pH-Linked Binding of Mn(II) to Manganese Peroxidase[†]

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ABSTRACT: The stability of Mn(II) binding to manganese peroxidase (MnP) has been studied as a function of pH by spectrophotometric and potentiometric titrations. The sensitivity of the potentiometric titrations allows collection of data that are consistent with a high-affinity and a low-affinity Mn(II) binding site on the peroxidase. The two sites differ in affinity by 4 to 900-fold between pH 4 and 6.5. The stability of Mn(II) binding to the high-affinity site increases with increasing pH, while the stability of Mn(II) binding to the low-affinity site decreases with increasing pH. Interestingly, at pH values above 5.0, the high-affinity site appears to be partially unavailable for binding Mn(II). A pH-dependent structural change in the Mn(II) binding site is proposed to account for this partial inactivation at elevated pH.

Manganese peroxidase (MnP) (EC 1.11.1.7) is a heme enzyme found in a variety of white rot fungi that catalyzes the oxidation of Mn(II) to Mn(III) by hydrogen peroxide (*1*). Interest in such enzymes has been stimulated by the observation that Mn(III) generated in this manner is effective as a relatively nonspecific oxidant in the degradation of lignin (*1*, *2*). The enzyme from *Pheanerochaete chrysosporium* has received extensive mechanistic and spectroscopic attention, and the three-dimensional structure of this enzyme has recently been determined by X-ray diffraction analysis (*3*, *4*).

Understanding the mechanism by which Mn(II) specifically binds to MnP during catalytic turnover is critical to the detailed characterization of MnP function. Based on the three-dimensional structure of the protein, a single binding site for Mn(II) has been proposed to exist on the surface of the protein near the γ-meso edge of the heme prosthetic group (*3*) (Figure 1). This binding site is acidic in character and is comprised of three carboxylate ligands provided by residues Glu35, Glu39, Asp179, a heme propionate oxygen, and by Wat520 and Wat441. The interaction with one of these water molecules is stabilized through hydrogen bonding to the second heme propionate group.

Although this structure addresses many critical issues concerning the mechanism of Mn(II) binding, it does not provide information concerning the stability of Mn(II) binding and the manner in which binding is affected by solution conditions. A previous spectrophotometric study provided initial information concerning the stability of Mn(II) binding (*5*), but these measurements were conducted at a single pH in the presence of organic chelators. In addition,

FIGURE 1: Structure of the proposed Mn(II) binding site of manganese peroxidase from *P. chrysosporium* (*3*). The manganese is bound in an octahedral geometry with ligands provided by three acidic amino acid residues, a heme propionate, and two water molecules. One of the coordinated water molecules is hydrogen bonded to the heme propionate group that is not bound to the manganese ion.

theoretical analysis reported prior to publication of the three-dimensional structure suggested the possible existence of multiple binding sites for Mn(II) of varying affinity (*6*, *7*). If such sites exist, they could be of sufficiently low affinity that they would have escaped detection in the crystallographic studies and might not have been detectable in earlier spectrophotometric experiments (*5*).

To characterize the interaction of Mn(II) with MnP in greater detail, we have extended the previous spectrophotometric analysis to consideration of the pH-dependence of

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Mn(II) binding, and we have performed parallel studies of Mn(II) binding to the peroxidase by potentiometric titrations. These latter studies provide a degree of sensitivity and precision not available with most other techniques and are ideally suited to evaluation of the electrostatic properties of Mn(II) binding.

EXPERIMENTAL PROCEDURES

Protein Purification. Manganese peroxidase (MnP) isozyme I was purified from the extracellular medium of acetate buffered cultures of *Pheanerochaete chrysosporium* strain OGC101 (*8*) as described previously (*9, 10*). Metal ions were removed from the enzyme by treatment with Chelex resin (BioRad) in sodium phosphate buffer (100 mM, pH 6.5) (*4*). The MnP treated in this manner retains its two calcium ions and was shown by atomic absorption spectroscopy to contain Mn(II) at <1% of the protein concentration. The concentration of enzyme was determined at 406 nm in 20 mM sodium succinate (pH 4.5, 25 °C) using an extinction coefficient of 129 mM^{-1} cm^{-1} (*9*).

