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Influence of Organic Acids on Kinetic Properties of Engineered *Phlebia radiata* MnP3 Enzymes (Wild-type and Mutants) during Mn (II) Oxidation

Usenobong F. Ufot[1,*], Imeh J. Okop[2], Dorcas U. Ufot[3], Aniefiok E. Ite[2], Monday I. Akpanabiatu[4]

[1]Department of Biochemistry, Faculty of Biological Sciences, Akwa Ibom State University, P.M.B. 1167, Uyo, Akwa Ibom State, Nigeria
[2]Department of Chemistry, Faculty of Biological Sciences, Akwa Ibom State University, P.M.B. 1167, Uyo, Akwa Ibom State, Nigeria
[3]Department of Biological Sciences, Akwa Ibom State Polytechnic, Ikot Osurua, P.M.B. 1200, Ikot Ekpene, Akwa Ibom State, Nigeria
[4]Department of Biochemistry, Faculty Sciences, University of Uyo, Akwa Ibom State, P.M.B. 1017, Uyo, Akwa Ibom State, Nigeria
*Corresponding author: usen_mboso@yahoo.com

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Abstract Organic acids are potent inhibitors or activators of enzymes and are involved in the catalytic cycle of peroxidase. This study investigated the influence of some organic acids on kinetic parameters during the oxidation of Mn (II) catalyzed by recombinant *Phlebia radiata* wild-type and mutant manganese peroxidases. For the wild-type, the highest affinity (low Km) and highest catalytic efficiency were obtained in the presence of lactate. The E40H, E44H, D186H and D186N rPr-MnP3 variants exhibited similar characteristics as the wild-type with highest catalytic efficiencies in the presence of lactate. Michaelis constant, Km for rPr-MnP3 mutants was far higher compared to wild-type indicating that enzymes with less affinity for Mn (II) were created by mutation of the Mn (II)-binding site. This resulted in gross decrease in catalytic efficiencies of the mutants relative to wild-type. While D186H was least efficient in tartrate, D186N showed equivalent catalytic efficiencies in both malonate (0.31 $mM^{-1}s^{-1}$) and tartrate (0.30 $mM^{-1}s^{-1}$). E40H/E44H was most effective in the presence of malonate. Our outcomes agree with the hypothesis that certain organic acids are involved in the catalytic cycle of manganese peroxidase. We conclude that mutation specific preferences for organic acid chelators have been created and histidine is a better alternative to asparagine in retaining the activity of Mn peroxidase. For practical applications, tartrate is the most for wild-type, E40H, E44H, D186H and D186N activity while malonate is the best for E40H/E44H mutant. Creation of more mutants and investigation of specific modulators of these mutant enzymes are on-going in our laboratory.

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1. Introduction

The manganese peroxidase (MnP), (EC 1.11.1.13), an extracellular oxidoreductase, effective in degenerating a variety of environmental aromatic pollutants [1,2]. This enzyme has been subjected to intensive studies in order to unravel it potential in lignin degradation [3,4,5,6,7]. Manganese peroxidase structural and kinetic properties have been extensively studied [8]. The active site of MnP contains a noncovalently bonded b-type heme, two calcium (II) binding sites one proximal and the other distal to the heme and five disulfide bridges [8,9]. The two calcium-binding sites and five disulfide bridges have been found to be important for maintaining protein stability and activity in peroxidases of class II and III [10,11,12].

The catalytic cycle of MnP resembles those of other heme peroxidases such as horseradish peroxidase (HRP) [13] and lignin peroxidase [14,15] and includes the native ferric enzyme as well as the reactive intermediates Compound I and Compound II [2,16,17]. Manganese peroxidase is so named because of its requirement for divalent manganese in carrying out peroxide-dependent oxidations [1,18]. It is therefore a well-known fact that Mn^{2+} is the preferred substrate for MnP.

