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The Roles of pH in the Modification of Wild-Type Recombinant *Phlebia radiata* Manganese Peroxidase 3 Activities and Stability of Secondary Structures

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Abstract

This investigation is aimed at understanding the specific role of pH and calcium ions on the activity and stability of wild-type recombinant *Phlebia radiata* manganese peroxidase 3 (rPr-MnP3). The pH-dependent cycle of reactions for rPr-MnP3 was evaluated by investigating time-dependent changes in the activity and electronic absorption spectrum of rPr-MnP3. The rPr-MnP3 had maximum efficacy (kcat/Km) for Mn (II) oxidation at pH 5.0 and 3.0 for oxidation of ABTS. Raising the pH of a solution of resting rPr-MnP3 from pH 6.7 (form XH) to pH 8.6 (form X^-), a rapid alkaline transition occurs. Leaving the X^- form of the enzyme at pH 8.6, it slowly becomes converted to a third form of the enzyme Y^-, which returned to the original XH form of the enzyme at pH 6.7. Recovery of form XH from form Y^- occurred through an intermediate Z form. The pH inactivation of rPr-MnP3 followed first-order kinetics. The rate of formation of XH from Z is pH-dependent and biphasic in nature, with measured rate constants (k) = 0.25 min^{-1}, and half-life ($T_{1/2}$) = 2.8 min. The pH-dependent properties observed may be indicative of a greater degree of conformational flexibility at rPr-MnP3 active site due to disruption of the haem-linked hydrogen-bonding network in the distal haem pocket. Calcium ions were observed to significantly stabilised the enzyme's spectral features and reduce the loss of activity during the alkaline pH transition.

Calcium ions enhance the recovery of the initial activity but cannot prevent the final time-dependent irreversible denaturation and aggregation.

Keywords

Phlebia radiata, Manganese Peroxidase, pH-Dependence, Inactivation, Conformation

1. Introduction

Phlebia radiata is a White-rot fungus that selectively and effectively degrades lignin [1] [2]. The lignin-degrading capacity of this fungus has been demonstrated in the biodegradation of synthetic lignins and lignin model compounds [3] [4]. *Phlebia radiata* has been shown under certain conditions to be a more effective lignin degrader than *P. chrysosporium* [5].

The unique capacity *Phlebia radiata* to efficiently degrade lignin is dependent on the production of their oxidative extracellular lignolytic enzyme system [6] [7]. This enzyme system primarily comprises lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, and hydrogen peroxide-generating oxidases [8]. These fungal peroxidases are heme proteins, which are mechanistically similar to plant and other microbial peroxidases [9].

Manganese peroxidase (MnP) (EC 1.11.1.13) is an extracellular oxidoreductase, a class II fungal haem-containing peroxidase belonging to the plant peroxidase-like protein superfamily [10]. MnP was first discovered in *Phanerochaete chrysosporium* [11] and thereafter in other Basidiomycota species, including *Panus tigrinus* [12], *Lenzites betulinus* [13], *Agaricus bisporus* [14], Bjerkandera sp. [15], *Nematoloma frowardii* and *Phlebia radiata* [16] [17]. MnP has also been found in Bacillus *pumilus*, *Paenibacillus* sp. [18], *Azospirillum brasilense* [19] and *Actinobacterium S. psammoticus* [20]. Manganese peroxidase is so named because of its requirement of divalent manganese in carrying out peroxide-dependent oxidations [11] [21].

Manganese peroxidase (MnP) is an effective degrader of dyes and phenol [22] as well as a variety of environmental aromatic pollutants [23]. MnP structural and kinetic properties have been widely studied [24]. The active site of MnP contains a noncovalently bonded b-type heme, two Calcium (II) binding sites, one proximal and one distal to the heme, and five disulphide bridges [24] [25] [26]. The two calcium-binding sites and five disulphide bridges have been found to be important for maintaining protein stability and activity in peroxidases of class II and III [27] [28].

The catalytic cycle of MnP resembles those of other heme peroxidases, such as horseradish peroxidase (HRP) [29] and lignin peroxidase [30] and includes the native ferric enzyme as well as the reactive intermediates Compound I and Compound II [31] [32].

The native MnP is oxidized by H_2O_2 to a highly reactive two-electron oxidized state, compound I. Compound I return to its resting state after two separate one-electron reductions by Mn (II), with compound II as an intermediate. A nonchelated Mn^{2+} ion acts as the one-electron donor for this porphyrin intermediate and is oxidized to Mn^{3+}. Compound II of MnP exhibits an absolute requirement for Mn (II) as an exclusive reductant essential for the completion of the catalytic cycle of the enzyme [33] [34]. The Mn (III) is believed to dissociate from the enzyme and form a diffusible oxidant complex with dicarboxylic acid chelators such as pyrophosphate [11], tartrate [35], oxalate [36] or lactate [33].

It is well known that peroxidase activity is strongly dependent on the presence of calcium ions [37] [38]. Calcium is essential for peroxidase activity and peroxidases have been shown to bind Ca^{2+} ions [39] [40], but there is a dearth of information on the direct dependence of recombinant *Phlebia radiata* MnP3 activity on free Ca^{2+}. Some peroxidases even have a calmodulin binding domain [37] [41] and calcium may switch a peroxidase between different modes of action [42].

The stability, activity and specificity of enzymes are the fundamental parameters required to develop enzymes for their optimal applications of enzymes in various industrial processes. The stability of enzymes in a non-natural environment is a critical issue in biotechnology. The stability of enzymes at high pH and temperature as well as ionic strength are very important factors affecting enzyme activity and key features in evaluating the applicability of enzymes in industrial processes. The protein surface is the point of contact with the solvent media; thus, the physical and chemical environment is sensed through the surface residues. Polar residues are influenced by pH and depending on their individual *pKa* values changes in their ionization state may interfere with enzymatic activity and/or alter enzyme thermo stability [43]. It has been shown that the activity of plants and other peroxidases may be reversibly controlled by pH induced conformational changes [44] [45].

Poor environmental conditions remain a critical factor in the large-scale use of peroxidase catalysis [46]. However, peroxidases have received extensive attention in recent years as biocatalysts for synthetic applications in biotransformation [47] [48]. In view of the biocatalytic potential of peroxidase [47] [48] and the fact that this enzyme's operational stability is of prime importance for any bioprocess characterisation of recombinant *Phlebia radiata* MnP3 (rPr-MnP3) stability under application-type conditions is therefore imperative.

