Crystal Structures of Substrate Binding Site Mutants of Manganese Peroxidase*

(Received for publication, March 6, 1997, and in revised form, March 24, 1997)

Munirathinam Sundaramoorthy‡, Katsuyuki Kishi§, Michael H. Gold§, and Thomas L. Poulos‡

From the ‡Department of Molecular Biology & Biochemistry and Physiology & Biophysics, University of California, Irvine, California 92697-3800 and §Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland, Oregon 97291-1000

Manganese peroxidase (MnP), an extracellular heme enzyme from the lignin-degrading basidiomycetous fungus, Phanerochaete chrysosporium, catalyzes the oxidation of MnII to MnIII. The latter, acting as a diffusible redox mediator, is capable of oxidizing a variety of lignin model compounds. The proposed MnII binding site of MnP consists of a heme propionate, three acidic ligands (Glu-35, Glu-39, and Asp-179), and two water molecules. Using crystallographic methods, this binding site was probed by altering the amount of MnII bound to the protein. Crystals grown in the absence of MnII, or in the presence of EDTA, exhibited diminished electron density at this site. Crystals grown in excess MnII exhibited increased electron density at the proposed binding site but nowhere else in the protein. This suggests that there is only one major MnII binding site in MnP. Crystal structures of a single mutant (D179N) and a double mutant (E35Q,D179N) at this site were determined. The mutant structures lack a cation at the MnII binding site. The structure of the MnII binding site is altered significantly in both mutants, resulting in increased access to the solvent and substrate.

White-rot basidiomycete fungi are capable of degrading the plant cell wall polymer, lignin (1–4), and a wide variety of aromatic pollutants (5–9). The best-studied lignin-degrading fungus, Phanerochaete chrysosporium, secretes two types of extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), which, along with an H2O2-generating system, are the major extracellular components of its lignin-degrading system (11–13). Moreover, MnP is produced by all white-rot fungi known to degrade lignin extensively (14–16).

P. chrysosporium MnP has been characterized by a variety of biochemical and biophysical methods (4, 17–24). In addition, the sequences of cDNA and genomic clones encoding several P. chrysosporium MnP isozymes (mnp1, mnp2, and mnp3) have been determined (4, 25–30). Biophysical studies and DNA sequences suggest that the heme environment and catalytic cycle of MnP are similar to those of other heme peroxidases, such as horseradish peroxidase and LiP (31, 32). However, MnP is unique in its ability to catalyze the one-electron oxidation of MnII to MnIII (18, 20, 23) in a multi-step reaction cycle (see Reactions 1–3).

MnP + H2O2 → MnP compound I + H2O (Reaction 1)
MnP compound I + MnII → MnP compound II + MnIII (Reaction 2)
MnP compound II + MnIII → MnP + MnIII + H2O (Reaction 3)

The enzyme-generated MnIII is complexed with a dicarboxylic acid such as oxalate, which is also secreted by the fungus (23, 33, 34). The MnIII-organic acid complex, in turn, oxidizes phenolic substrates, including lignin model compounds (35), lignin (11), chlorinated phenols (9), and mediators (13, 22).

Recently, the crystal structures of both LiP and MnP have been reported (36–39). Both enzymes have the same tertiary fold and share topology with other heme peroxidases (39). These structures also confirm that the heme environments of LiP and MnP are similar to those of cytochrome c peroxidase, plant, and fungal peroxidases (38, 39). However, MnP has a unique cation binding site consisting of Glu-35, Glu-39, Asp-179, and one of the heme propionates, and this site has been proposed as the MnII binding site (39, 40). The recent characterization of MnP site-directed mutants at Asp-179, Glu-35, and Glu-39 (41, 42) suggests that these residues form the manganese binding site. In the present study, we have crystallized MnP in the presence of various amounts of MnII to further probe the MnII binding site of this protein. In addition, we have solved and refined the crystal structures of a single mutant (D179N) and a double mutant (E35Q,D179N) of amino acid ligands at the MnII binding site.