Spectrophotometric Titrations. The binding of Mn(II) to MnP was monitored by following the difference absorption maximum at 405 nm that is produced upon binding of Mn(II) to MnP (*5*). $MnSO_4$ solutions were prepared for these measurements gravimetrically. Data were obtained in 10 mM potassium acetate with 100 mM KCl at MnP concentrations of 1.5−3.0 μM. Spectra were recorded at 25 °C with a Shimadzu UV-260 spectrophotometer. Apparent dissociation constants for Mn(II) binding were calculated with use of the relationship described by Wariishi et al. (*5*; eq 6), which is the linearized expression for binding at a single site. Low-affinity complex formation was assumed in this analysis by setting the concentration of unbound Mn(II) equal to the initial concentration of Mn(II).

Potentiometric Titrations. The binding of Mn(II) to MnP was studied by potentiometric titrations with a Radiometer ABU93 Triburet operated under computer control as previously described by Mauk and co-workers (*11*). In these experiments, ∼7.5 mL of the titrant solution (0.9−4.0 mM $MnCl_2$ prepared by dilution from a manganese standard solution (Titrisol, EM Science) with KCl added to adjust ionic strength to 0.1 M) was transferred to the argon-flushed, water-jacketed titration vessel maintained at 25 °C, and the pH was adjusted to the desired value with dilute, CO_2-free HCl/KCl or KOH/KCl solution ($\mu = 0.1$ M). Once the pH was stable, the $MnCl_2$ was transferred anaerobically to a sealed bottle that was continuously flushed with argon and loaded into one of the burets. The titration vessel was then washed with 5 mM HCl/0.1 M KCl to remove adsorbed Mn(II). Manganese peroxidase was exchanged into 0.1 M KCl by centrifugal ultrafiltration with a Centriprep 10 (Amicon) before being transferred to the argon-flushed titration vessel. Approximately 2.5 mL of the titrand MnP solution (25−70 nmol) was adjusted to the same pH value as the titrant $MnCl_2$ solution. When the pH was stabilized, the titration was started.

Titrations were carried out under computer control in an interactive manner. Each titration curve consisted of 40−70 points that correspond to the volume of standardized acid or base required to maintain the pH at its starting value versus the volume of $MnCl_2$ solution added at each point. These data were converted to mole fraction of exchanged protons, H, versus the ratio of $[MnCl_2]/[MnP]$ for nonlinear regression analysis using the program SCIENTIST (MicroMath, Salt Lake City, UT) to obtain the association constant(s), K_A, and the saturating value of H_e^+, q. The potentiometric data were analyzed by fitting the data to either a one-site model (*11*) or to a two-site model (*12*) as described previously.

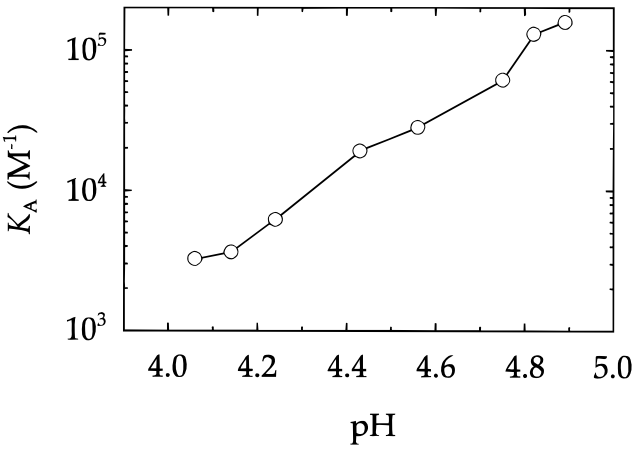

FIGURE 2: Dependence of the association constant for Mn(II) binding to manganese peroxidase on pH as determined by electronic absorption spectroscopy.

RESULTS

Spectrophotometric Titrations. Binding of Mn(II) to manganese peroxidase isozyme I between pH 4.0 and 4.9 was monitored by difference spectroscopy. Data were not obtainable at higher pH values because the high-affinity of the enzyme for Mn(II) required the use of sufficiently low enzyme concentrations that the absorbance change accompanying binding of Mn(II) were too small to determine reliably. The association constants determined from the slopes of double reciprocal plots (ΔA^{-1} vs $[Mn(II)]^{-1}$) are shown in Figure 2. The assumptions inherent in the analysis used can result in an underestimation of association constants, particularly at higher pH where MnP affinity for Mn(II) is greater.