The kinetic studies of wild-type recombinant enzyme and mutant variants generated by site-directed mutagenesis have been reported [49]. The expression, refolding, purification and characterisation of *Phlebia radiata* MnP3 (rPr-MnP3) have been reported [50] [51]. The suitability of engineered Pr-MnP 3 for dye-decolouration is reported [52]. Also, the dependence of manganese (II)oxidation reaction on pH by recombinant wild-type and mutants *Phlebia radiata* manganese perox-

idase 3 (rPr-MnP3) enzymes has also been reported [53]. In this investigation, we use UV-visible studies to characterize the various states of recombinant Pr-MnP occurring during pH-dependence deactivation and renaturation by calcium. Our aim is to understand the role of calcium ions on the activity and stability of the secondary structure elements of rPr-MnP3. The present results will enable the complete characterization and analysis of the pH-dependent conformational changes of the enzyme that have strong implications for understanding its structure-function relationship.

2. Materials and Methods

2.1. Materials

All chemicals used in this study were obtained from Fisher or Sigma-Aldrich chemicals, UK. Spectroscopic measurements were carried out using a Shimazdu-UV-2401 spectrophotometer. All restriction enzymes and their appropriate buffers were supplied by New England Biolabs Ltd, UK. *E. coli* cells, TOPO10 and DH5α (Thermo Fisher Scientific) were applied for cloning, and *E. coli* strain W3110 (ATCC27325) cells for production of recombinant proteins adopting the expression vector pFLAG1 (International Biotechnologies Inc, UK). 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 GeneBank accession number for the cDNA encoding peroxidase Pr-MnP3 is AJ 566200. The Pr-MnP3 cDNA was present in vector pCR 2.1. TOPO.

2.2. Enzyme Production, Activation and Purification

The recombinant *Phlebia radiata* MnP3 (rPr-MnP3) enzyme was produced in *E. coli* (W3110 strain) after transformation with a corresponding plasmid [54]. Cells were grown in Terrific Broth until the absorbance at 500 nm was 1.0 before induction with 1 mM Isopropyl-*β*-D-thiogalactopyranoside (IPTG) and further grown for 3.5 hours. The apoenzyme accumulated in inclusion bodies, as shown by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was recovered using 6 M urea. *Invitro* folding was performed using 0.15 M urea, 5 mM $CaCl_2$, 0.5 mM oxidized-glutathione, 0.1 mM dithiothreitol ratio, 20 µM hemin, 50 mM Tris-HCl, pH 9.5 and 200 µg/ml rPr-MnP3. Active enzyme was purified using anion-exchange column (Mono-Q FPLC System; Pharmacia LKB Biotechnology Ltd), Sweden [55].

2.3. Measurement of rPr-MnP3 Activities

Direct oxidation of Mn^{2+} was estimated by the formation of a Mn^{3+}-tartrate complex (ε238 = 6.5 $mM^{-1}{\cdot}cm^{-1}$) using 1 mM $MnSO_4$ for the rPr-MnP3. Hydrogen peroxide (0.1 mM) was included in the assay buffer (100 mM sodium tartrate buffer pH 5.0). All enzymatic activities were measured at 25˚C using a Shimadzu UV-2401 spectrophotometer.

2.4. Determination of the pH Optima for Mn (II) and ABTS Oxidation by Wild-Type Recombinant *P. radiata* MnP3 (rPr-MnP3) Enzyme

The optimum pH values for the oxidation of Manganese (II) and 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) by rPr-MnP3 were determined by assaying enzyme activity in 100 mM tartrate buffers of varying pH. In Mn (II) oxidation, assay mixtures contained 1 mM $MnSO_4$, 0.1 mM H_2O_2, and 0.023 μM enzyme and a pH range from 3.5 to 10.0 was used. In ABTS oxidation, the assay mixtures contained 1.6 mM ABTS, 0.1 mM H_2O_2, and 0.006 μM enzyme and a pH range of 3.0 to 5.5 was used. The total volume of all assay mixtures was 1 ml and the assays were performed at 25˚C. The reaction was initiated by the addition of hydrogen peroxide and the increase in absorbance at 238 nm or 414 nm, for Mn (II) and ABTS, respectively, were measured using a UV/Vis spectrophotometer (Shimadzu UV-2401 PC).

2.5. The pH Cycle of Recombinant *P. radiata* Manganese Peroxidase 3 (rPr-MnP3)

The UV-vis spectra were recorded on a Shimadzu UV-2401 spectrophotometer using a 1 cm path-length quartz cuvette. A high enzyme concentration (117 μM) was used in this experiment to enable sufficient absorbance at wavelengths greater than 450 nm. A mixed buffer containing 10 mM MOPS and 10 mM Bicine pH 6.7 was used to record a spectrum of an initial low pH enzyme sample (form XH). This sample was then diluted with an equal volume of 50 mM MOPS and 50 mM Bicine pH 9.05, resulting in a final pH of 8.6. The sample was scanned within 1 min of adjusting the pH and the spectrum of an initial high pH form of the enzyme (form X^-) was recorded. The sample was then incubated for one hour at 25˚C and a spectrum of the final high pH form of the enzyme (form Y^-) was taken. Next, the sample was further diluted with an equal volume of buffer containing 170 mM MOPS and 170 mM Bicine, pH 5.95 resulting in a final pH of 6.7. The sample was scanned within 1 min of pH adjustment and the spectrum of the initial product (form Z) of this downward pH jump was recorded. The sample was then further incubated for 24 hours at 25˚C and a spectrum for the recovered form (XH'), similar to the initial low pH form of the enzyme produced. Finally, after 24 hrs of incubation at pH 6.7, 0.4 mM $CaCl_2$ was added to the protein sample in the cuvette to see if the extent of recovery could be enhanced. Small samples of each enzyme form were taken and frozen immediately in liquid nitrogen for activity assays later.

A control experiment using rPr-MnP3 was also set up as described above. After raising the pH to 8.6, a spectrum of the initial high pH form of the enzyme (form X^-) was taken, and then 5 mM $CaCl_2$ was added to enzymes form X^-. Rapid scans were then taken at two minutes interval for one hour. Then the same protocol as before was followed.