MATERIALS AND METHODS

Enzyme Preparation—Wild-type MnP isozyme 1 was purified from the extracellular medium of acetate-buffered, agitated cultures of P. chrysosporium strain OGC101, a derivative of strain BKM-F-1767, as described (17, 21). The enzyme concentration was determined at 406 nm using an extinction coefficient of 129 mM−1 cm−1 (17). In an attempt to remove the enzyme-bound MnII ion, MnP was applied to a Chelex 100 (Bio-Rad) column (1.0 × 20 cm), equilibrated with 100 mM sodium phosphate buffer (pH 6.5) at room temperature, and eluted with the same buffer. The protein was desalted by ultrafiltration. The MnII and CaII content of the Chelex-treated MnP (MnP*) was determined by atomic absorption spectroscopy.

Site-directed Mutagenesis and Purification—Site-directed mutagenesis was carried out by overlap extension (43) using the polymerase chain reaction as described (41, 42). Transformation of P. chrysosporium mutants was carried out as described (42, 44). Production and purification of variant proteins were as described previously (41, 42).

* This research is supported by National Science Foundation Grants MCB-9405128 (to T. L. P.) and MCB-9405978 (to M. H. G.) and by U.S. Department of Energy, Division of Energy Biosciences Grant DE-FG06-93ER20093 (to M. H. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure of the MnP1 crystal structure (code 1MN1) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

‡ To whom correspondence should be addressed: Dept. of Molecular Biology & Biochemistry, University of California, Irvine, CA 92697-3900. Tel.: 714-824-7020; Fax: 714-824-3280; E-mail: poulos@uci.edu.

§ The abbreviations used are: LiP, lignin peroxidase; MnP, manganese peroxidase; polyethylene glycol MnP*, Chelex-treated MnP; MnP*(EDTA), MnP* crystallized in the presence of EDTA; MnP*(Mn), MnP* crystallized in the presence of manganese; Wat, water molecule.
The RMS deviations of bond parameters represent the root mean square deviations from expected values. Engh and Huber parameters (52) were used in the refinement.

### Crystallographic data collection summary

| Crystal   | MnP\(^{\text{II}}\) | MnP\(^{\text{II}}\)(Mn) | MnP\(^{\text{II}}\)(EDTA) | E35Q-D179N | D179N |
|-----------|---------------------|-------------------------|--------------------------|------------|-------|
| Data observed | 67,431             | 98,031                  | 73,221                   | 91,040     | 96,757|
| Number of reflections | 18,298             | 31,351                  | 20,888                   | 27,424     | 27,414|
|\(R_{\text{sym}}\) (%) | 13.93              | 9.12                    | 11.04                    | 10.61      | 10.35 |
| Highest resolution | 2.3                | 1.9                     | 2.2                      | 2.0        | 2.0   |
|\(I/\sigma(I)\) at highest resolution | 2.3                | 1.9                     | 2.3                      | 1.0        | 1.4   |
| Completeness | 100                | 96                      | 99                       | 98         | 98    |

\(\frac{|I_i - \langle I_i \rangle|}{\langle I_i \rangle}\), where \(I_i\) is the intensity of the \(i\)th observation and \(\langle I_i \rangle\) is the mean intensity.

### Crystallographic refinement summary

| Crystal   | MnP\(^{\text{II}}\) | MnP\(^{\text{II}}\)(Mn) | MnP\(^{\text{II}}\)(EDTA) | E35Q-D179N | D179N |
|-----------|---------------------|-------------------------|--------------------------|------------|-------|
| Resolution range (Å) | 8.0–2.3             | 8.0–2.0                  | 8.0–2.2                   | 8.0–2.0    | 8.0–2.0|
| Reflections measured | 16,384              | 25,562                   | 18,753                    | 25,117     | 25,321|
| Reflections used, \(F > 2 \sigma(F)\) | 14,511              | 23,907                   | 16,495                    | 22,248     | 23,362|
|\(R\) factor | 0.182              | 0.200                    | 0.182                     | 0.187      | 0.213 |
| RMS deviation of\(B\) | 1.431              | 1.388                    | 1.420                     | 1.359      | 1.398 |

\(R = \frac{|F_o - |F_c||}{|F_o|}\). The RMS deviations of bond parameters represent the root mean square deviations from expected values. Engh and Huber parameters (52) were used in the refinement.

