Mutagenesis of the Mn^{2+}-Binding Site of Manganese Peroxidase Affects Oxidation of Mn^{2+} by both Compound I and Compound II†

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ABSTRACT: The present study investigates whether compound I and compound II of manganese peroxidase from the white-rot fungus *Phanerochaete chrysosporium* utilize the same Mn-binding site for catalysis. Manganese peroxidase was expressed from its cDNA in *Escherichia coli* and refolded from inclusion bodies to yield fully active enzyme. Three mutants of the enzyme were generated by site-directed mutagenesis. Each of the three amino acid residues proposed to be involved in Mn^{2+} binding, E35, D179, and E39, was mutated. The acidic side chains of E35 and E39 were shortened by one carbon to the acidic group D, and the acidic side chain of D179 was shortened by one carbon to the alkyl group A. These mutants, E35D, D179A, and E39D, were used to determine whether Mn^{2+} reacts at the same site with both compound I and compound II of manganese peroxidase and to determine whether phenolic substrates for the enzyme react at this site. Our results conclusively demonstrate that E35 and D179 residues are involved not only in Mn^{2+} binding but also in electron transfer from Mn^{2+} to the enzyme for both compound I and compound II. In contrast, E39 is not critically important to either process. None of the three residues is involved in reactions with phenolic substrates or with H_2O_2.

Manganese peroxidases (MnPs)[1] are extracellular heme-proteins secreted by white-rot fungi under conditions of secondary metabolism (*1*, *2*). These enzymes are part of the lignin-degrading systems of these fungi (*3*, *4*). Manganese peroxidase catalyzes the oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} in turn acts as a diffusible oxidant that enters the lignin polymer, bringing about its oxidative depolymerization (*2*, *5*). Manganese peroxidases have been found in all lignin-degrading white-rot fungi that have been tested for their presence (*6*). The manganese peroxidase from the white-rot fungus *Phanerochaete chrysosporium* is able to depolymerize lignin *in vitro* (*2*, *7*, *8*). MnP undergoes a classical peroxidase reaction cycle. It first reacts with H_2O_2 to yield a two-electron-oxidized enzyme intermediate, compound I (*9*). Compound I is reduced by Mn^{2+} to generate Mn^{3+} and a one-electron-oxidized enzyme intermediate, compound II (*10*). Compound II is also reduced by Mn^{2+}, returning the enzyme to its resting state and generating another Mn^{3+}.

The reactions of compound I have a number of significant differences with respect to those of compound II. While compound I can be reduced by free noncomplexed (hexaaquo) Mn^{2+}, compound II must be reduced by Mn^{2+} complexed to a chelator (*10*). Compound I reacts with free Mn^{2+} at a considerably faster rate than complexed Mn^{2+} reacts with compound II (*10*). The reaction of compound II with complexed Mn^{2+} is the rate-limiting step of the MnP catalytic cycle. Compound I can also be reduced by a variety of phenolic molecules, though at a considerably slower rate that it is reduced by Mn^{2+} (*9*), while phenolic compounds reduce compound II at a rate so slow as to be insignificant under steady-state conditions.

The X-ray crystal structure for MnP from *P. chrysosporium* has been solved (*11*). A region of electron density was modeled as a Mn^{2+} ion bound to the surface of the protein. The modeled Mn^{2+} was in a position to interact with a heme propionate group, two water molecules, and three amino acid residues, E35, E39, and D179. The authors proposed that this is the Mn^{2+}-binding site for the enzyme. Subsequent site-directed mutagenesis of these sites (single mutants D179N, E35Q, and E39Q and the double mutant D179N/E35Q) essentially abolished the steady-state activity (to less than 1 s^{-1} from 300 s^{-1}) (*12*, *13*). These mutants were characterized by steady-state kinetics. Whereas the reactivity of compound II was characterized in these mutants, no experiments were performed on the reactions of compound I with Mn^{2+}.

Our kinetic data with wild-type MnP had already shown differences in the reactivities of compound I and compound II (see above). Furthermore, EPR data indicated the presence of more than one Mn-binding site in MnP (*14*). For these reasons, we investigated whether compound I and compound II of MnP utilize the same Mn-binding site for catalysis. Using a recently developed bacterial expression system for this enzyme (*15*, *16*), we have used site-directed mutagenesis to create three mutants of MnP. We have individually mutated each of the three amino acid residues proposed to be involved in Mn^{2+} binding. In each mutant, one of the

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[1] Abbreviations: MnP, manganese peroxidase; DMP, 2,6-dimethoxyphenol; NHE, normal hydrogen electrode; CV, cyclic voltammogram; ET, electron transfer; λ, reorganization energy; *E*, electrochemical driving force.

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Mn-binding side chains was shortened by one carbon, resulting in an acidic D side chain, in the case of E to D mutants, or resulting in an alkyl A side chain, in the case of the D to A mutant. By generating, purifying, and kinetically characterizing mutants E35D, E39D, and D179A, we addressed the possibility of whether Mn^{2+} reacts with compound I and compound II at the same site and whether phenolic and peroxide substrates for compounds I and II of MnP react at this site.

MATERIALS AND METHODS

Chemicals. Hydrogen peroxide was purchased from Fisher Scientific. Oxidized glutathione, ampicillin, chloramphenicol, $CaCl_2$, phenylmethanesulfonyl fluoride, Pharmalytes, and oxalic acid were purchased from Sigma Chemical Co. Ultrodex was purchased from Pharmacia. IPTG was purchased from Promega Biotech. Oligonucleotide synthesis was done on an Oligo 100 apparatus from Beckman Instruments (Fullerton, CA). The concentration of H_2O_2 was determined spectrophotometrically at 240 nm using an extinction coefficient of 39.4 M^{-1} cm^{-1} (*17*).