Potentiometric Titrations. At pH 4.5, the pH at which catalytic activity of MnP is optimal, the binding of Mn(II) to MnP results in the liberation of protons (Figure 3). Although the titration data obtained under these conditions appear to be fit relatively well by the assumption of 1:1 binding stoichiometry (Figure 3A), a small, systematic deviation of the data from the fitted curve is apparent that suggests that this simple model is inadequate. On the other hand, analysis of the same data by the assumption that the peroxidase binds two Mn(II) ions eliminates any indication of a systematic deviation from the fitted curve (Figure 3B) and significantly improves the statistical quality of the fit (data not shown). The inadequacy of the 1:1 model in describing Mn(II) binding to MnP is more readily apparent when this experiment is repeated at other values of pH. For example, at pH 6.0, the necessity of invoking higher order complex formation is evident from the shape of the proton release curve (Figure 3C).

In fact, curve fitting analysis of potentiometric titration data obtained for formation of the Mn(II)−MnP complex between pH 4.0 and 6.5 revealed that at pH 5 and above,

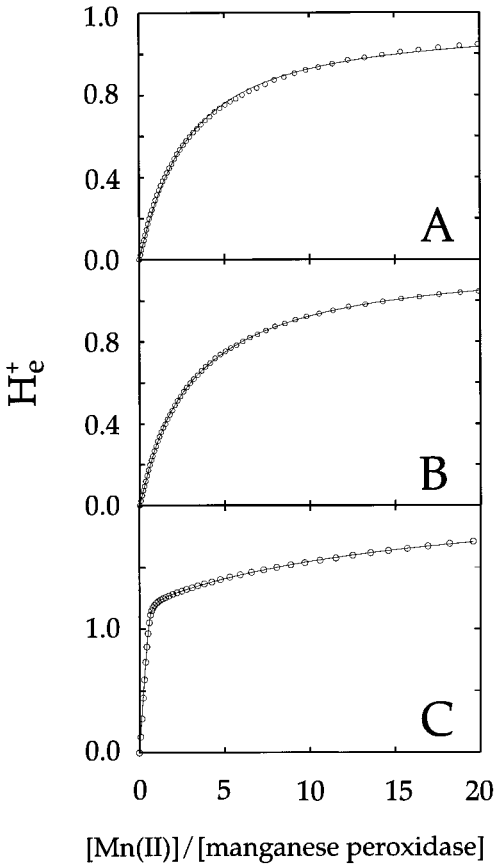

FIGURE 3: Representative potentiometric titration data and fits obtained for Mn(II) binding to manganese peroxidase (25 °C, μ = 0.1 M). (A) pH 4.5; data were fit assuming a 1:1 binding stoichiometry. (B) pH 4.5; data were fit assuming a 2:1 binding stoichiometry. (C) pH 6.0; data were fit assuming a 2:1 binding stoichiometry with reduced availability of the high affinity site. Positive values of H_e^+ indicate that protons are liberated upon Mn-(II) binding to the peroxidase.

even the two-site model was not sufficient to fit the data adequately. The only way that consistently acceptable fits to the data collected at the higher range of pH could be obtained was to allow the possibility that a fraction of the high-affinity site is unavailable for binding added Mn(II). For example, at pH 5.23, we estimate that the high-affinity site is not available for binding added metal in ~22% of the enzyme, while at pH 6.5 this site is inaccessible in ~58% of enzyme. The parameters derived from numerical analysis of the potentiometric data obtained in this study are summarized in Table 1. The magnitude of the q values for both sites exhibits a general trend toward a greater number of protons released upon Mn(II) binding with increasing pH values. The structural implications of this relationship cannot be assigned at this time. It should be noted that for titrations without distinct break points (i.e., weak binding interactions), the values q and K are highly correlated and, therefore, difficult to assess (*13*).

The effect of pH on the stability of Mn(II) binding to the high- and low-affinity sites is shown in Figure 4. Clearly, the affinity of the high-affinity site increases with pH. Although the general trend of the response of the low-affinity site to increasing pH is less clear, it seems most likely that its binding affinity decreases with increasing pH. This conclusion becomes more reasonable once it is recognized that despite the manner in which this drawing is, by necessity,