### Comparison of Mn\(^{\text{II}}\) binding in different crystals

| Crystal   | MnP\(^{\text{II}}\) | Native MnP | MnP\(^{\text{II}}\)(EDTA) |
|-----------|---------------------|------------|--------------------------|
| Resolution range (Å) | 8.0–2.3             | 12.6       | 10.7                     |
| Number of common reflections | 13,508            |            | 7.6                      |
| Peak height in \(F_o - F_c\) | 31.47               | 33.53      | 43.60                    |
|\(B\) factor of Mn\(^{\text{II}}\) (Å\(^2\)) | 8.48                | 9.60       | 9.57                     |
| Occupancy | 0.63                | 0.57       | 0.49                     |

\(a\) The overall \(B\) factors were calculated from the Wilson plot using the common reflections observed in the individual data sets.

### Crystallization

Crystallization—Crystals of MnP\(^{\text{II}}\), the D179N single mutant MnP and the E35Q-D179N double mutant MnP, were grown by the hanging drop vapor diffusion method as described (45). Approximately 5 μl of the protein solution (9–19 mg/ml) were mixed with an equal volume of 30% polyethylene glycol 8000, 0.2 mM ammonium sulfate, and 0.1 M sodium cacodylate buffer (pH 6.5) and equilibrated against 1 ml of the same buffer for 1–2 days. The protein solution drops were micromanipulated by first touching the crystals of native MnP crystals with a very thin metal wire and then touching the protein solution drops. For macroseeding, the small seed crystals grown by touch seeding were washed successively in solutions containing 35, 32.5, and 30% polyethylene glycol 8000. A washed crystal was added to a freshly pre-equilibrated solution. Details of the data collections were provided in Table I.

### Refinement

Refinement—The native MnP crystal structure reported earlier (39) was used as the starting model for refinement in all cases. With MnP\(^{\text{II}}\), MnP\(^{\text{II}}\)(EDTA), and MnP\(^{\text{II}}\)(Mn), a 50-cycle positional refinement with \(B\) factors set at 15 Å\(^2\) was carried out, followed by 10 cycles of group \(B\) factors and 20 cycles of individual \(B\) factors for all non-hydrogen atoms using X-PLOR (47) (Table II). Objective estimates of the relative occupancies of the Mn\(^{\text{II}}\) site were obtained by refining the models using the common reflections observed in all the data sets in the 8.0–2.3-Å resolution range with a \(F > 2 \sigma(F)\) cutoff.\(F_o - F_c\) omit electron density maps were generated by removing Mn\(^{\text{II}}\) from the models, followed by 20 rounds of positional refinement. When Mn\(^{\text{II}}\) was included in the refinement, the \(B\) factor for Mn\(^{\text{II}}\) was set equal to the overall temperature factor obtained from the Wilson plot for the same set of reflections, and only the occupancy of Mn\(^{\text{II}}\) was refined in the last round of refinement. The refinements converged typically in 6–7 cycles. Alternatively, the occupancy for Mn\(^{\text{II}}\) was set to unity, and the \(B\) factor was refined for all the data sets. Various measurements of the relative occupancies of the Mn\(^{\text{II}}\) site, i.e. \(F_o - F_c\), electron density, \(B\) factor, and occupancy, are listed in Table III for all data sets.