Expression and Refolding of MnPs. All the MnPs used in this study were recombinant enzymes, expressed in *Escherichia coli* as previously described (*16*), using the pET21aH4looped(+) vector and *E. coli* strain BL21(DE3)-pLysS (Novagen, Madison, WI). Recombinant MnPs were refolded to active holoenzyme as described previously (*16*). To produce the E35D, E39D, and D179A mutants, oligonucleotide-directed mutagenesis was used according to the procedure of Kunkel (*18*). CJ236 was the dut^-ung^- strain of *E. coli* used. The oligonucleotides used to create the E35D, E39D, and D179A mutations were TGGGCATCA-TCGCCACA, GACGGATGACATCGTGG, and CGTC-GACCTTGGCCGCA, respectively (for each oligonucleotide, the mutant codon is underlined). Sequencing was used to identify successfully mutated transformants (results not shown). The mutant pET21aH4looped(+) vectors were isolated from positive clones and transformed into BL21-(DE3)pLysS cells for expression.

Purification of Recombinant MnPs. A modification of the previously described scheme (*16*) was used to purify the recombinant MnPs. Refolded preparations of recombinant MnP were dialyzed exhaustively against 50 mM Tris-HCl at pH 8.0 and 4 °C. Upon dialysis, a precipitate formed. The dialyzed sample was centrifuged at 10000*g* for 15 min at 4 °C, and the supernatant was loaded onto a DEAE-BioGel A column (1.5 cm × 20 cm) in 50 mM Tris-HCl at pH 8.0. The enzyme was eluted with 300 mL of a linear 0 to 0.15 M $CaCl_2$ gradient in column buffer. Fractions exhibiting the highest specific activity were pooled and dialyzed against distilled, deionized H_2O. The preparation was then further purified by preparative flatbed isoelectric focusing using a Pharmacia/LKB Multiphor II apparatus (Pharmacia, Piscataway, NJ). Gels were made of Ultrodex, and a pH 4.2 to 8.0 gradient was established using Pharmalytes. After isoelectric focusing, the enzyme was dialyzed against distilled, deionized H_2O and stored until it was assayed. The MnP preparations typically exhibited Rz values (the ratio of absorbances at 407 and 280 nm) in the 3−4 range, characteristic of highly purified MnP.

Steady-State Methods. The determinations of k_{cat}, K_m-(H_2O_2), and K_m(Mn^{2+}/malonate) were done in 50 mM sodium malonate at pH 4.5 and 28 °C. The oxidation of Mn^{2+} to Mn^{3+} was monitored at 270 nm. An extinction coefficient of 8.5 mM^{-1} was used for the Mn^{3+}/malonate complex (*10*). It was necessary to use malonate as the Mn^{2+} chelator instead of oxalate, the probable physiological chelator used by MnPs, because oxalate is unable to chelate Mn^{2+} in a 1:1 complex at the high Mn^{2+} concentrations required to saturate the Mn-binding mutants. At these Mn^{2+} concentrations, there is inefficient oxalate to form the 1:1 complex with Mn^{2+} that is required to react with the enzyme (*10*). Malonate is capable of chelating Mn^{2+} in a 1:1 complex at Mn^{2+} concentrations in the range used for these studies (*10*).

MnP concentrations were quantitated spectrophotometrically at 407 nm using an extinction coefficient of 127 mM^{-1} for MnP H4 (*19*). The K_m for H_2O_2 was determined with saturating $MnSO_4$ (0.2 mM for the wild-type MnP and for the E39D mutant and 15 mM for the E35D and D179A mutants). The K_m for Mn^{2+}/malonate was determined at 0.05 mM H_2O_2. Due to H_2O_2 inhibition of the enzymes, the initial velocity data were analyzed according to the method of Cleland (*20*), as discussed previously (*16*).

Transient-State Methods. The stopped-flow apparatus was purchased from KinTek Instruments (State College, PA) and contained a 2.6 cm light path. Each kinetic trace is composed of the average of three shots. All reactions were run at 28 °C.

MnP is a heme-containing peroxidase and undergoes a classical peroxidase reaction cycle. The resting enzyme is also known as the ferric enzyme as its heme iron is in the ferric state. During the MnP reaction cycle, the ferric enzyme first reacts with peroxide and is oxidized by two electrons to form compound I, in which the heme iron is in a ferryl state. Compound I is reduced by two successive one-electron reductions to first generate a one-electron-oxidized intermediate, compound II, and then return the enzyme to its resting, ferric state.

The rate of compound I formation from the reaction of ferric enzyme with H_2O_2 was monitored at 397 nm, the isosbestic wavelength between compound I and compound II (*10*). The rate of compound I formation was studied with 0.7−1.0 μM recombinant MnP and varying concentrations of H_2O_2 in 20 mM sodium tartrate at pH 4.5.

The serial conversion of compound I to compound II to compound III by H_2O_2 was monitored at 417 nm, as described previously (*21*). Varying amounts of H_2O_2 were added to typically 1 μM enzyme in 10 mM sodium succinate at pH 4.5. Because the rate of compound III formation was significantly slower than the rate of compound II formation, the resulting curves could be fitted to a double-exponential equation to obtain both rate constants for this two-step process.

Reactions of compound I and compound II with Mn^{2+} and DMP were studied under single-turnover pseudo-first-order conditions with the stopped-flow apparatus in the double-mixing, three-syringe mode. The stopped-flow apparatus is designed with a stepper motor and without a stopping syringe to allow for these double-mixing experiments as previously described (*10*). Briefly, to generate compound I, ferric enzyme was mixed with 1 equiv of H_2O_2. The solution was then aged in the delay line for 1.5 s to allow for complete formation of compound I. The second push from the stepper motor forced the mixing of the resultant compound I with

the contents of the third syringe which contained the reducing substrate (Mn^{2+} or DMP).

The reduction of compound I to compound II by Mn^{2+} was monitored at 417 nm, the isosbestic wavelength between resting ferric enzyme and compound II. Typically, 1 μM enzyme was used with varying Mn^{2+} concentrations in 20 mM sodium tartrate at either pH 2.5 or 4.5, as indicated in the Results.

The reduction of compound II by Mn^{2+}/malonate was monitored at 426 nm, the isosbestic wavelength between the resting ferric enzyme and compound I. Typically, 1 μM enzyme was used with varying Mn^{2+} concentrations in 20 mM sodium malonate at pH 4.5.