3. Results

3.1. Optimum pH for Mn (II) and ABTS Oxidation

Figure 1(a) and Figure 1(b) show the respective optimum pH of Mn (II) and ABTS oxidation by rPr-MnP3. A strong acidic pH preference was observed for wild-type MnP3 activity. The highest rPr-MnP3 turnover number for Mn (II) oxidation was found to be at pH 5.0. The enzyme was observed to be moderately active at alkaline pH, showing similar activity of approximately 18% of the maximum in the pH range of 6.5 to 8.5 with a sharp decrease towards no activity

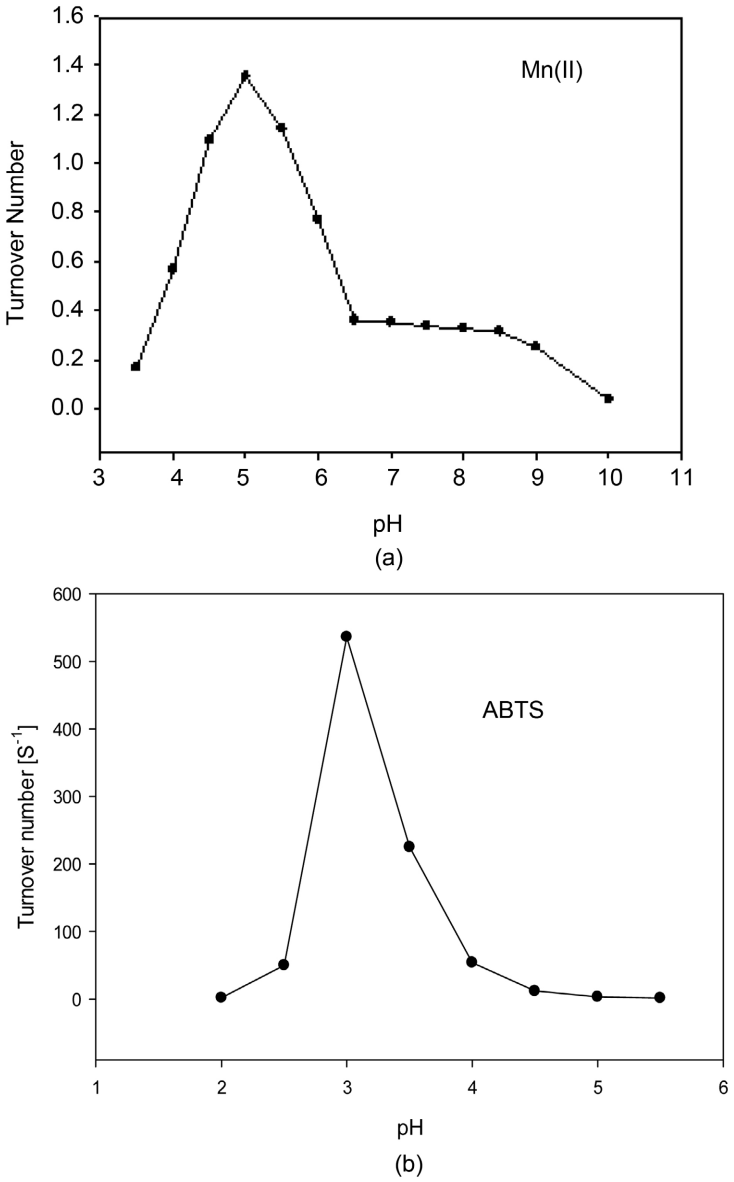


Figure 1. (a) and (b): pH optima for Mn (II) and ABTS oxidation by recombinant *P. radiata* MnP3 enzyme. The reactions were performed in 100 mM sodium tartrate buffer at different pHs, using 0.1 mM H_2O_2, 1 mM $MnSO_4$ and enzyme concentrations of 0.023 and 0.006 μM for Mn (II) and ABTS (1.6 mM) assays respectively. Assays were conducted at 25˚C.

at pH 10.0. The activity for ABTS oxidation by recombinant *Phlebia radiata* MnP3 was highest at pH 3.0 and decreased to zero at pH 5.5 (Figure 1(b)).

3.2. Spectroscopic Properties and Activity Changes during pH Transitions of Recombinant *Phlebia radiata* MnP3

The spectral and activity changes in the study in which rPr-MnP3 was caused to undergo a cycle of pH-induced and incubation-induced changes are summarised in Figure 2(a) and Figure 2(b) and Table 1 and interpreted in Scheme 1. Forms that had a distinct UV/Vis profile were designated XH, X^-, Y and Z respectively. The individual steps of the cycle are considered subsequently (Scheme 1 below).

3.3. Effect of Increasing pH from 6.7 to 8.6

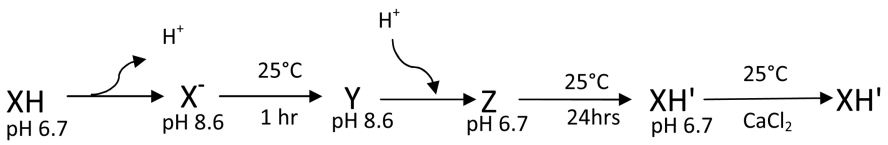
Figure 2(a) shows the UV/Vis spectra of different forms of wild-type rPr-MnP3 during the pH cycle illustrated in Scheme 1 above. The rPr-MnP3 enzyme in buffer (10 mM MOPS and 10 mM Bicine), pH 6.7 was designated XH.

On increasing the pH to 8.6, the enzyme changed to form X^-. Prolonged incubation of enzyme form X^- at pH 8.6 resulted in more spectral changes as the enzyme slowly converted to form Y (green spectrum). After one hour of incubation at pH 8.6, the Soret peak had red-shifted to 412 nm, becoming much less intense (54%) and gaining a shoulder at 368 nm, and new peaks corresponding to α and β bands at 559 and 531 nm had appeared. Activity had also decreased to 10% (Table 1). Five isosbestic points at 358, 421, 474, 521, and 615 nm, respectively, (Figure 2(a)) were observed during this alkali-induced change.