In the refinement of the mutant structures, the Mn\(^{\text{II}}\) and its ligands, with the exception of the heme, were omitted from the refinement, and electron density maps were calculated. The changes around the Mn\(^{\text{II}}\) were discernible in the difference Fourier maps. The side chains and solvent structure around the mutated sites were rebuilt guided by the 2F\(_o\) – F\(_c\) and F\(_o\) – F\(_c\) maps using the TOM FRODO graphics software (48), followed by refinements of the model iteratively until the maps were fitted satisfactorily and \(B\) factors converged. At the final stage, omit maps were calculated excluding the remodeled side chains and solvent molecules from 25 cycles of positional refinement. The details of refinement are provided in Table II.

### RESULTS

The crystal structure of the native MnP was reported earlier, and the proposed Mn\(^{\text{II}}\) binding site was based on this structure (39). The obligatory substrate for the enzyme, Mn\(^{\text{II}}\), binds to a heme propionate and is coordinated to five other ligands in an octahedral geometry (Fig. 1). Three of the Mn\(^{\text{II}}\) ligands are acidic amino acid side chains, Glu-35, Glu-39, and Asp-179, and the remaining two are oxygen atoms of water molecules. The
site is at the surface of the protein and is accessible to the solvent.

**Crystal Structure of MnP** in the Presence of EDTA or Excess Mn**II**—When grown at room temperature, the crystals of the Chelex-treated protein, MnP**, bleached before growing to a size suitable for diffraction. However, the crystals were stable at 7 °C and grew to full size, although at a slower rate. The data set for MnP** extended to a slightly lower resolution compared with the average resolution of the native MnP crystals previously obtained (Table I) (39). The difference Fourier map calculated with the MnII excluded from the structure showed a reduced, but significant, electron density peak at the Mn II site indicating that Mn II was not removed completely (Table III). Crystals grown in the presence of EDTA [MnP*(EDTA)] were similar to MnP** crystals but exhibited a much lower peak in the \( F_o^2 - F_c \) electron density map, which was close to the average peak height of water (Fig. 2A and Table III). However, there was no change in the orientation of the Mn**II** ligands, suggesting that the MnP**(EDTA)** crystal still might have a cation bound, either Mn**II** with much lower occupancy or possibly another cation such as sodium.

MnP** crystals grown in the presence of excess Mn**II** [MnP**(Mn)**] showed approximately the same peak height at the Mn**II** binding site as observed in the initial MnP structure determination (Fig. 2B and Table III) (39), indicating that the proposed Mn**II** site in the enzyme was at least partially occupied by Mn**II** ion throughout the purification process. Importantly, the addition of excess Mn**II** to MnP** did not lead to a new electron density peak, strongly suggesting that there is no other major Mn**II** binding site in MnP.

To gain some insight into relative occupancies and disorder, we used two different refinement approaches, considering only the common reflections in the 8.0- to 2.3-Å resolution range for all of the crystals. First, the occupancies were held constant at 1.0 for all non-hydrogen atoms, including the Mn**II** ion, and the crystallographic temperature or \( B \) factors were refined. The \( B \) factor of the Mn II ion refined to a value of 33.5 Å\(^2\) compared with 8.4 and 10.0 Å\(^2\) for the two calcium sites in the native data set. On the other hand, the \( B \) factor for the Mn II site for MnP**(EDTA)** data increased to 58.0 Å\(^2\). In the second set of refinements, the \( B \) factor of the MnII site was fixed at the value determined from Wilson statistics, and the occupancy of the Mn**II** site was refined. The occupancy fell well below 1.0 in all cases, the lowest being 0.37 for MnP**(EDTA)**. These results suggest that the MnP**(Mn)** and native MnP crystals were more fully occupied with Mn**II**, whereas crystals grown in the absence of Mn**II** or in the presence of EDTA were only partially occupied with Mn**II** or possibly occupied with another cation such as sodium.