Manganese peroxidase is oxidized by H_2O_2 to a highly reactive two-electron state, compound I. Compound I returns to its resting state after two separate one-electron reductions by Mn (II), with compound II as an intermediate. A monochelated Mn^{2+} ion acts as

one-electron donor for this porphyrin intermediate and is oxidized to Mn^{3+}. Compound II of MnP exhibits an absolute requirement for Mn^{2+} as an exclusive reductant essential for the completion of the catalytic cycle of the enzyme [19,20]. The Mn^{3+} is believed to dissociate from the enzyme and form a diffusible oxidant complex with dicarboxylic acid chelators such as pyrophosphate [1], tartrate [21], oxalate [1,22] or lactate [23].

Manganese activity is also known to be stimulated by simple organic acids, which chelate the Mn (III) ion product of catalytic activity. The relative stabilities of the Mn (III)-lactate complex and Mn (III)-malonate complex have been documented [2]. In MnP reactions, organic acids chelators have been found to facilitate the dissociation of Mn^{3+} from the manganese-enzyme complex and also to stabilize Mn^{3+} in aqueous solution with a relatively high redox potential [1,19,20].

After a successful generation of recombinant *Phlebia radiata* MnP3, a new MnP enzyme, an attempt was made to investigate the effect of organic acids on its activity of wild-type and mutant enzymes. Here we report on the effect of organic acids on kinetic properties of engineered *Phlebia radiata* MnP3 enzymes during Mn (II) oxidation.

2. Materials and Method

2.1. Enzyme Preparation

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. Restriction enzymes were supplied by NEBiolabs while the *Escherichia coli* expression vector pFLAG1 was obtained from International Biotechnologies Inc, UK. The engineered *Phlebia radiata* MnP3 enzymes, wild-type and mutants, 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 [24]. All chemicals used in this study were obtained from Sigma-Aldrich, UK and Fisher Scientific, UK.

2.2. Enzyme Assays

The Activities of the enzymes were determined using each of the organic acids (tartrate, malonate and lactate) as buffers. The aim was to determine which buffer gave the highest activity with rPr-MnP3. The steady-state kinetics of Mn (II) oxidation were assayed at 238 nm, and 25°C in 100 mM of appropriate sodium (Na) (tartrate, malonate and lactate) buffers at pH 5.0. Reaction mixtures contained Mn (II) as $MnSO_4$ with concentration range 0.2 – 1.2 mM for wild-type enzyme, and 1 – 45 mM for all the mutant enzymes. The fixed concentration of H_2O_2 was 0.1 mM and the enzyme concentrations were approximately 0.2 µM for the mutant enzymes and 8.0 nM for wild-type enzyme. The MnP activity was measured as the formation of the Mn (III)-tartrate complex (ε_{238} = 6500M^{-1} cm^{-1}) [2], Mn (III)-malonate complex (ε_{238} = 8500$M^{-1}cm^{-1}$) [22] and Mn (III)-lactate complex (ε_{238} = 3500$M^{-1}cm^{-1}$) [22]. All assays were done in triplicate and spectroscopic measurements were carried out using ultraviolet spectrophotometer (UV-2401 PC, Shimadzu Scientific Instruments, Addison, IL). Data analysis, plotting and manipulations were carried out using the solver tools of Microsoft Excel and the statistics analysis software, SigmaPlot for Windows V4.01 (SPSS UK Ltd, Woking, UK). From the K_m (Michaelis-Menten constant) and the first-order rate constant (K*cat*) values obtained, specificity constants (k_{cat}/K_m) were calculated.

The rPr-MnP3 enzymes specificities were examined in the presence of different dicarboxylic acids. The results showed that the rPr-MnP3 (wild-type and mutants) enzyme's activity varied depending on the enzyme and the organic acid used. The wild -type rPr-MnP3 and mutants (E40H, E44H, D186H, D186N in Figures 1(a, b, c, e, f) and Table 1) showed organic acid preference for lactate. Figure 1 (a, b, c, e, f) exhibited the highest activity in the presence of lactate, while the double mutant, E40H/E44H was most active in the presence of malonate (Figure 1 and Table 1).