The reduction of compound I and II by DMP were monitored sequentially at 426 nm, the isosbestic wavelength between the resting ferric enzyme and compound I. Typically, 1 μM enzyme was used with varying DMP concentrations in 50 mM sodium tartrate at pH 4.5. Because the rate of compound II reduction was significantly slower than the rate of compound I reduction, the resulting curves could be fitted to a double-exponential equation to obtain both rate constants for this two-step process.

Cyclic Voltammetry. Electrochemical determinations were carried out on a PAR Model 273A Potentiostat/Galvanostat operated using the model 270 software on a Gateway 486 IBM-compatible computer. Approximately 3−4 mL of 15−40 μM enzyme in 100 mM sodium sulfate and 40 mM potassium phosphate at pH 7.0 was placed in an anaerobic spectrochemical cuvette, with airtight ports for a carbon working electrode, a platinum wire counter electrode, and an Ag/AgCl reference electrode. The carbon electrode was polished with 0.05 μM diamond paste prior to each use. The anaerobic cuvette contained a stopcock, and the enzyme solutions were made anaerobic by alternating regimens of vacuum evacuation and purging with oxygen-free argon gas. At scan rates slower than 1000 mV/s, the charge transfer to MnP was irreversible. The Fe^{3+}/Fe^{2+} couple of the heme group could be reduced, but could not be reoxidized. As faster scan rates and higher enzyme concentrations were used, the charge transfer become quasi-reversible. The cyclic voltammogram (CV) of each enzyme solution was obtained by subtracting the CV of the electrolyte solution from the CV of the electrolyte plus enzyme solution. The midpoint potential ($E^{\circ\prime}$) was calculated as described by Bard and Faulkner (*22*). To obtain an $E^{\circ\prime}$ with respect to the normal hydrogen electrode (NHE), 197 mV was subtracted.

RESULTS

Steady-State Parameters. The three MnP mutants, E35D, E39D, and D179A, were expressed in a bacterial host, purified, and compared kinetically to the wild-type enzyme, also expressed in the bacterial host. The wild-type MnP as expressed in a bacterial host is spectrally and kinetically identical to the native wild-type enzyme isolated from *P. chrysosporium* (*16*). The steady-state kinetic values for the wild-type MnP and the three mutants are shown in Table 1. The steady-state kinetics were determined using 50 mM sodium malonate at pH 4.5 as both a buffer and a Mn^{2+} chelator. This ensured that Mn^{2+} remained in the 1:1 ion/chelator complex preferred by the compound II form of the enzyme, even at the high Mn^{2+} concentrations required to saturate the E35D and D179A mutants. Kuan et al. (*10*) showed that, when present in excess of the Mn^{2+} concentrations used in this study, oxalate, the probable physiological chelator used by the enzyme, forms a 2:1 Mn^{2+}/chelator complex which does not react with compound II.

Table 1: Steady-State Kinetic Parameters of Wild-Type and Mutants of MnP[a]

enzyme	$K_m(H_2O_2)$ (μM)	$K_m(Mn^{2+})$ (mM)	k_{cat} (s^{-1})	$k_{cat}/K_m(H_2O_2)$ ($M^{-1}\ s^{-1}$)	$k_{cat}/K_m(Mn^{2+})$ ($M^{-1}\ s^{-1}$)
wild type	147	0.033	508	3.5×10^6	1.4×10^7
D179A	2.8	1.1	3.1	1.1×10^6	2.8×10^3
E35D	2.1	1.4	5.3	2.5×10^6	3.8×10^3
E39D	54	0.036	410	7.6×10^6	1.1×10^7

[a] All enzymes were expressed in *E. coli* as described in Materials and Methods. Assays are as described in Materials and Methods. The initial velocity data were analyzed according to the method of Cleland (*20*).

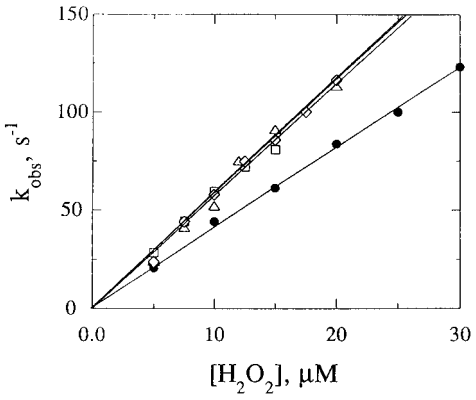


FIGURE 1: Compound I formation from the reaction of ferric enzyme with H_2O_2. Data were obtained from stopped-flow experiments at 28 °C using 0.7−1.0 μM MnP, 20 mM sodium tartrate (pH 4.5), and varying concentrations of H_2O_2. The symbols are as follows: wild-type MnP (●), E35D (△), E39D (□), and D179A (◇).

The E39D mutant is, within experimental error, kinetically identical to that of the wild-type MnP. However, both the E35D mutant and the D179A mutant show significant differences. Both showed a 100−150-fold decrease in k_{cat}, a 30−40-fold increase in K_m for Mn^{2+}, and a 30−40-fold decrease in K_m for H_2O_2. The increase in K_m for Mn^{2+} for these two mutants is reflected in the 10^4-fold decrease in the k_{cat}/K_m (Mn^{2+}) values. This value is a measure of the reactivity of the enzyme with the substrate. However, the $k_{cat}/K_m(H_2O_2)$ values for these two mutants are not radically changed. This indicates that, despite the decrease in the K_m for H_2O_2 (which is really just a ratio of k_{cat} to k_{cat}/K_m, the two fundamental kinetic constants for the enzyme), the reactivity of MnP toward H_2O_2 has not been changed by either the E35D mutation or the D179A mutation.

Reactivity of Ferric Enzyme. This k_{cat}/K_m value for H_2O_2 is in agreement with results obtained from stopped-flow studies (Figure 1 and Table 2). The rate of compound I formation is not significantly changed by any of the mutations. This is consistent with the lack of change in the $k_{cat}/K_m(H_2O_2)$ for the mutants.