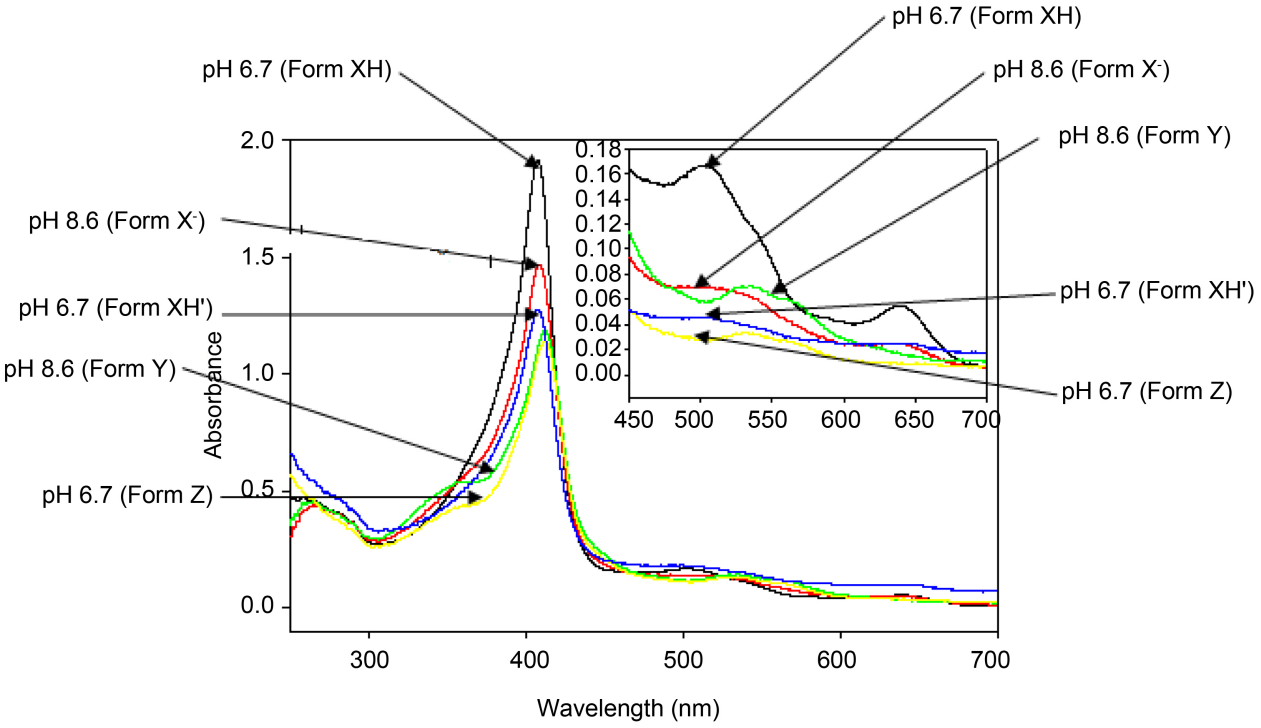
3.4. Effect of Reduction of pH from 8.6 to 6.7

When the pH was lowered from 8.6 to 6.7, a reactivation process was initiated which resulted in enzyme form X (yellow spectrum). The spectrum of form X^- (recorded within the first minute of return to pH 6.7) showed no change in Soret position at 412 nm, the charge transfer (CT) bands I and II did not reappear while the α and β bands at 536, 559 nm and the shoulder at 368 nm, respectively were retained. Only 5% of activity was recovered (Table 1).

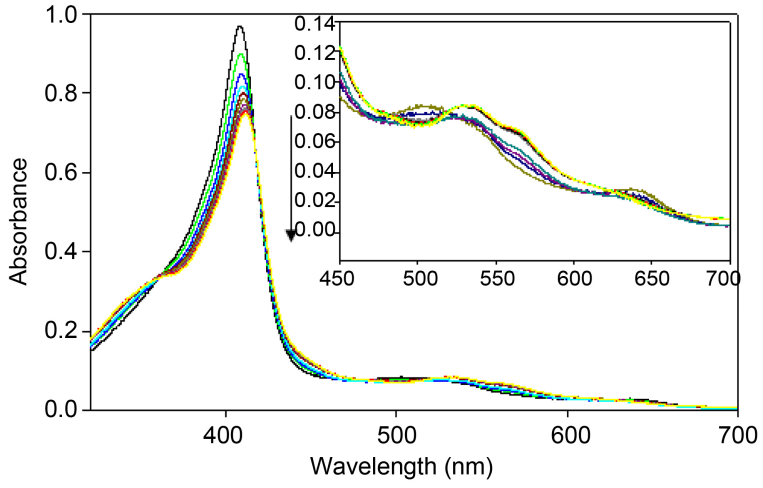
However, on a more prolonged incubation at pH 6.7, the enzyme slowly returned to a resting-state enzyme form, XH' (blue spectrum), but the return was incomplete. The rate of reactivation and conversion of form X^- to form XH' was monitored by repeat spectroscopic scans of the sample and was a very slow process. During the incubation at pH 6.7, Soret absorption maximum blue shifted from 412 to 409 nm, the α and β bands at 559, 536 and the shoulder at



Scheme 1. A scheme showing stages in pH cycle of recombinant rPr-MnP3.



(a)



(b)

Figure 2. (a): UV/visible spectra of the pH cycle of rPr-MnP3 transitional forms. The spectra of X^-, Y^-, Z and XH' have been corrected for dilution caused by pH adjustment. XH, pH 6.7(—); X^-, pH 8.6 (—); I^-, pH 8.6 (—) after 1 hour at 25˚C; X, pH 6.7(—), XH' pH 6.7 (—) after 24 hours at 25˚C and XH' + $CaCl_2$, pH 6.7 (spectrum not shown). (b): UV/visible spectra showing the conversion of wild-type MnP3 form X^- to form Y^-, after pH jump from pH 6.7 to pH 8.6. The enzyme was incubated for 40 mins at 25˚C and scans repeated every 2 mins. Spectra showed gradual change from high-spin to low-spin ferric iron in the haem group.

368 nm disappeared, while the CT bands reappeared at 638 nm (CT I) and 517 nm (CT II). This indicated a restoration of the high-spin haem state. The pH adjustment from 8.6 to 6.7 and incubation for 24 hours resulted in a recovery of approximately 74% of the initial activity of the rPr-MnP3 enzyme (Table 1).

