{2+} (36.0 ± 2.0). The respective k_{cats} and catalytic efficiencies for the E40H, E444H and E40H/E44H mutants are presented in Table 3.

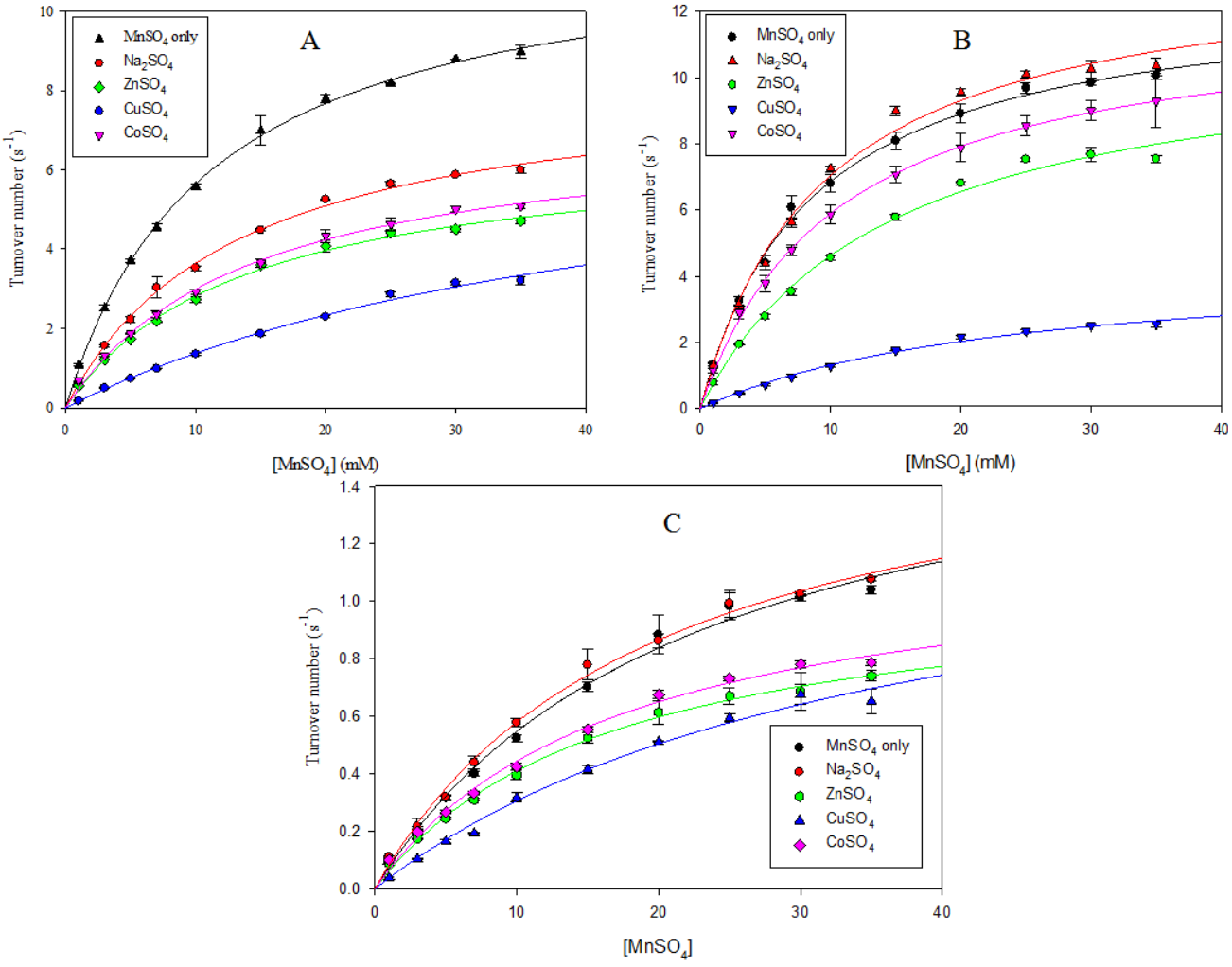

Figure 4. Effect of metal ion addition on Mn (II) oxidation of rPr-MnP3 mutant enzymes (E40H, E44H and E40H/E44H. (—) 0.8 mM $ZnSO_4$, (—) 0.2 mM $CuSO_4$, (—) 0.8 mM $CoSO_4$ (—) 0.8 mM $NaSO_4$. The activity assay performed in 100 mM Na tartrate, pH 5.0 at 25°C using varying concentrations $MnSO_4$ and fixed concentration 0.1 mM H_2O_2