Reactivity of Compound I. Also consistent with the steady-state results, the E39D mutation did not significantly alter the reactivity of compound I toward Mn^{2+} (Figure 2A). In contrast, the D179A and E35D mutants show significant differences. The kinetic alterations introduced by the D179A and E35D mutations are confined to the reactivities of the

Table 2: Transient-State Kinetic Parameters for Wild-Type and Mutants of MnP[a]

	rate for reactions of ferric (M^{-1} s^{-1})	rate for reactions of compound I (M^{-1} s^{-1})			
enzyme	H_2O_2 pH 4.5	Mn^{2+} pH 2.5	Mn^{2+} pH 4.5	DMP pH 4.5	H_2O_2 pH 4.5
wild type	4.0×10^6	4.2×10^4	$>10^7$	2.5×10^4	2.0×10^4
D179A	6.0×10^6	6.6×10^2	3.0×10^5	5.2×10^4	6.0×10^4
E35D	6.1×10^6	1.9×10^2	1.1×10^6	1.9×10^4	6.9×10^4
E39D	6.0×10^6	1.8×10^4	$>10^7$	1.8×10^4	1.8×10^4

[a] All enzymes were expressed in *E. coli* as described in Materials and Methods. Assays are as described in Materials and Methods.

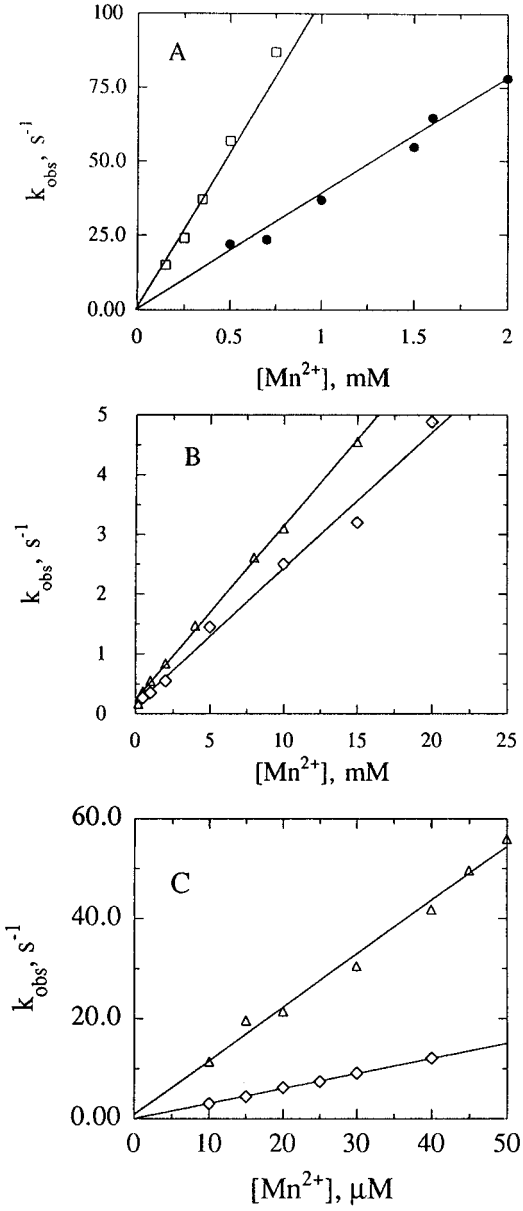


FIGURE 2: Reactions of compound I with Mn^{2+}. (A) Wild-type MnP (●) and E39D (□) at pH 2.5. (B) E35D (△) and D179A (◇) at pH 2.5. (C) E35D (△) and D179A (◇) at pH 4.5. Data were obtained from stopped-flow experiments at 28 °C using approximately 1 μM MnP, an equimolar concentration of H_2O_2, 20 mM sodium tartrate (pH 2.5 or 4.5, as indicated), and varying concentrations of Mn^{2+}.

mutant enzymes toward Mn^{2+}. At pH 4.5, the pH optimum for this enzyme, compound I of wild-type MnP reacts with Mn^{2+} at a rate that is too fast to measure in the stopped-flow spectrophotometer. As rate constants up to approximately 10^7 M^{-1} s^{-1} can be measured for peroxidases under pseudo-first-order conditions with this instrument, the rate constant at which compound I is reduced to compound II by Mn^{2+} must be greater than this value. [For cytochrome *c* peroxidase, rate constants for this step have been successfully determined using a second-order treatment (*39*, *40*), but comparable experiments were not attempted in this study.] At the nonoptimal pH of 2.5, this rate constant is slowed in the wild-type enzyme to 4.2×10^4 M^{-1} s^{-1} (Figure 2A). The D179A and E35D mutations both reduced the rate constant for the reduction of compound I to compound II by Mn^{2+} at pH 2.5 by 2 orders of magnitude, to 6.6×10^2 M^{-1} s^{-1} for the D179A mutant and to 1.9×10^2 M^{-1} s^{-1} for the E35D mutant (Figure 2B). These mutations also slowed this process at pH 4.5 sufficiently so that the rate constant could now be measured. At pH 4.5, the rate constant for the reduction of compound I to compound II by Mn^{2+} was 0.3×10^6 M^{-1} s^{-1} for the D179A mutant and 1.1×10^6 M^{-1} s^{-1} for the E35D mutant (Figure 2C).

Compared to the oxidation of Mn^{2+}, the mutations had little effect on the reactivity of compound I toward H_2O_2 (Figure 3) or DMP (Figure 4). Compound I of MnP can be reduced to compound II by H_2O_2 and by phenols, such as DMP, though at rates much slower than the reduction of compound I by Mn^{2+}. The rate constants for the reduction of compound I to compound II by DMP for the D179A and

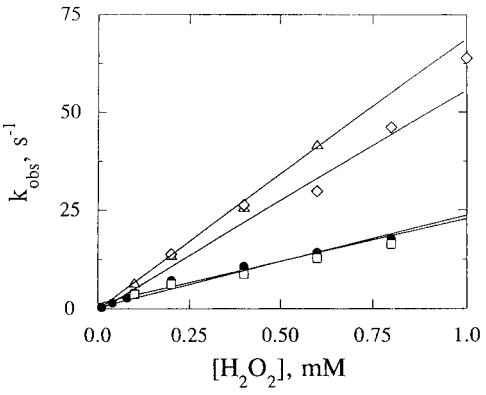


FIGURE 3: Reactions of compound I with H_2O_2. Data were obtained from stopped-flow experiments at 28 °C using approximately 1.0 μM MnP, 20 mM sodium succinate (pH 4.5), and varying concentrations of H_2O_2. The symbols are as follows: wild-type MnP (●), E35D (△), E39D (□), and D179A (◇).