Table 1. Mn (II) oxidation by wild-type and mutant rPr-MnP3 with different organic acid Mn (III) chelators. As described in section 2.0. Oxidations of Mn (II) was carried out using 100 mM of appropriate buffer (sodium tartrate, sodium malonate or sodium lactate) pH 5.0. Reaction mixtures contained 0.03 – 0.2µM MnP3, 0.1mM H_2O_2 and 0.2 -1.2 mM $MnSO_4$ for wild-type and 1 – 45 mM $MnSO_4$ for mutant enzymes. Results are the average of three determinations with the standard errors indicated

	Tartrate			Malonate			Lactate		
MnP3	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Wild-type	0.17 ± 0.01	175 ± 3.0	1029.4	0.31 ± 0.01	240 ± 3.0	774	0.13 ± 0.02	300 ± 11	2308
E40H	11 ± 0.4	12.0 ± 0.2	1.0	10 ± 2	6.3 ± 0.4	0.63	2.0 ± 0.2	9 ± 0.20	4.50
E44H	8.5 ± 0.5	12 ± 0.3	1.4	8.0 ± 1	8.0 ± 0.4	1.00	2.3 ± 0.2	12 ± 0.30	5.20
E40H/E44H	20 ± 3	1.7 ± 0.1	0.09	9.2 ± 2	5.0 ± 0.4	0.54	7.0 ± 1.1	0.9 ± 0.04	0.13
D186H	23 ± 4	0.84 ± 0.1	0.04	5.3 ± 0.8	4.0 ± 0.2	0.75	8.0 ± 0.7	10 ± 0.30	1.25
D186N	30 ± 4	8.0 ± 0.5	0.30	16.0 ± 2	5.0 ± 0.2	0.31	7 .0 ± 0.4	8.4 ± 0.13	1.20
MnP[a]		176[b]			220[c]			211[d]	

a *P. chrysosporium* MnP
b [25] 50 mM sodium tartrate, pH 4.5, 0.02 µM MnP, 0.1mM H_2O_2 and 0.2 mM $MnSO_4$.
c [22] 40 mM sodium malonate, pH 4.5 0.1µM MnP, 50 µM H_2O_2 and 0.2 mM $MnSO_4$.
d [22] 40 mM sodium lactate, pH 4.5 0.1µM MnP, 50 µM H_2O_2 and 0.2 mM $MnSO_4$.