Table 1: Parameters Derived from Analysis of Potentiometric Titration of MnP with Mn(II)

pH	primary site	secondary site (100% available)	K_1/K_2
4.01	100% available		
	$K_1 = 1.8 \pm 0.3 \times 10^4$ M^{-1}	$K_2 = 4.1 \pm 0.2 \times 10^3$ M^{-1}	4.4
	$q_1 = -0.017 \pm 0.006$	$q_2 = 0.245 \pm 0.005$	
4.50	100% available		
	$K_1 = 1.6 \pm 0.4 \times 10^5$ M^{-1}	$K_2 = 1.31 \pm 0.07 \times 10^4$ M^{-1}	12
	$q_1 = 0.46 \pm 0.04$	$q_2 = 0.75 \pm 0.03$	
4.50[a]	100% available		
	$K = 2.65 \pm 0.03 \times 10^4$ M^{-1}		
	$q = 1.155 \pm 0.004$		
4.92	$100 \pm 4\%$ available		
	$K_1 = 3.3 \pm 0.8 \times 10^5$ M^{-1}	$K_2 = 1.5 \pm 0.3 \times 10^4$ M^{-1}	22
	$q_1 = 0.51 \pm 0.04$	$q_2 = 0.39 \pm 0.02$	
5.23	$78 \pm 4\%$ available		
	$K_1 = 6.0 \pm 0.1 \times 10^5$ M^{-1}	$K_2 = 3.0 \pm 0.5 \times 10^3$ M^{-1}	200
	$q_1 = 1.428 \pm 0.005$	$q_2 = 0.48 \pm 0.05$	
6.00	$57 \pm 3\%$ available		
	$K_1 = 2.7 \pm 0.2 \times 10^6$ M^{-1}	$K_2 = 3.0 \pm 0.2 \times 10^3$ M^{-1}	900
	$q_1 = 1.229 \pm 0.004$	$q_2 = 0.46 \pm 0.02$	
6.51	$42 \pm 7\%$ available		
	$K_1 = 2.4 \pm 0.1 \times 10^6$ M^{-1}	$K_2 = 3.0 \pm 0.3 \times 10^3$ M^{-1}	800
	$q_1 = 0.988 \pm 0.005$	$q_2 = 0.85 \pm 0.05$	

[a] Titration data were analyzed in terms of a single-site model. The fit produced by this model indicated a pronounced nonrandom deviation from the data.

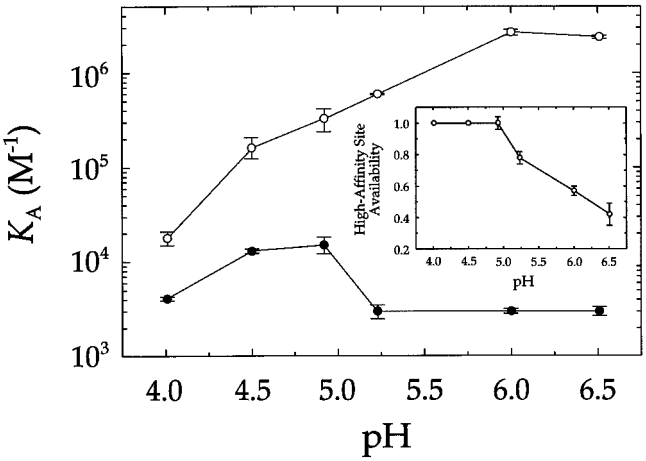

FIGURE 4: The pH-dependence of the association constants for Mn-(II) binding to the high-affinity and low-affinity sites of manganese peroxidase (25 °C, μ = 0.1 M): (○) high-affinity site (from potentiometric titrations), (●) low-affinity site (from potentiometric titrations). The inset shows the fraction of the high-affinity site available for Mn(II) binding.

prepared, we cannot determine which association constant is produced by which site at the lowest pH employed in this study. If, in fact, the relative affinities of the two sites reverse at this low pH, then the conclusion that the stability of Mn-(II) binding at the low-affinity site decreases with increasing pH becomes more consistent with the observed values. The divergence in affinity of the two sites for Mn(II) as a function of pH is shown in Table 1 as the ratio K_1/K_2. At pH 4.0, the two sites exhibit only a 4-fold difference in affinity, while a maximum difference of 900-fold is observed at pH 6.0.

DISCUSSION

Under lignolytic conditions, *P. chrysosporium* secretes several organic acids; therefore, the kinetics of Mn(II) oxidation to Mn(III) by MnP is routinely studied in the presence of a chelator. At the optimum pH for steady-state

turnover, pH 4.5, the reaction of compound I with Mn(II), either free or chelated, is too rapid to be measured by stopped-flow methods while the reaction of Mn(II) with compound II is facilitated by oxalate, lactate, and malonate (*14*, *15*). The mechanism proposed by Kuan et al. (*14*) requires Mn(II) to be chelated to support steady-state turnover of MnP. While chelators are required for maximal activity of MnP (*15*), the crystallographic studies (*4*) and earlier binding studies (*5*) establish that the presence of a chelator is not a requirement for Mn(II) binding to the enzyme. The latter studies may suggest the involvement of a ligand displacement mechanism.