Subsequent treatment of the pH cycled XH' enzyme (form X^-) with 0.4 mM

Table 1. Spectral characteristics and activities of the different forms of wild-type MnP3 from *P. radiata* with and without 5 mM $CaCl_2$ added to X^- form of the enzyme during pH transitions. The experiment was carried out at 25˚C. The standard Mn (II) assay conditions were used. Extinction coefficients were calculated using the concentrations of the various enzyme forms. Characteristics were extracted from spectra depicted in **Figure 2(a)** and **Figure 2(b)**. Soret, *β*, *α*, CT II, CTI, shoulder denote the type of absorption band of the spectra. HS = high spin, LS = low spin. Sample with 5 mM $CaCl_2$ added to X^- form of the enzyme is indicated with 5 mM $CaCl_2$ in bracket.

pH	Predominant form	Percentage remaining activity	Soret (nm)	Extinction coefficient ($mM^{-1}\cdot cm^{-1}$)	*β* (nm)	*α* (nm)	Soret Shoulder (nm)	CT II (nm)	CT I (nm)	Spin state
6.7	XH	100	408	173	-	-	-	504	638	HS
	XH (5 mM $CaCl_2$)	100	408	173	-	-	-	506	642	HS
8.6	X^-	73	408	148	-	-	-	507	636	HS
	X^- (5 mM $CaCl_2$)	83	408	152	-	-	-	505	640	HS
8.6	Y^-	10	412	103	531	559	368	-	-	LS
	Y^- (5 mM $CaCl_2$)	60	409	126	-	-	-	508	636	HS
6.7	Z	15	412	79	536	559	368	-	-	LS
	Z (5 mM $CaCl_2$)	96	407	130	-	-	-	508	641	HS
6.7	XH'	74	409	99	-	-	-	517	638	HS/LS
	XH' (5 mM $CaCl_2$) 12 hours Aggregation	76	408	150	-	-	-	507	640	HS
6.7	XH' 24 hours + 0.4 mM $CaCl_2$	81	409	103	-	-	-	503	638	HS
	XH' (5 mM $CaCl_2$) 24 hours Aggregation	73	408	163	-	-	-	501	641	HS

$CaCl_2$ at pH 6.7 did not significantly alter the spectrum after 24 hours of incubation but restored an additional 7% of the enzyme's activity (**Table 1**). The scattering observed in the final XH' spectrum could be caused by the irreversible denaturation of some of the enzyme after the long incubation time and is consistent with the inability to completely restore the enzyme's activity even with calcium addition.

3.5. The Effect of Addition of 5 mM $CaCl_2$ to Form X^- during the pH Cycle for rPr-MnP3

The response of alkali treatment on the engineered rPr-MnP3 enzyme to Ca^{2+} addition at an earlier stage in the pH cycle was observed when 5 mM $CaCl_2$ was added to form X^- at pH 8.6. The aim was to see if calcium addition would stabilise the enzyme and encourage more reversible behavior.

Form XH was treated with buffer to produce pH 8.6, as above. This resulted in the conversion of form XH to form X^- (**Figure 3(a)**, **Table 1**). In addition to 5 mM $CaCl_2$ and incubation of form X^- at pH 8.6 for 1 hr, the enzyme form was

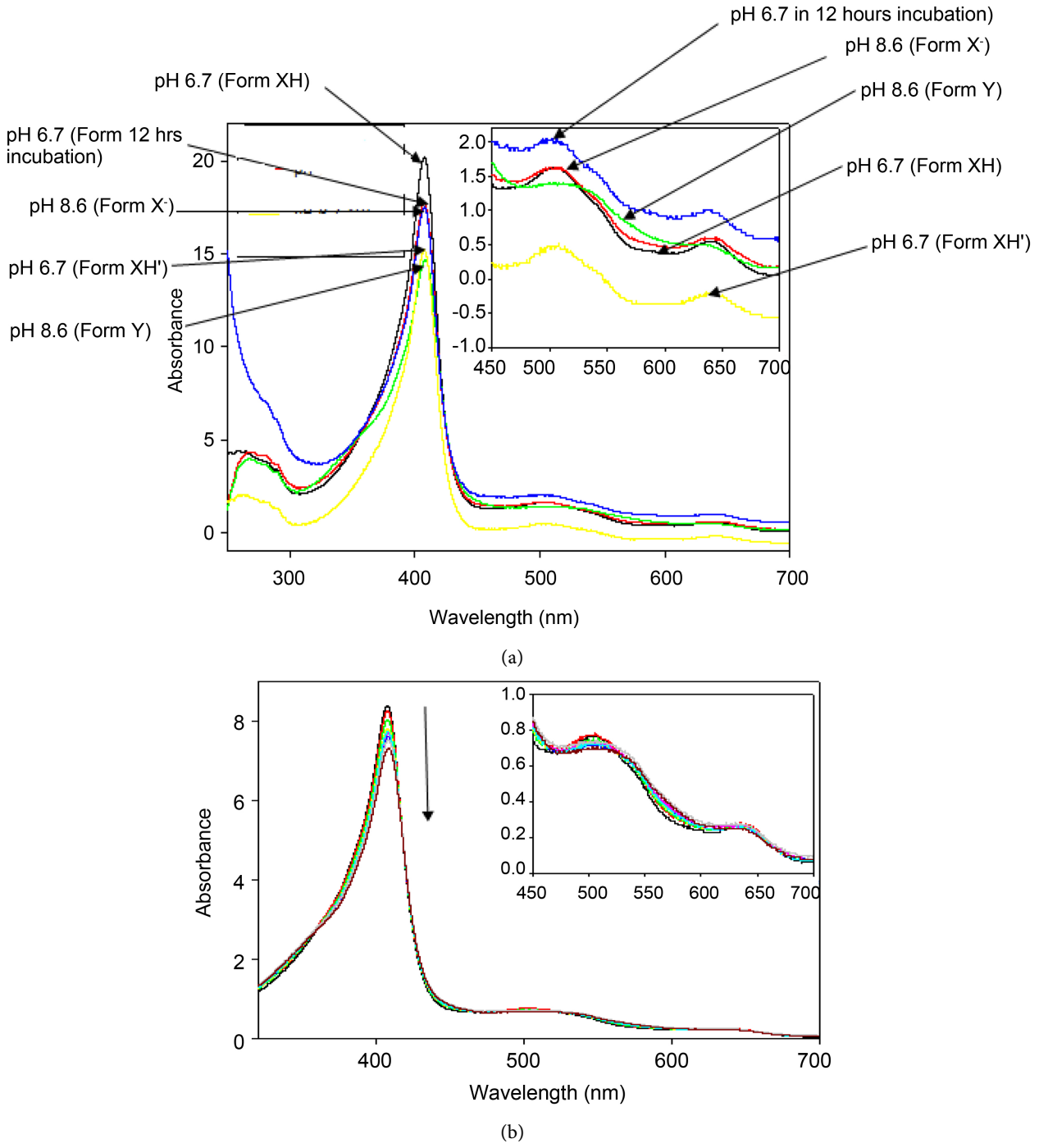


Figure 3. (a): UV/visible spectra of pH cycled forms of wild-type MnP3 with 5 mM $CaCl_2$ added. The spectra of X^-, Y^-, Z and XH' have been corrected for dilution caused by pH adjustment. XH, pH 6.7 (—); X^-, pH 8.6 (—); Y^-, pH 8.6 (—) after 1 hour at 25˚C; Z, pH 6.7 (—), XH' pH 6.7 (—) after 12 hours at 25˚C pH 6.7. (b): UV/visible spectra of rPr-MnP3 showing the conversion of form X^- to form Y^- in the presence of 5 mM $CaCl_2$. The enzyme was incubated for 40 mins at 25˚C and scans repeated every 2 mins. Spectra show a gradual change from purely high-spin to a mixture of high spin and low spin ferric iron in the haem group.

partially and slowly converted to form Y^- (**Figure 3(a)**). The Y^- spectrum had a slight red shift in the Soret peak to 409 nm and a 27% decrease in Soret extinction coefficient. A corresponding 40% decrease in activity was also seen. The CT

I was also slightly blue shifted to 636 nm, and CTI II was slightly red-shifted to 508 nm.