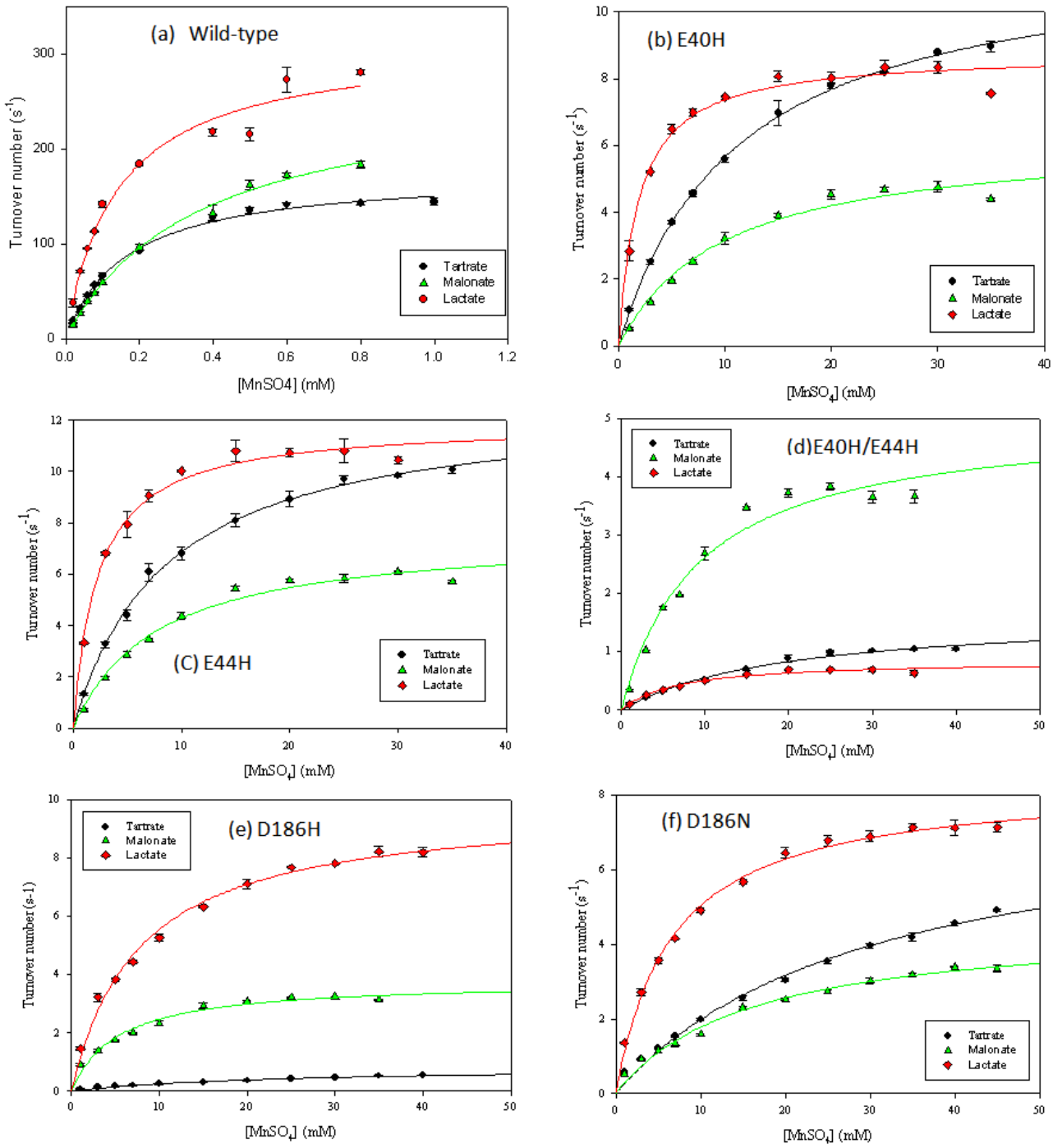

Figure 1.

3. Discussion

In this investigation, we discussed the characteristic effect of organic acid chelators on the Mn (II) oxidation activity of *P. radiata* MnP3. The choice of organic acids was based on reports of their ability to act as Mn (III) chelators [20]. The results obtained from this investigation are summarized in Figure 1 and Table 1. Worthy of note is that oxalate, earlier proposed to be the physiological chelator for Mn (III) in *P. chrysosporium* [22] was used in this study, but no activity was observed at pH 5.0 (optimum pH for Pr-MnP3), meaning that no significant data could be obtained. Hence, work on oxalate was suspended. The no activity observed when oxalate was used as buffer for Mn^{2+} oxidation by *Phlebia radiata* MnP3 could be pH related. However, it is difficult to explain the reason for this behaviour of oxalate with recombinant *Phlebia radiata* MnP3 enzymes.

The Km values obtained for the wild-type rPr-MnP3 for Mn (II) oxidation using tartrate, malonate and lactate at pH 5.0 were found to be 0.17 ± 0.01 mM, 0.31 ± 0.01 mM and 0.13 ± 0.02 mM, respectively. These results show that out of the three organic acids, lactate has the highest effect on the activity of the wild-type rPr-MnP3.