Table 3. Kinetic Parameters for Manganese (II) Oxidation by Recombinant *Phlebiaradiata* MnP3 Mutants (E40H, E44H, E40H/E44H) in the Presence or Absence of Metal Ions [Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Na^{+} (control)]. Activity assays were performed in 100 mM Na tartrate, pH 5.0 at 25°C using varying concentrations $MnSO_4$ and fixed concentrations 0.1 mM H_2O_2. Apparent kinetic parameters are estimated from the best fits to the Michaelis-Menten equation

Metals Added	K_m (mM)			k_{cat} (s^{-1})			k_{cat}/K_m ($mM^{-1}s^{-1}$)		
	E40H	E44H	E40H/E44H	E40H	E44H	E40H/E44H	E40H	E44H	E40H/E44H
$MnSO_4$	11.0 ± 0.4	9.0 ± 0.5	20.0 ± 2.0	12.0 ± 0.2	13.0 ± 0.3	1.70 ± 0.10	1.0	1.5	0.08
0.8mM Na_2SO_4	13.2 ± 0.8	10.0 ± 1.0	19.0 ± 2.0	8.5± 0.2	14.0 ± 0.5	1.70± 0.07	0.6	1.5	0.09
0.8mM $ZnSO_4$	14.0 ± 1.0	15.0 ± 1.4	17.0 ± 1.0	7.0 ± 0.2	11.0 ± 0.5	1.10 ± 0.04	0.5	0.8	0.07
0.2 mM $CuSO_4$	47.0 ± 7.0	24.0 ± 3.0	36.0 ± 2.0	8.0 ± 0.8	5.0 ± 0.3	1.4 ± 0.2	0.2	0.2	0.04
0.8 mM $CoSO_4$	14.3 ± 1.0	1.0 ± 0.4	17.0 ± 2.0	7.3 ± 0.2	12.0 ± 0.2	1.20 ± 0.06	0.5	1.2	0.07

3.4. Effects of Metal Ions on Mn (II) Oxidation by rPr-MnP3 Mutant Enzymes (D186H)

The kinetic parameters for Mn(II) oxidation by rPr-MnP3 mutant enzyme, D186H, in the presence or absence of Na^{2+}, Zn^{2+}, Cu^{2+} or Co^{2+} are shown in Figure 5 and Table 4. The Sensitivity of Mn (II) oxidation by rPr-MnP3 mutant, E40H to other metal ions was studied to see if the change of ligand set at the Mn (II) oxidation site will result in changes to metal ion specificity. The results of this study showed that for E40H, the K_m, k_{cat}, k_{cat}/K_m values for Mn^{2+} oxidation alone and Mn^{2+} oxidation in the presence of other metal ions were far lower than that of the wild-type rPr-MnP3 (Figure 6 and Table 5). The effect of Na, Zn, Cu and Co ions on activity of rPr-MnP3 mutant (E40H) slightly increased apparent K_m while apparent k_{cat} decreased. The inhibition of Mn (II) oxidation by these metals was more of non-competitive in nature. In this study, it was observed that Cu increase the apparent K_m by ~ 300 %, and decrease the apparent k_{cat} by 36 % when compared with Mn^{2+} oxidation in the absence of exogenous metal ions. These findings indicated a mixed type of inhibition effects with K_i values of 0.06 mM (strongly competitive) and 0.4 mM (non-competitive). For the E40H mutant, the apparent K_m was observed to increase while the k_{cat} decreased for all metal ions studied. Subsequently, there was a recorded decrease in specific activity in the presence of the monovalent and divalent ions, suggestive of mechanistic inhibition. The most significant decreased in specific activity was observed in the presence of Cu^{2+}.

Table 4. Kinetic Parameters for Manganese (II) Oxidation by *Phlebia radiata* MnP3 Mutant, D186H in the Presence or Absence of Metal Ions

Metals Added	K_m(mM)	k_{cat}(s^{-1})	k_{cat}/K_m(mM^{-1} s^{-1})
$MnSO_4$ only	23 ± 4	0.84 ± 0.1	0.04
0.8 mM Na_2SO_4	14 ± 2	1.0 ± 0.1	0.07
0.8 $ZnSO_4$	14 ± 2	0.4 ± 0.2	0.03
0.2 mM $CuSO_4$	22 ± 5	0.7 ± 0.1	0.03
0.8 mM $CoSO_4$	26 ± 4	0.9 ± 0.1	0.04