4. Discussion

This study investigated the effect of different pH conditions (acid and alkaline) on the stability of recombinant Pr-MnP3 enzyme with respect to spectral properties and activity. All UV-vis spectra were recorded spectrophotometrically from 750 nm to 250 nm, while activity assays were conducted using standard Mn (II) oxidation assay conditions as contained in the methodology section.

pH is a determining factor in the expression of enzymatic activity, which may depend on changes in the ionization states of amino acid side chains or ionization of the substrate. The active site of enzymes is frequently composed of ionized groups and that must be in the proper ionic form in order to maintain the conformation of the active site.

Figure 1(a) and **Figure 1(b)** showed the pH optima for Mn (II) and ABTS substrates oxidation by rPr-MnP3. rPr-MnP3 showed a strongly acidic pH preference with both substrates. In light of this observation, A non-specific interaction site for ABTS in the general region of the Mn (II) binding site cannot be excluded, implying that protonation events are also required for ABTS oxidation. Hence, the behavior of the enzyme activity at different pH values gives information on the identities of the phototropic groups at the active site [56].

Scheme 1 explains the results of the alkaline transition experiment. As can be seen, the cycle contains four distinct detectable intermediates. For ease, the enzyme sample in each step is referred to as if it were a single form XH, X^-, Y or Z, even though each form definitely consists of an equilibrium mixture of these species instead of a single species, with the distinct form dominating. To confirm the interpretation, the final spectrum obtained in **Figure 2(a)** which corresponds to the enzyme after one complete cycle, referred to as XH' is similar to that of the original enzyme XH, with 43% recovery of the Soret absorption coefficient. The high recovery of the original form of the enzyme implies that the large increase in ionic strength experienced by the enzyme solution during the pH cycle is not the cause of any of the observed effects.

The alkaline transition for recombinant *Phlebia radiata* MnP3 enzyme is characterised by a major change in the UV/visible spectrum of the enzyme similar to that of [R38K] HRP-C [45]. It has been observed that the spectrum of rPr-MnP3 undergoes marked changes in the pH range of 6.7 - 8.6. These changes are both time and pH dependent and involve both spectral changes and parallel changes in the percentage activity that is largely reversible.

The changes observed during the pH jump from pH 6.7 to pH8.6 suggest that the haem ion of the alkaline-inactivated enzyme was now in a low-spin form [57]. The spectral changes observed are possibly indicative of a mixed population of largely high-spin 6-coordinate enzyme and some low-spin 6-coordinate haem iron. According to the studies of [58] and [59] using electronic absorption,

NMR and resonance Raman spectroscopy at neutral pH, unusual mixed-spin haem states (mixture of high-spin (S = 5/2) and intermediate spin (S = 3/2) have been observed to be common to three Class III peroxidases, HRP isoenzyme A2, soybean peroxidase, and barley peroxidase at room temperature.

Lowering the pH to 6.7 caused a rapid change in Soret intensity and enzyme activity. The spectrum of the X form of rPr-MnP3 (recorded within the first minute of adjusting the pH back to 6.7) showed the Soret peak blue shifted to 407 nm with CT I and CT II bands at 641 and 508 nm, respectively. A restoration to 96% of initial enzyme activity was observed. The spectrum and activity of the enzyme therefore closely resembled that of the resting state, XH.

Interestingly, the activity of the X form was observed to gradually decrease with time (XH' form). Within the first minute of return to pH 6.7, activity was restored to 96% of the initial activity. After one hour the activity was observed to drop to 85%, while incubation for 24 hours resulted in a decrease to 73%. The gradual activity loss and the inability of the Soret absorbance to fully return to its initial value may be attributed to enzyme loss through aggregation, a process that apparently showed that the presence of $CaCl_2$ cannot protect against.

Therefore, the addition of 5 mM $CaCl_2$ at an early stage has significantly stabilised the enzymes spectral features and reduced the loss of activity during the alkaline pH change as shown in **Figure 3(b)** above. The transition of enzyme form X^- to Y is strongly affected by the presence of calcium. Calcium has been observed to prevent the majority of the enzyme from changing to the low-spin, largely inactive form. The X^- to Y transition therefore involves the loss of one or more Ca^{2+} ions from the enzyme. Calcium was also able to promote the recovery of most of the initial activity (reversible inactivation) but is not able to prevent the final irreversible denaturation and aggregation that is time dependent.

4.1. Effect of Calcium on Alkali-Treated rPr-MnP3

The alkali-treated rPr-MnP3 enzymes at pH 6.7 responded to the addition of exogenous calcium (5 mM $CaCl_2$). It was observed that lowering the pH from 8.6 back to 6.7 without the addition of calcium most partially restored the activity of the enzyme. However, incubating the enzyme with exogenous calcium at acidic pH for 24 hours restored the spectral features of the enzyme, *i.e.*, high-spin compared to low spin, but still did not fully restore 100% enzyme's activity. [39] showed that the extent of recovery of activity was inversely dependent upon the duration of exposure to the extreme condition, suggesting that other structural rearrangements were possible within the protein, which predisposed the enzyme to irreversible inactivation.

Successful stabilization of peroxidase activity and electronic structure in alkaline pH was obtained by adding Ca ions to the sample (**Figure 3(b)**). This observation is in agreement with the results of *P. chrysosporium* lignolytic enzyme [60]. The addition of 5 mM $CaCl_2$ to X^- forms of the enzymes showed that alkaline inactivation of rPr-MnP3 could be avoided. The key point is that the addi-

tion of exogenous Ca^{2+} prevents the transition from X^{-} to Y, *i.e.*, high-spin transition to low-spin. The enzyme can then almost completely be reactivated at the return to low pH provided Ca^{2+} is present. The effect of Ca^{2+} on rPr-MnP3 in this study is consistent with previous reports [61] [62] [63].