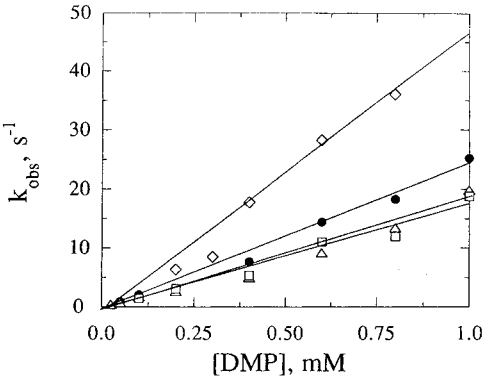


FIGURE 4: Reactions of compound I with DMP. Data were obtained from stopped-flow experiments at 28 °C using approximately 1.0 μM MnP, an equimolar concentration of H_2O_2, 20 mM sodium tartrate (pH 4.5), and varying concentrations of DMP. The symbols are as follows: wild-type MnP (●), E35D (△), E39D (□), and D179A (◇).

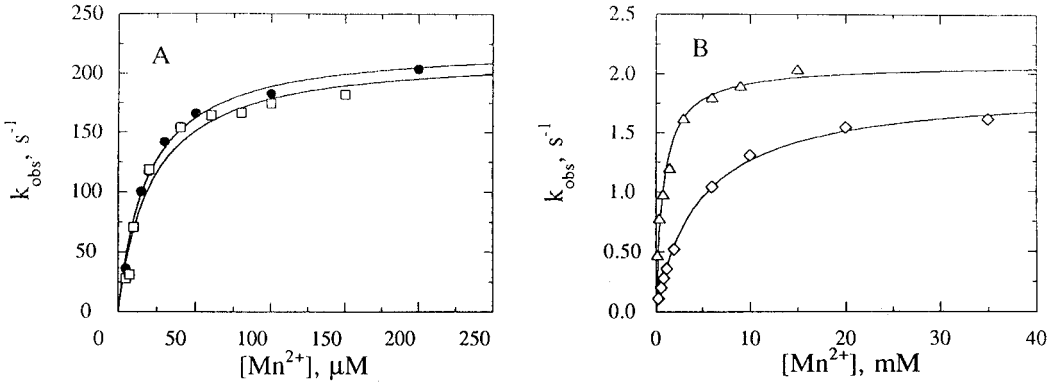


FIGURE 5: Reactions of compound II with Mn^{2+}/malonate at pH 4.5. (A) Wild-type MnP (●) and E39D (□). (B) E35D (△) and D179A (◇). Data were obtained from stopped-flow experiments at 28 °C using approximately 1 μM MnP, an equimolar concentration of H_2O_2, 20 mM sodium malonate (pH 4.5), and varying concentrations of Mn^{2+}.

Table 3: Transient-State Kinetic Parameters for Compound II of Wild-Type and Mutants of MnP[a]

enzyme	K_d for Mn^{2+} (μM)	k for Mn^{2+} (s^{-1})	rate for DMP ($M^{-1}s^{-1}$)	rate for H_2O_2 ($M^{-1}s^{-1}$)
wild type	20	225	92	2.3×10^3
D179A	4800	1.9	130	0.56×10^3
E35D	850	2.1	49	0.81×10^3
E39D	22	217	85	1.8×10^3

[a] All enzymes were expressed in *E. coli* as described in Materials and Methods. Assays are as described in Materials and Methods.

E35D mutants were not significantly different from the corresponding rate constant for the wild-type enzyme (Table 2). While the rate constants for the conversion of compound I to compound II by H_2O_2 were approximately 3-fold higher in the E35D and D179A mutants than in the wild-type enzyme, these changes were not nearly as dramatic as the rate changes seen with Mn^{2+} as the reducing substrate.

Reactivity of Compound II. The rate at which compound II is reduced to the ferric (resting) state of MnP by Mn^{2+}/malonate was not drastically affected by the E39D mutation (Figure 5A) but was affected by the D179A and E35D mutations (Figure 5B). The rate of reduction was decreased by 2 orders of magnitude, to 1.9 s^{-1} for the D179A mutant and to 2.1 s^{-1} for the E35D mutant. Unlike compound I, compound II can be saturated with Mn^{2+}, though the Mn^{2+} must be complexed to a chelator in order to see reactivity; hence, a K_d for the Mn^{2+}/chelator complex can be obtained. The K_d of compound II for Mn^{2+}/malonate is 20 μM for the wild-type enzyme but 4.8 mM for the D179A mutant and 0.85 mM for the E35D mutant, increases of 240- and 42.5-fold, respectively (Table 3).

In single-turnover experiments, DMP can reduce compound II of MnP to the ferric form of the enzyme. Similar to the reactivity of compound I, the D179A and E35D mutations did not affect the rate constant for this process for compound II (Figure 6 and Table 3). Compound II of MnP can be oxidized by H_2O_2 to a noncatalytic enzyme intermediate, compound III, also known as the oxycomplex (*9*). The D179A and E35D mutations had no significant effect on the rate constant for this process (Figure 7).