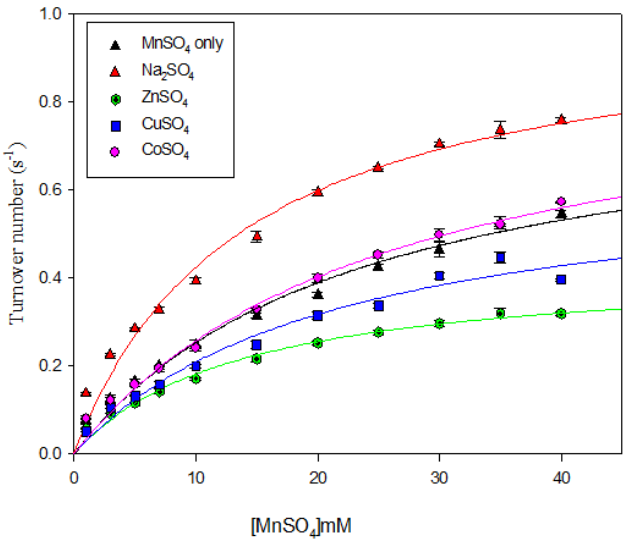

Figure 5. Plots showing effect of metal ion effectors Mn (II) oxidation by rPr-MnP3 mutant enzyme, D186H. (—) 0.8 mM $ZnSO_4$, (—) 0.2 mM $CuSO_4$, and (—) 0.8 mM $CoSO_4$, and (—) 0.8 mM NaSO4 (as control) Activity assays performed in 100 mM Na tartrate, pH 5.0 at 25°C using varying concentrations $MnSO_4$ and fixed H_2O_2 concentration (0.1mM)

Steady-state kinetics of engineered Pr-MnP3 mutant (E44H) in the presence and absence of Na, Zn, Cu and Co showed that the K_m and the apparent k_{cat} during Mn (II) oxidation were not different from that of Na_2SO_4. The calculated percentage increases in the K_m of E44H when $ZnSO_4$, $CuSO_4$ and $CoSO_4$ were added was higher in the presence of $CuSO_4$ compared to $ZnSO_4$. However, $CoSO_4$ had the least K_m value indicating enhance binding which in turn result in higher catalytic efficiency. For E44H mutant, $CoSO_4$ may be an alternative substrate to $MnSO_4$. The results obtained in this present study indicated that $ZnSO_4$ and $CuSO_4$ are possible mixed function inhibitors of E44H with $CuSO_4$ having the strongest inhibitory effect. The sensitivity of Mn (II) oxidation by rPr-MnP3 mutant (E40H/E44H) in the absence and presence of added metal ion was also investigated. The results obtained in this study showed that $ZnSO_4$ and $CoSO_4$ had no appreciable effect on K_m but appeared non-competitive with inhibition constants of ~ 1.6 and 2.1 mM, respectively. However, it was observed that $CuSO_4$ significantly increased K_m of E40H/E44H. This effect of $CuSO_4$ on kinetic parameters of double mutant (E40H/E44H) shows that $CuSO_4$ is a more competitive inhibitor with K_i ~ 0.2 mM. In a direct comparison, the effect of Cu on the activity of E40H/E44H was consistent with that obtained for wild-type rPr-MnP3 and other mutants (E40H and E44H) in this study.

Furthermore, the result of the sensitivity of Mn (II) oxidation byrPr-MnP3 mutant (D186H) to metal ions in the absence of added metal ion, the K_m and the k_{cat} were 23 ± 4 mM and 0.84 ± 0.07 s^{-1}, respectively and the catalytic efficiency was 0.036 $mM^{-1}s^{-1}$. In addition, the

effects of addition of Na, Zn (II), Cu (II) and Co (II) sulphates show Na_2SO_4 activated D186H activity strikingly with approximately ~ 41 % decrease in K_m while apparent k_{cat} increased by 20 % (Figure 5 and Table 4). In contrast, it was observed that both $ZnSO_4$ and $CuSO_4$ inhibited D186H enzyme activity competitively with reduced K_m (41%, for Zn) and k_{cat} (49 % for Cu), respectively while $CoSO_4$ compared effectively with $MnSO_4$. This present study further showed that the strong competitive inhibitory effect of Cu^{2+} was largely removed in D186H mutant enzyme. In this caseit could be argued that Cu and Zn behaved in a similar way, unlike the wild-type and other variants. The introduction of $NaSO_4$ resulted in the highest catalytic efficiency, indicating $NaSO_4$may be a preferable substrate for D186H mutant. Therefore, both $CoSO_4$and $MnSO_4$ may be an alternative substrate due to their similar behaviour.