Steady-state kinetic analysis employing variants of MnP in which the anionic charge at position 35, 39, or 179 has been removed (E35Q, E39Q, D179N, and the double variant E35Q/D179N) yielded K_m values for the substrate Mn(II) that were ~50-fold or more greater than the corresponding value for the wild-type enzyme and k_{cat} values for Mn(II) oxidation that were ~300-fold or more lower than that for wild-type MnP (*16*, *17*). Crystallographic studies (*4*) of the D179N variant and the double variant did not detect the presence of a cation in the vicinity of the Mn(II) binding site that was identified in the structure of the wild-type enzyme. This result strongly suggests that the Mn(II) binding site described in the three-dimensional structure (*3*) is also the productive substrate oxidation site both for MnP compound I and MnP compound II oxidation of Mn(II) (*17*, *18*; M. D. Sollwijn, H. Youngs, K. Kishi, and M. H. Gold, in preparation).

The binding of Mn(II) to MnP appears to enhance the thermal stability of the enzyme. Crystallization experiments (*4*) indicated that MnP in the presence of 2 mM Mn(II) was less prone to denaturation and crystal bleaching at ambient temperatures than was MnP crystallized in the absence of added Mn(II). Our preliminary studies in which the thermal stability of MnP is evaluated by monitoring ellipticity at 222 nm indicate that the melting temperature (T_m) of MnP can be increased by more than 5 °C in the presence of a 50-fold molar excess of Mn(II) (data not shown). Characterization of the Mn(II) affinity of this metal binding site was initiated by Wariishi et al. (*5*). In that spectrophotometric titration study, the binding of Mn(II) to MnP was found to involve formation of a 1:1 complex at pH 4.5. The present study involving two titration techniques employing similar solution conditions describes a marked effect of pH on the affinity of the enzyme for Mn(II). The potentiometric titrations reported here identify a high-affinity and a low-affinity site, while the experiments employing electronic difference spectroscopy detected only a single site for Mn(II) binding. Potentiometric titration is a technique that is well suited to detection of a low-affinity binding site in the presence of a high-affinity site (*12*). The inclusion of chelating agents in the difference spectroscopy experiments and in the kinetic studies may preclude the ability of these experiments to detect the low-affinity site owing to competition for Mn(II) by the chelator. However, the nature of the potentiometric titrations rules out the inclusion of a chelator in these experiments.

It is interesting to compare the relative effects of pH on the activity of MnP and the affinity for Mn(II) binding. As noted previously (*9*), the pH optimum for MnP is 4.5. More recent work (*15*) defines two acid dissociation constants (pK_as of 6.1 and 3.4) that appear to be important in regulating the activity of MnP. At pH 4.5, we find a 12-fold difference in Mn(II) affinity for the two sites. As seen in Figure 4, the stability of Mn(II) binding to MnP at pH 4.5 is not maximal for either the high-affinity or low-affinity site. Comparison of the results from the potentiometric titrations and from the spectrophotometric titrations, which presumably reflect the behavior of the crystallographically defined Mn(II) binding site (Figure 1), suggests that the curves shown in Figure 4 do, in fact, cross over at low pH so that the site for Mn(II) depicted in Figure 1 has a lower affinity for Mn(II) than that of the secondary site at very low pH.

At pH 4.5, intermediate affinity of both sites is observed, consistent with the properties required for optimal catalytic turnover. Presumably catalytic turnover is optimal under conditions where Mn(II) binding is sufficiently great to permit facile assembly of an enzyme−substrate complex, but not so great that the enzyme−product complex will fail to dissociate at an adequate rate. The resulting requirement for a balance between affinity and release appears to be fully consistent with the results reported here for Mn(II)−MnP complex formation. Under physiological conditions, of course, the Mn(II) would be chelated by oxalate, a consideration that will also contribute to the relative stability of Mn(II) binding to the two types of binding site and to turnover of the enzyme.