**Structure of the Single Mutant**—The \( F_o - F_c \) maps for the D179N mutant data set, calculated using the native MnP coordinates, including and excluding the Mn**II** and its ligands, suggested the absence of a cation in the Mn**II** site (Fig. 3A). The maps are very noisy in this region, indicating large changes in the structure as a result of the mutation. The mutated residue, Asn-179, undergoes very little conformational change from its...
native position, except a small rotation of $\sim 30^\circ$ about the C$_{\text{b}}$–C$_{\text{g}}$ bond. However, the other two Mn$^{II}$ ligands, Glu-35 and Glu-39, undergo large changes (Fig. 4A). Both Glu-35 and Glu-39 turn away from the Mn$^{II}$ site and, consequently, the solvent structure in this region rearranges. The Glu-35 side chain rotates almost 110° about the C$_{\text{b}}$–C$_{\text{g}}$ bond and becomes solvent-exposed. This leaves a void that fills with two solvent molecules (Wat-653 and Wat-441). Wat-653 is about 1.5 Å from the Mn$^{II}$ site and still interacts with the side chains of Asn-179 and Glu-39 and with the propionate. Wat-520, bridging the two propionates and a ligand to Mn$^{II}$ in the native structure, moves about 2.0 Å. In its new position, this water forms a hydrogen bond interaction with the side chain amide of Asn-179.

Structure of the Double Mutant—Similar to the single mutant, the difference Fourier map calculated using the E35Q,D179N double mutant data set and the coordinates of native MnP, including and excluding Mn$^{II}$ and its non-heme ligands, do not show any significant positive density that can be interpreted as a cation in the vicinity of the Mn$^{II}$ site. On the other hand, the difference map calculated using the complete set of native MnP coordinates showed a large negative peak in the Mn$^{II}$ site (Fig. 3B), indicating the absence of a cation or only partial occupancy of this site. The refined structure of the mutant around the Mn$^{II}$ site is shown in Fig. 4B. One of the mutated residues, Gln-35, is disordered and appears to be in multiple conformations. Gln-35 was modeled in two conformations, one pointing outward and the other pointing inward as in the native conformation, with 50% occupancy of each. In one of the two conformations, Gln-35 retains a hydrogen bond with the side chain of Arg-177. In the conformation pointing outward, the void is occupied by a water molecule (Wat-653) which is about 1.5 Å from the Mn$^{II}$ site. This is similar to the single mutant structure (Fig. 4, A and B). The other mutated residue, Asn-179, undergoes little conformational change from the native position of Asp-179. There is a $\sim 30^\circ$ rotation about the C$_{\text{b}}$–C$_{\text{g}}$ bond, which does not alter its position or local interactions significantly. The side chain carbonyl oxygen of Asn-179 retains the hydrogen bond with the backbone amide of Ala-187 and the side chain amino group hydrogen bonds with the invariant solvent molecule (Wat-459), analogous to the native structure. In the double mutant protein, Glu-39 undergoes a dramatic conformational change in the absence of a cation in the proposed Mn$^{II}$ site, swinging out and away from the Mn$^{II}$ site. One of the side chain carboxylate oxygens of Glu-39 in the mutant forms a weak hydrogen bond (3.2–3.3 Å) with both conformations of the Gln-35 side chain. In the absence of the cation in the double mutant, one of the solvent ligands, Wat-520, moves by 2.0 Å out of the plane formed by the heme propionates, while still bridging the heme propionates as was observed in the single mutant. Another feature of the mutant structure is the large movement of the distal Arg-42 toward the peroxide binding pocket, so as to form a hydrogen bond with the distal Wat-556. This Arg is invariant in non-mammalian heme peroxidases and has been implicated as an important residue in cleavage of the H$_2$O$_2$ O–O bond during the formation of...
such movement of the distal Arg has been observed in the crystal structure of compound I of CcP (50).