Electrochemistry. The midpoint potentials of the Fe^{3+}/Fe^{3+} couple of each of the MnP mutants were determined by cyclic voltammetry at pH 7.0. Figure 8 shows the voltammograms of the wild type and the three mutant enzymes. The cyclic voltammograms were acquired using

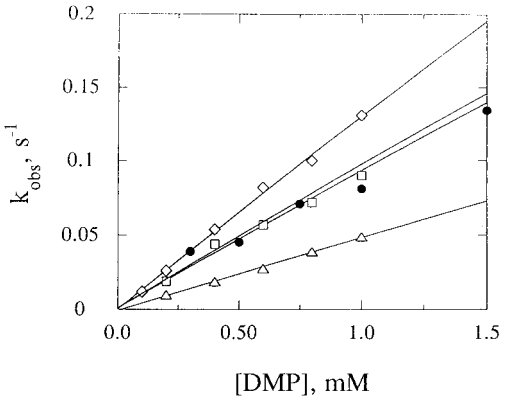


FIGURE 6: Reactions of compound II with DMP. Data were obtained from stopped-flow experiments at 28 °C using approximately 1.0 μM MnP, an equimolar concentration of H_2O_2, 20 mM sodium tartrate (pH 4.5), and varying concentrations of DMP. The symbols are as follows: wild-type MnP (●), E35D (△), E39D (□), and D179A (◇).

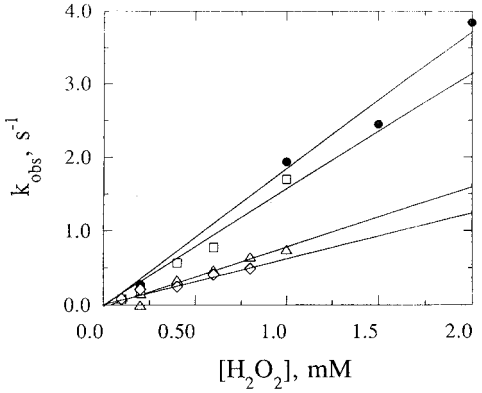


FIGURE 7: Reactions of compound II with H_2O_2. Data were obtained from stopped-flow experiments at 28 °C using approximately 1.0 μM MnP, 20 mM sodium succinate (pH 4.5), and varying concentrations of H_2O_2. The symbols are as follows: wild-type MnP (●), E35D (△), E39D (□), and D179A (◇).

carbon electrodes. When corrected for the difference between Ag/AgCl and normal hydrogen electrodes (NHEs), wild-type recombinant MnP gave an $E^{\circ\prime}$ of −96 mV vs the NHE. This is in close agreement with the $E^{\circ\prime}$ of −93 mV vs the NHE determined for the native MnP H4 by potentiometric titrations (*19*). The midpoint potentials of the three MnP mutants are shown in Table 4. None of the mutants shows any significant changes with respect to wild-type MnP in the redox potential of its Fe^{3+}/Fe^{2+} couple.

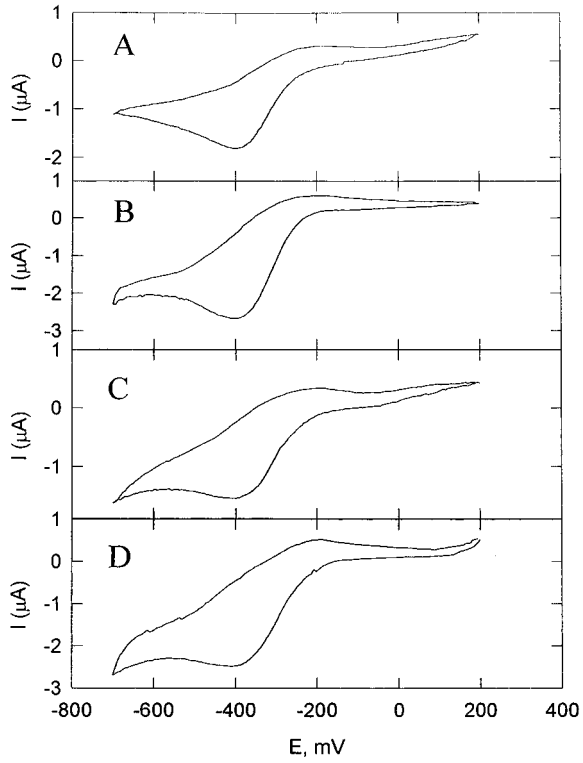


FIGURE 8: Cyclic voltammograms at 1000 mV/s of (A) wild-type MnP, (B) E35D, (C) E39D and (D) D179A. Cyclic voltammograms were performed at pH 7.0 as described in Materials and Methods using a Ag/AgCl reference electrode. Positive current is anodic, and negative current is cathodic.

Table 4: Midpoint Potentials of the Fe^{3+}/Fe^{2+} Couple[a]

enzyme	$E^{\circ\prime}$ [b] (mV)
wild type	−96
D179A	−101
E35D	−93
E39D	−98

[a] All enzymes were expressed in *E. coli* as described in Materials and Methods. [b] Cyclic voltammetry was performed in 100 mM sodium sulfate and 40 mM potassium phosphate at pH 7.0 using a carbon working electrode and a Ag/AgCl reference electrode. All $E^{\circ\prime}$ values are given with respect to the NHE to ease comparisons with earlier work.

DISCUSSION

The Mn-binding site of MnP (Figure 9) was first identified by X-ray crystallography (*11*) and confirmed by site-directed mutagenesis (*13*, *23*). The ligands involved in Mn binding were identified as D179, E35, E39, a heme propionate, and two water molecules. Kusters-van Someren et al. (*13*) created the D179N mutant of MnP and found that the D179N mutation reduced the k_{cat} of MnP 265-fold and increased the K_m of Mn^{2+} 50-fold. In the transient state, the authors only analyzed the reduction of compound I by cresol, a phenolic substrate of the enzyme, and the reduction of compound II by Mn^{2+}. They found that the reaction of compound I with cresol was unaffected by the mutation and that the reaction of compound II with Mn^{2+} was severely impaired. The K_d of compound II for Mn^{2+} was 100-fold higher in the D179N mutant, and the rate of reduction was 200-fold lower.