The observation that MnP binds two Mn(II) ions with different affinities in a pH-dependent fashion is reminiscent of the behavior that we have recently reported for substrate binding by cytochrome *c* peroxidase (*12*). In both cases, the peroxidases involved catalyze the oxidation of 2 equiv of substrate through facilitation of two one-electron transfer reactions. At least three possibilities can be suggested at this time for the catalytic significance of the second site. First, the low-affinity site may simply represent nonspecific binding that is irrelevant to catalysis. Second, it may be that substrate binding at one site is optimal for transfer of an electron to the radical center of the compound I intermediate (*19*), while substrate binding at the other site is optimal for transfer of an electron to the iron center of compound II. Recent steady-state kinetic studies (*18*; M. D. Sollwijn, H. Youngs, K. Kishi, and M. H. Gold, in preparation), however, argue that compounds I and II are reduced by Mn(II) bound at a single site. Finally, perhaps substrate bound at only one of these sites is effective at electron transfer, and binding of substrate at the second site facilitates displacement of the oxidized substrate from the functional binding site by reducing diffusion of the second equivalent of substrate to this site from three dimensions to two dimensions.

While we have clear evidence for the binding of a second equivalent of Mn(II) to MnP, our experiments do not provide information concerning the location of the second binding site. Modeling studies by Loew and co-workers (*6*, *7*) based on a simulated structure for MnP suggested the existence of two Mn(II) binding sites with interaction energies less favorable than that of the primary Mn(II) binding site at Glu35, Glu 39, Asp179, and a heme propionate. These sites merit further consideration now that the three-dimensional structure of MnP has been determined. Banci et al. (*20*) detected the binding of several equivalents of Mn(II) to MnP by EPR spectroscopy. Although the locations of these

binding sites were not determined, comparison with results obtained from similar studies with lignin peroxidase led to the suggestion that they are in the carbohydrate attached to the protein.

The ^{1}H NMR study of Banci et al. (*20*) places the binding site for a single equivalent of Mn(II) on the 8-methyl side of the heme. This suggestion appeared to be in agreement with the model proposed by Harris et al. (*21*), which found that adducts at the δ-meso carbon of the heme inhibit Mn(II) oxidation. However, the distances between the Mn(II) and the heme 3-methyl and 8-methyl groups calculated by Banci et al. (*20*) are in reasonable agreement with distances obtained from the crystallographically determined structure [Mn(II) to 3-methyl group, 14.9 Å; Mn(II) to 8-methyl group, 9.9 Å] with the Mn(II) bound to residues 35, 39, 179, and the heme 6-propionate. Therefore, it seems likely that the Mn(II) binding site detected by ^{1}H NMR spectroscopy is presumably the site detected in the spectrophotometric titrations (*5*), the crystallographic studies (*3, 4*), the studies of mutants (*17*), and the high-affinity site observed in our potentiometric titrations. A possible explanation for the low affinity constant for Mn(II) (10^4 M^{-1} at pH 6.5, 0.1 M phosphate buffer, 0.5 mM MnP) reported by Banci et al. (*20*) is that electrostatic screening effects arising from the high protein concentration required for NMR measurements reduce the metal ion binding affinity (*22*).

Perhaps the most unusual observation arising from the current study concerns the apparent unavailability of the high-affinity binding site for Mn(II) above pH 5.0. The recent crystallographic study of Sundaramoorthy et al. (*4*) suggests that significant levels of Mn(II) are present in the Mn(II) binding site of crystals prepared at pH 6.5 from Chelex-treated MnP, even though atomic absorption spectroscopic analyses indicated that the protein contained <0.2% Mn. This observation correlates well with our finding that the high-affinity Mn(II) site is not quantitatively available for binding of added Mn(II) under certain solution conditions. It therefore appears that at pH 6.5, the Mn(II) binding site in a portion of the MnP molecules may not permit bound Mn(II) to exchange with solvent. As the pH is decreased to pH 5, the MnP becomes uniform in its ability to exchange free and bound Mn(II). From the pH-dependent nature of this behavior, it seems reasonable to suggest that some pH-linked structural change in the Mn(II) binding region of the peroxidase may be responsible and that one or more His residues may be involved.

Examination of the proposed Mn(II) binding site (*3*) reveals that His 38 and 340 are, in fact, located in close proximity to the proposed Mn(II) binding site. As a result, we tentatively suggest that as one or both of these His residues is deprotonated with increasing pH, the structure or the dielectric properties of the Mn(II) binding site changes in a fashion that prevents the function of this site in the free exchange of Mn(II). Alternative origins for this effect, of course, are equally plausible in the absence of additional information.

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