In this study, the general changes in the K_m and k_{cat} with respect to the effect of Cu (II) on rPr-MnP3 activity, reveals the presence of both competitive and non-competitive kinetics (mixed inhibition). The strong inhibitory effect caused by Cu (II) is an indication of a higher peroxidase affinity for Cu^{2+} compared to the affinity for Mn^{2+}. Although $ZnSO_4$ exhibited a significant increase in both the K_m and k_{cat}, it was observed that $CoSO_4$ exhibited a contrasting effect compared to Cu (II). This study showed that Cobalt exhibited a significant decrease in K_m and increase in k_{cat} of rPr-MnP3, an observation made for the first in the kinetics of MnP enzyme. From the results obtained in this study, the selectivity between Mn^{2+} and Cu^{2+}has been demonstrated and the results has shown that Mn^{2+} oxidation is strongly inhibited by Cu^{2+}. The antagonistic interaction observed in this study particularly between Cu and Mn is similar to that reported by Sunda and Huntsman [59]. Although the exact mechanism of inhibition of Mn (II) by Cu^{2+} and Zn^{2+} is not clearly understood, the observed effect could be attributed to the potential competition between Cu^{2+} and Mn^{2+} for the binding to the enzyme active site [59,60,61].

This results obtained in this study generally confirmed that metal ions of Cu, Zn, Co and occasionally Na (I) compete with Mn for the binding to the Mn (II) site in rPr-MnP3. This contrasting resultssuggest that the Mn binding site in rPr-MnP3 binds Cu and Zn more strongly than it does for Mn. The observation may be attributed to the low reactivity of Mn toward binding to organic ligands compared to reactivity of Cu and Zn – the so called Irving-Williams order of affinity [59]. These inherent differences in reactivity may make it impossible for a Mn porter to have a higher affinity for Mn^{2+} than it does for more reactive, similarly sized, divalent metal ions such as Cu^{2+}. However, these metal ions (Cu, Zn, Co, Mn, and Na) are capable of forming coordination complexes with a variety of organic functional groups, which may be ionic covalent type. Since the degree of filling of the 3^{rd} electron orbital differs in these metals, the coordination complexes they form may differ in electron configuration and type of linkages. It is therefore conceivable that the differences among these metals as to the rate of rPr-MnP3 activation or inhibition may depend on the configuration and stability of coordination complexes of the metallic ion with substrate or protein. It might be predicted that these transition metal ions might show a certain degree of similarity in their ability to serve as activating or inhibiting ions for rPr-MnP3.

With regard to ligand preferences for these metal ions, the manganese (II) ion accepts ligands with either oxygen or nitrogen donor atoms, but the bivalent metal ions to the right of the transitions series show an increasing preference for ligands with nitrogen donor atoms[62]. Generally, the Co (II), Cu (II) and Zn (II) are known to show an increasing preference for ligands with nitrogen or sulphur ligands [63,64]. This suggests that inhibition of rPr-MnP3 by metal ions is dependent on the preferred ligand types and the co-ordination number of the ion, the former perhaps being the dominant factor. The strongest inhibition of the enzyme by Cu (II) compared to Zn (II) and Co (II) may be a consequence of its tetragonal coordination preference. The rPr-MnP3 inhibition by copper may be attributed to increase in the number of bonding interactions or effective binding of the copper to the enzyme as a result of the shorter and stronger bonds. This behaviour may also be that copper have fewer constraints on the co-ordination geometries they adopt and can easily occupy sites of low symmetry. In addition, their complexes may be labile, allowing an easy exchange of ligands.

4. Conclusion

The results obtained in this study have shown that the engineered rPr-MnP3 mutant variants have been created in which the metal binding functionality has been largely lost. The result of this present study agrees with the previous findings [25] that altering any of the amino acid ligands, Glu40, Glu44 and Asp186 at the Mn (II) binding site drastically affects the oxidation of it substrate, most probably by decreasing the affinity of the enzyme for Mn (II) as well as other substrates. The results have also showed that the Mn^{2+} binding site of rPr-MnP3 consisting Glu40, Glu44 and Asp186 is the protective catalytic site and that these ligands are essential for Mn (II) coordination and catalysis. Although single histidine substitutions retained a strong selectivity for Cu (II) as competitive inhibitor, the binding of Zn was however observed to be more non-competitive and this suggest the involvement of sites other than the Mn (II) binding site.

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