Catalytic activity of rMnP3 from *P. radiata* as in other peroxidases depends on Ca^{2+} ions embedded in the enzyme. Calcium release has previously been observed both during thermal and alkaline inactivation of MnP and LiP [27] [39] [60]. A previous inactivation model proposed an increase in distal histidine flexibility after Ca^{2+} loss, leading to a low-spin haem and to activity inhibition [25] [27]. In addition, [28] suggested that for Ca^{2+}-depleted HRP, other structural changes around the proximal histidine could occur and influence the catalytic performance of the enzyme.

The cause-consequence relationship between inactivation and Ca^{2+} loss has been established by reconstituting peroxidase activity in the presence of $CaCl_2$. In this study, the preservation of Soret absorption, and a high-spin haem, as well as a partial restoration of activity, corroborate the direct dependence of catalytic activity on structural Ca^{2+} ions.

In this work, calcium loss has been shown to play an important role in the pH inactivation of rPr-MnP3. The data collected provide a viable explanation that calcium ion plays a role in conferring structural stability to the haem environment and in retaining the enzyme active site geometry. Calcium also functions in the conversion of the haem iron from a low-spin state to a high-spin state following reversible inactivation.

4.2. Kinetics of the pH Induced X^{-} to Y Conversion

In peroxidases, activity is dependent on the presence and correct conformation of the haem group and the residues forming the catalytic pocket. Conformational changes in the protein are likely to affect enzyme activity [64].

A slow pH-dependent spectral transition in such haem proteins as horseradish peroxidase [65] and turnip peroxidase [66] has been reported. Though the transitions previously reported were termed "slow", the time scale referred to is generally the order of milliseconds, that is, slow relative to many spectral changes but much faster than the changes reported in this work. It is interesting to note that one isozyme of peroxidase from turnip root (P1) exhibited a very slow spectral change after a pH jump, with T1/2 = ~0.05 S^{-1} [66]. However, this change accounted for less than 10% of the total spectral alterations, most of which were orders of magnitude faster.

As observed (**Figure 4(a)**), the rPr-MnP3 enzyme in this study had spectral changes which are essentially first order but also show evidence of a second slow phase. Thus rPr-MnP3 appears to possess a unique mechanism of haem-protein interaction, involving both catalytic and spectral properties of the enzyme and occurring in a physiologically significant pH and time context.

The decrease in Soret absorbance has been used as a convenient probe for the

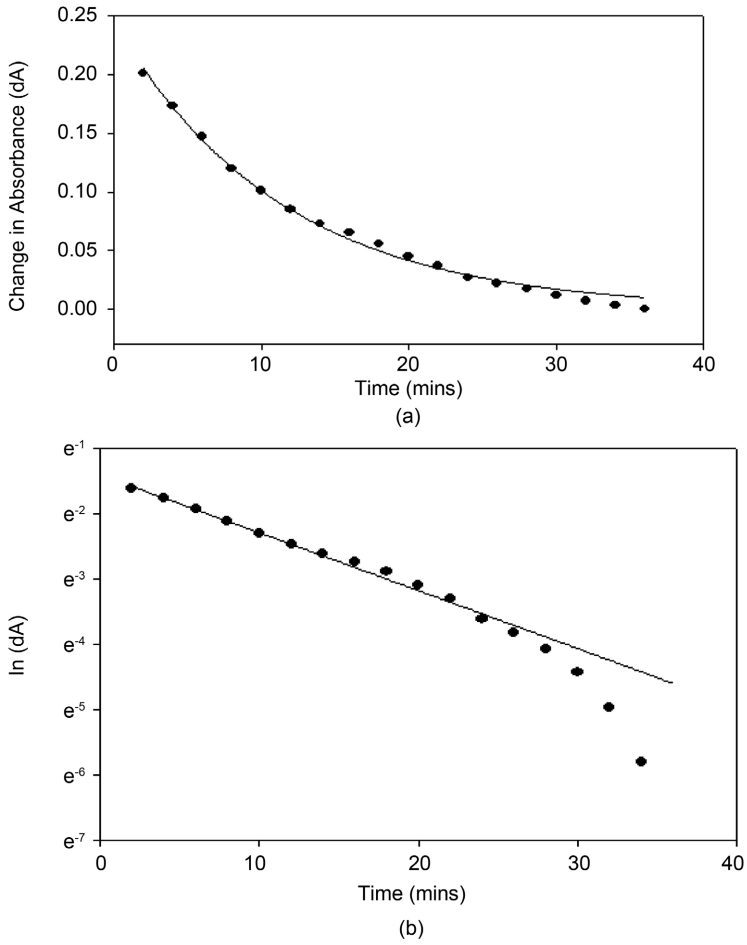


Figure 4. (a): Time- of change in absorbance at 408 nm during the conversion of enzyme form X^- to Y of rPr-MnP3 enzymes. (b): Semi-log plots used to determine the first-order rate constants for the loss of haem at 408 nm during the conversion of enzyme form X^- to Y of rPr-MnP3. The determined decay rate constants ($_{Kd}$) extracted from the gradients of the straight lines for rPr-MnP3 enzyme is 0.25 ± 0.01 min^{-1} with Half-life ($T_{1/2}$) of 2.8 min.

conformational stability of *Coprinus cinereus* peroxidase (CiP) [53] [67] during high pH, urea, and heat treatments. This assumption was also employed by [68] to determine the rate of irreversible unfolding of wild type and mutant peanut cationic peroxidase using guanidium chloride. The remaining fraction of native peroxidase (RP) at time t is described by the equation thus:

$$\mathrm{RP} = [\mathrm{native}]_t / [\mathrm{native}]_0 = (A_t - A_\infty) / (A_0 - A_\infty)$$

where A_t is the absorbance at time t (during the unfolding reaction), A_0 is the initial absorbance at time 0, and A_∞ is the absorbance at the end of the reaction.

The pH inactivation of rPr-MnP3 followed first-order kinetics. Therefore, a plot of In (ΔA) versus time gave a straight line, **Figure 4(b)** from where the rate constant of inactivation reaction (k) can be deduced and half-life ($t_{1/2}$) calculated. The loss of absorbance occurs with a rate constant (k) = 0.25 min^{-1}, and half-life ($T_{1/2}$) = 2.8 min.