Subsequently, Kishi et al. (*23*) generated and characterized the E35Q and E39Q mutants of MnP. The authors also created and characterized a E35Q/D179N double mutation

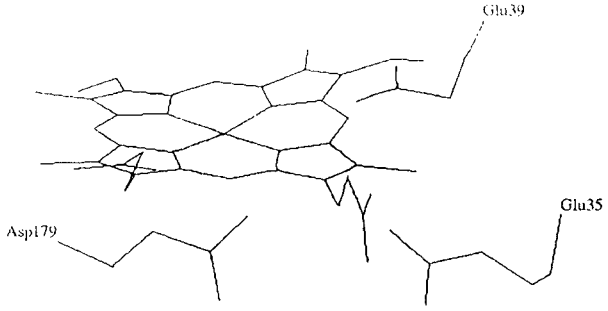


FIGURE 9: Mn binding site. The diagram shows the four acidic residues involved in Mn binding. Three of the residues are from amino acids Asp and Glu, whereas the fourth is the heme propionate. Adapted from ref 11.

in the enzyme. Again, these mutants exhibited an increase in the K_m for Mn^{2+} and drastically reduced k_{cat} values. Transient-state kinetic studies on the Mn^{2+} reaction were performed only with compound II, not compound I. These mutations affected the rates at which compound II reacted with Mn^{2+} but not the rates at which compound II reacted with *p*-cresol or ferrocyanide, thus confirming that the proposed sites were specific for Mn^{2+} binding by compound II of MnP.

The present mutagenesis study was initiated not to solely address the location of the Mn-binding site but to address whether the Mn-binding site identified by crystallography is the site of electron transfer for both compounds I and II. This is not a trivial question because EPR studies indicated the presence of more than one binding site for Mn. Banci et al. (*14*) monitored the EPR signal of Mn^{2+} in the presence of MnP. A 2 mM solution of Mn^{2+} was titrated with MnP, and the EPR signal of the free ion decreased in intensity. When the molar ratio of MnP to Mn^{2+} was 1:9, the EPR signal was less than 3% of the original signal, indicating that the protein has several binding sites. Furthermore, ^{1}H-NMR studies on MnP suggested the existence of a Mn-binding site close to the heme with a K_d for Mn^{2+} of 10^{-4} M, whereas equilibrium binding studies suggested the existence of a binding site with a much lower K_d value, in the range of 50×10^{-6} M for Mn^{2+} complexes (*24*). Our transient-state kinetic data clearly show that mutations at the putative Mn-binding site affect the reaction of Mn^{2+} with both compound I and compound II of MnP. In the E35D mutant, the reactions of compound I and compound II with Mn^{2+} are reduced 63- and 118-fold, respectively. In the D179A mutant, there are 220- and 107-fold decreases in the reactions of compound I and compound II with Mn^{2+}, respectively. Both mutants also exhibited a much higher K_d for Mn^{2+}. The E35D and D179A mutations increased the K_d for Mn^{2+} of MnP 42- and 240-fold, respectively. Clearly, the reactions of both enzyme intermediates are compromised by these mutations, indicating identical binding sites for both compound I and II.

Other results presented here are largely in accord with those of Kusters-van Someren et al. (*13*) and Kishi et al. (*23*) with some minor exceptions that are worthy of mention here. First, we noted a marked decrease in the K_m of the mutant enzymes for H_2O_2 which was not observed by either Kusters-van Someren et al. or Kishi et al. This at first surprised us because it implied that the mutations we generated increased the affinity of the enzyme for H_2O_2 whereas the mutants of Kusters van-Someren et al. and Kishi

et al., at the same residues, had no effect. However, closer examination indicates that the K_m for H_2O_2 *should* change in such mutants. As Cleland has pointed out (*25*), K_m is not a fundamental constant of enzymes. The two independent fundamental constants revealed by steady-state studies are k_{cat}, which is the turnover number of the enzyme, and k_{cat}/K_m, which provides a lower limit for the apparent first-order rate constant of the enzyme's reaction with substrate at low substrate concentrations. Changes in the Michaelis constant, K_m, of an enzyme simply reflect changes occurring independently in the k_{cat} and k_{cat}/K_m of that enzyme. Our k_{cat} values were markedly decreased by the mutations, as were the k_{cat}/K_m values for Mn^{2+}. However, we observed no change in the k_{cat}/K_m values for H_2O_2. This is consistent with the proposed mechanism, indicating that the mutations in the Mn-binding site should have no effect on the apparent first-order rate constant for H_2O_2. Poulos and Kraut (*26*) have clearly shown that H_2O_2 binds at the distal pocket of peroxidases, which is far removed from the Mn^{2+}-binding site of MnP. Thus, with respect to the reactions of H_2O_2 with MnP, a decrease in the k_{cat} of the enzyme and no change in $k_{cat}/K_m(H_2O_2)$ have to result in a decrease in K_m which is the ratio of these two fundamental constants. We do not understand how K_m was not changed in the mutants studied by Kusters-van Someren et al. (*13*) or Kishi et al. (*23*) because this would indicate that changes in Mn^{2+} binding would result in a change in the reactivity of the enzyme toward H_2O_2.

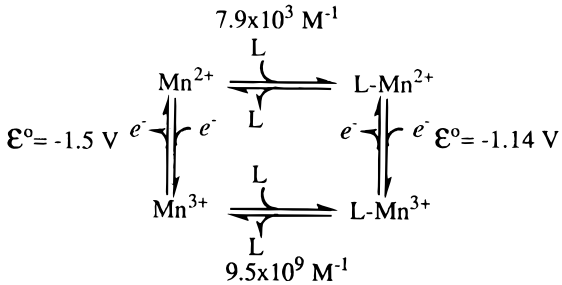
A more significant difference between our results and those of Kishi et al. (*23*) is the fact that we found that the mutating E39 residue had no effect on MnP. That group mutated E39 to Q. The kinetic consequences of this E39Q mutation were essentially identical to the kinetic consequences of the E35Q mutation. The results of Kishi et al. (*23*) clearly show that E39 is involved in the Mn-binding site. The results of this study suggest, however, that this residue is not as intimately involved as the other two resides, E35 and D179, in Mn binding. Merely repositioning the carboxylate functional group, which occurred in the E39D mutation, did not affect the kinetic properties of the enzyme; replacing the carboxylate group with an amide group, however, which occurred in the E39Q mutation, impaired catalysis dramatically. It may be that E39 binding is intrinsically weak and that E39 makes only a minor contribution to Mn^{2+} binding so that the repositioned carboxylate group in the E39D mutant is oriented so as to continue to make the minor contribution to binding made by the E39 carboxylate of the wild-type enzyme. Alternatively, instead of coordinating Mn^{2+} directly, Glu39 might be H bonded to a water molecule that in turn is coordinated to the Mn^{2+} ion, in an interaction analogous to the manner in which the heme propionate group was proposed by Sundaramoothy et al. to interact with Mn^{2+} (*11*). If this were the case, the D39 carboxylate group of the E39D mutant might still be able to fulfill the function of the E39 carboxylate group of the wild-type enzyme, H bonding the important water molecule.