The kcat value obtained when the reactions were conducted in tartrate was however lower than in malonate or lactate. Consequently, the results show that wild-type rPr-MnP3 has the highest catalytic efficiency in oxidizing Mn (II) 2308 $mM^{-1}s^{-1}$, 1029.4 $mM^{-1}s^{-1}$, and 774 $mM^{-1}s^{-1}$

when the reaction takes place in lactate, tartrate and malonate, respectively.

Comparing the kcat values of recombinant *P. radiata* MnP3 with MnP of *P. chrysosporium*, *the results show* that for these two enzymes tartrate and malonate values are very similar but the lactate values vary significantly (see Table 1). These results are also different from those observed for MnP from *A. terreus LD-1* where lactate inhibited the MnP activity completely [26]. However, the observed Km values for MnP3 with the three organic acid systems are all relatively high, compared to the values previously reported for *P. chrysosporium* [22,27].

Similarly, the results of the steady-state kinetic parameters for Mn (II) oxidation were also determined for the five rPr-MnP3 mutants using the three organic acids as described in the methods section. The results in Figure 1 and Table 1 show that Km values for all the rPr-MnP3 mutant were relatively higher and kcat relatively lower compared to the wild-type values when reactions occurred in tartrate, malonate or lactate. The increase in Km of all the rPr-MnP3 mutant implies that mutations of the Mn (II)-binding site have generated enzymes with less affinity for Mn (II) as evident in gross decrease in their catalytic efficiencies relative to the wild-type rPr-MnP3. E40H and E44H MnP3 mutant variants behaved similarly to wild-type when activity assays were conducted in tartrate, malonate and lactate buffers with these two enzymes being most effective in lactate buffer with catalytic efficiencies of 4.5 and 5.2 $mM^{-1}s^{-1}$ respectively, and showing least effectiveness in malonate buffer (Figure 1 (b and c) and Table 1).

The double mutant, E40H/E44H behaved differently from the wild-type in the presence of these organic acid by being most effective in malonate buffer with catalytic efficiency of 0.54 $mM^{-1}s^{-1}$ and least effective in tartrate buffer, having kcat/Km ratio of 0.09 $mM^{-1}s^{-1}$. Hence, the replacement of Glu 40 and Glu 44 with histidines at the manganese–binding site of rPr-MnP3 created a far less active enzyme, thereby showing the importance of Glu 40 and Glu 44 in the binding and oxidation of Mn (II) by rPr-MnP3 of *P. radiata*.

The D186H and D186N MnP3 mutant variants also behaved differently from the wild-type MnP3 enzyme in the organic acid buffers. While the D186H was most efficient in lactate and least efficient in tartrate buffer, the D186N also showed the most effectiveness in lactate and equivalent catalytic efficiencies in both malonate (0.31 $mM^{-1}s^{-1}$) and tartrate (0.30 $mM^{-1}s^{-1}$) buffers. However, D186N had the largest increase in the Km value among the five rPr-MnP3 mutant enzymes studied, with much lower kcat values in the buffers used. This resulted in the catalytic efficiency of D186N being reduced by 3400, 2500 and 1920 – fold in tartrate, malonate and lactate respectively, compared to the wild-type. The implication is that the D186N mutant was least favoured with histidine being a somewhat better alternative and also showed the lowest Mn (II) specificity constant in malonate buffer. Clearly mutation specific preferences for organic acid chelating agents have been created and histidine remained a better alternative to asparagine in terms of retaining significant Mn peroxidase activity.

4. Conclusion

The effects of some organic acids on the oxidation of Mn (II) catalyzed by *Phlebia radiata* manganese peroxidase 3 was studied. Results obtained are in agreement with the postulate that certain organic acids are involved in the catalytic cycle of manganese peroxidase. On the basis of the present results, we propose that malonate and tartrate seem to be the most effective chelator for the recombinant *Phlebia radiata* manganese peroxidase activity. A search for other organic acid chelators that may better satisfy the roles described above is in progress in our laboratory.

Conflict of Interest

The authors have declared no conflict of interest.

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