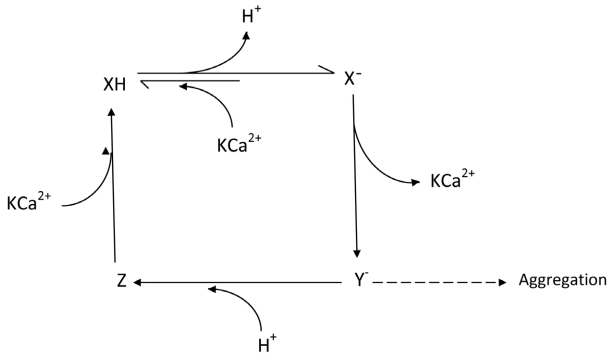
The comparison of the stability of peroxidases has shown that many of these enzymes have similar stabilities while some extracted from other plant sources have also been proved sometimes even superior, compared to traditional horseradish peroxidase (HRP) [69] [70]. The Reasons for the high stability of these peroxidases are not yet clear.

The effect of pH on rPr-MnP3 stability is shown in **Figure 2(a)** and **Figure 2(b)** and **Table 1** above. Identification of the various conformational forms of the enzymes in this work uses data based solely on the enzyme's activity and the UV/visible spectra. It has been observed that the rPr-MnP3 enzyme responds to alkaline transition by activity loss and conformational change is evidenced in the spectral features. The presence of calcium was also observed to either prevent or minimise the extent of alkaline inactivation of these enzymes implying that calcium has a significant capacity to stabilise the enzyme's haem environment and prevent activity loss.

It was possible to detect four distinct spectroscopic species associated with enzyme state, which interconvert in a reversible manner between pH 6.7 and 8.6 during rPr-MnP3 pH transitions. The outstanding conformational enzymes forms were designated as XH, X^-, Y^-, and Z. The electronic spectra alone do not allow quantification of the relative proportion of these forms but our results indicate that the high spin haem state is favoured at acidic pH and that the low spin haem form is favoured at alkaline pH.

The results suggest that forms XH and X^- are in rapid pH-dependent equilibrium. Since all activity measurements were carried out at the enzymes optimum pH 5.0, it can be seen from the current data that form X^- is less active relative to form XH. The current data is also suggestive of forms Y^- and Z as enzymatically inactive. Undoubtedly, X^- to Y^- conversion involves a conformational change. The appearance of β and α band, as well as a shoulder n, the Soret peak is suggestive of Y^- being a low-spin form of the enzyme.

The following **Scheme 2** extended from that of [57], is to explain the basic pH cycle of rPr-MnP3.



Scheme 2. A scheme describing the pH-dependent changes in the spectrum of recombinant *P. radiata* MnP3 (rPr-MnP3) enzyme. Reactions shown by solid arrow symbolise changes taking place when the pH is altered. Reaction indicated with broken arrow represent unfolding and aggregation following prolong incubation at alkaline pH. (Where K = 1 or 2).

1) It is hypothesised that states Y^- and Z have a collapsed haem pocket of some kind and it is not known whether the Y^- or X^- states have lost one or two Ca^{2+} ions.

2) It is possible that the weaker site is depleted in the X^- state and the more tightly bound site in the Y^- form.

3) It is possible that protonation of the distal histidine in Y^- drives the partial restoration of Soret features observed in Z.

In view of the spectral properties of the enzyme forms described and in comparison, to those of other haem proteins [71] [72] [73] [74]. It is possible to imagine the nature, in the different enzyme forms, of the ligand occupying the sixth coordination position of the haem iron. At alkaline pH a transition to six-coordinate low spin has been observed where the sixth ligand is OH^-; hydrogen bonded directly to distal arginine, and through a water molecule to distal histidine [74]. The alkaline transition of rPr-MnP3 is therefore proposed to be an abstraction of a hydrogen atom from a water molecule in the distal cavity with subsequent binding of OH^- group to the haem iron, rather than deprotonation of an amino acid side chain. Hence, it is suggestive that, in form XH, a molecule of H_2O occupies the sixth coordination position. The possibility that an amino acid residue deprotonates in alkaline solution and becomes the sixth iron ligand cannot be excluded by the data obtained in this study.

With the apparently simple relationship that exists between XH and X^-, it is assumed that in form X^-, the ligand should be OH^-. This would make form X^- structurally analogous to the high-pH form of HRP-C [75] [76], though evidently from the spectrum of form X^- (**Figure 2(a)**), there is comparatively little low-spin character. A β-absorption band closer to 540 nm favours the coordination of hydrogen bonded OH^- ligand rather than an amino acid residue around 525 nm [74]. The conversion of form Y^- to form Z is considerably more rapid than that of form X^- to form Y^- but still appears slower than the XH to X^- interconversion. It may likely involve a conformational change in addition to a protonation, which might prompt a further conformational change. As observed, the formation of Z is undoubtedly followed by further slower conformational changes, which result in the regeneration of XH designated XH'. The sample of Z designated in **Figure 2(a)** and **Table 1** is likely to contain a considerable number of other forms of the enzyme, particularly XH, as the time required for the acquisition of the spectra in **Figure 2** was approximately 2 min. Thus, the prediction of its sixth ligand from these data is most likely. With this background, it could be postulated that in alkaline pH, rPr-MnP3 is deprotonated. Protonation of rPr-MnP3 at low pH may preclude or perturb such stabilizing interactions and thereby favor the high spin form of the enzyme.

5. Conclusion

The behavior of enzymes in acid-base environments is an important subject of study due to its prominent role in enzyme catalysis, substrate binding and en-

zyme conformation. The transition of rPr-MnP3 from X^- to Y^- is strongly affected by the presence of calcium. When $CaCl_2$ was added to the X^- form of this enzyme, only slight changes in the spectral features were observed; hence, the high-spin state of the enzyme was maintained. We therefore conclude that Calcium ions have significantly stabilised the enzymes spectral features and reduced the loss of activity during the alkaline pH change. Also, calcium promotes the recovery of most of the initial activity (reversible inactivation) but is not able to prevent the final irreversible denaturation and aggregation that is time-dependent. Therefore, while calcium is depleted following pH inactivation, irreversible inactivation can be prevented by the presence of exogenous Calcium ions. This has potential implications for the use of this enzyme industrially. The insight gained in this study can be useful in several more complex cases involving a pH dependent transition of great biochemical interest.

Author Contributions

UU performed the experiments and processed and analysed the raw data. MA and IO wrote the first draft of the manuscript. MU and IA discussed and wrote sections of the manuscript. All authors contributed to the manuscript revision, read and approved the submitted version.

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Conflicts of Interest

The authors declare that they have no competing interests.

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