While the E35D and the D179A mutants appear to be impaired in their ability to react with Mn^{2+} as a substrate, both mutants can be saturated with Mn^{2+} at high (in the millimolar range) concentrations of that substrate. However, even when the binding site is saturated with Mn^{2+}, the catalytic efficiency of the enzyme is impaired. The k_{cat} observed for both mutant enzymes is dramatically lower than that of the wild-type enzyme. At the same time, E35 and D179 do not appear to influence the redox potential of the heme center. Neither of these mutations, nor the E39D mutation, affected the midpoint potential of the Fe^{3+}/Fe^{2+} couple. Although the Fe^{3+}/Fe^{2+} couple is not involved in the enzymatic oxidations and reductions catalyzed by MnP, it is likely that any change in the midpoint potential of the catalytically relevant Fe^{3+}/Fe^{4+} couple would be reflected in changes in the midpoint potential of the Fe^{3+}/Fe^{2+} couple. Indeed, it has been demonstrated for a wide variety of metalloporphyrins that the sum of one-electron oxidation and one-electron reduction potentials is a constant (*27*). Thus, the finding that mutants exhibit redox potentials equivalent to those of the wild type for the Fe^{3+}/Fe^{2+} reduction argues strongly that the redox potentials for the mutant and native Fe^{4+}/Fe^{3+} couples are extremely similar, if not identical. Accordingly, differences in observed electron transfer (ET) kinetics for Mn^{2+} oxidation must result from changes in the Mn^{2+} part of the ET couple. There are three obvious ways in which this can be manifested: through a change in the redox potential, a change in the distance between redox sites, and/or a change in the reorganization energy. According to Marcus theory (*28*, *29*), the rate of an ET reaction can be described by

$$\text{rate} \propto H_{AB} e^{-\Delta G^*/kT}$$

where H_{AB} is the electron transfer matrix element (which scales inversely with distance), k is Boltzmann's constant, T is the temperature, and ΔG^* is the activation energy.

Marcus showed that $\Delta G^* = (\lambda + \Delta G^\circ)2/4\lambda$, where λ is the reorganization energy and ΔG° is the free energy change for the ET reaction. A change in ligation can lead to a change in redox potential via the equation $-\Delta G^\circ = nFE^\circ$, where n is the number of electrons transferred, F is Faraday's constant, and E° is the electrochemical driving force (i.e. the difference in redox potentials for the balanced half-reactions). To illustrate this principle, consider the following thermodynamic cycle for the oxidation of Mn^{2+}/oxalate using published binding constants for Mn^{2+} and Mn^{3+} (*30*−*32*) and the published reduction potential of hexaaquo Mn^{3+}/Mn^{2+} (*33*). A decrease of 0.36 V in the reduction potential is predicted from the tighter binding of oxalate to Mn^{3+} than to Mn^{2+}:



Thus, changes in the metal-binding site can lead to changes in the redox potential.

Moreover, such changes can also lead to changes in λ. This is most easily illustrated by considering the effect of a D to A mutation. In the case of high-spin Mn^{2+}, the ligand field stabilization energy is zero (*34*), so loss of a coordinating ligand is easily accommodated; Mn^{2+} often exhibits coordination numbers of 5 (or 7). However, there is a net

ligand field stabilization for octahedral, high-spin Mn^{3+}, indicating a preference for a six-coordinate environment. In the absence of a sixth ligand, a water could coordinate to satisfy this requirement. The formation of a bond in conjunction with a redox reaction leads to a significant change in λ, which could then result in altered kinetics.

It is important to point out that the effects on $E°$ and λ are, in this system, inseparable. Since neither the Fe^{4+}/Fe^{3+} nor the Mn^{3+}/Mn^{2+} redox potentials are known, and since the relationship between the ET rate and $\Delta G°$ is parabolic, it is not possible to know which effect dominates (or in fact if both effects are simultaneously operating). We merely point out the fact that both effects could be responsible for a reduction in the ET rate.

Finally, even if the coordination environment does not change, a change in rate can still be observed by a difference in H_{AB}, the electron transfer matrix element. For example, in the E35D mutant, if the D coordinates to Mn^{2+}, there would necessarily be a change in the geometry corresponding to the loss of a methylene on the side chain. This could in turn slightly alter either the position or the orientation of the metal ion relative to the heme, with either effect capable of inducing a change in the ET rate, either through the classical distance dependence expression for ET (*35*) or, in line with more current thinking, via altered through-bond pathways (*36*−*38*).

Excess H_2O_2 will react with compound I of MnP, yielding compound II, and will also react with compound II, yielding compound III, the oxycomplex form of the enzyme. The site at which H_2O_2 reacts with compound I and compound II has not been established, but it is not unreasonable to assume that it would be at the same general site as its reaction with the resting ferric enzyme, somewhere near the distal histidine and the heme iron. Certainly, altering the Mn-binding site of MnP did not have a dramatic effect on the reactions of H_2O_2 with compound I or compound II. Electron transfer from Mn^{2+} was severely impaired, but the reactions of H_2O_2 were only moderately affected. If H_2O_2 were interacting with compounds I and II at the Mn-binding site, one would expect a similar impairment of the catalytic process. It is more likely that the mutations were producing minor structural perturbations that were producing minor effects at the actual site where H_2O_2 was reacting.

Finally, E35, E39, and D179 do not appear to be involved in the reactions of DMP, a phenolic compound, with MnP.

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