**DISCUSSION**

MnP is a unique heme peroxidase that oxidizes Mn$^{II}$ to Mn$^{III}$ (18, 21, 23). The enzyme-generated Mn$^{III}$, complexed with an organic acid such as oxalate, oxidizes either the terminal phenolic substrate (18, 35) or a mediator (13, 22). Our earlier crystallographic study (39), as well as homology modeling of MnP (40), predicts a Mn$^{II}$ binding site close to the surface of the protein, consisting of three acidic amino acid residues, Asp-179, Glu-35, and Glu-39 and one of the heme propionates. Site-directed mutagenesis studies on the amino acid ligands in the manganese binding site demonstrate that this is the productive binding site (41, 42). In contrast, earlier work by Harris et al. (51) and Banci et al. (24) suggested a Mn$^{II}$ binding site close to the $\delta$-meso edge of the heme.

The crystals of MnP* and of MnP*(EDTA) exhibit reduced electron density at the proposed Mn$^{II}$ binding site, indicating reduced Mn$^{II}$ occupancy. However, our results indicate that the Mn$^{II}$ is not completely removed from the MnP* or MnP*(EDTA) crystals, although atomic absorption spectroscopic analyses indicate that these proteins contain less than 0.2% Mn$^{II}$ ion (data not shown). Since MnP has a higher affinity for Mn$^{II}$ at pH 6.5 (the pH of the cacodylate buffer used for crystallization) than at the physiological pH of 4.5 (data not shown), a trace amount of contaminating Mn$^{II}$ in the buffer may bind to MnP during crystallization. MnP* crystals grown in the presence of excess Mn$^{II}$ exhibit sharply increased electron density at the proposed binding site, suggesting that the electron density at this site is, indeed, due to Mn$^{II}$ (Table III). Furthermore, crystals grown in the presence of excess Mn$^{II}$ exhibit no additional large positive peaks in the electron density map, indicating that there is no other strong Mn$^{II}$ binding site in MnP.

Characterization of site-directed mutations at the Mn$^{II}$ binding site of MnP, including the D179N, E35Q, and E39Q single

---

**FIG. 4. Stereo representations of refined structures and interactions around the Mn$^{II}$ binding site in the D179N single mutant (A) and in the E35Q,D179N double mutant (B).** In E35Q,D179N, Gln-35 is modeled in two conformations, and Wat-653 is present only in the open conformation of Gln-35. In D179N Wat-653 is fully occupied, and the extra space left by the movement of Glu-35 is occupied by Wat-441. Wat-653 forms hydrogen bond interaction with the heme propionate, Glu-39, Asn-179, and a solvent (Wat-441 in the single mutant and Wat-650 in the double mutant).
mutations and the D179N,E35Q double mutations, strongly suggests that this is the productive Mn$^{III}$ binding site of MnP (41, 42). Kinetic analyses of the single mutants, E35Q, E39Q, and D179N, yielded $K_m$ values for the substrate Mn$^{II}$ that were ~50-fold greater than the corresponding $K_m$ value for the wild-type enzyme. Similarly, the $k_{cat}$ values for Mn$^{III}$ oxidation were ~300-fold lower than that for the wild-type MnP. The E35Q,D179N double mutant had a $K_m$ value for Mn$^{III}$ that was ~120-fold greater and a $k_{cat}$ value that was ~1000-fold less than those for the wild-type MnP. Transient-state kinetic analysis for the reduction of MnP compound II by Mn$^{II}$ allowed the determination of the equilibrium dissociation constants ($K_D$) and first-order rate constants for the mutant proteins. The $K_D$ values were approximately 100-fold higher for the single mutants and approximately 200-fold higher for the double mutant, as compared with the wild-type enzyme. The first-order rate constants for the single and double mutants were 200- and ~4000-fold less, respectively, than that for the wild-type enzyme. In contrast, the $K_m$ values for H$_2$O$_2$ and the rates of compound I formation were similar for the mutant and wild-type MnPs. Thus, these mutants affect both binding and electron transfer from Mn$^{III}$ to compound II but do not affect the formation of compound I (41, 42).

The present study provides a structural basis for understanding the functional consequences of mutating the Mn$^{II}$ ligands. The structures of Chelex-treated MnP (MnP*) and MnP$^{III}$ crystals grown in the presence of EDTA exhibit greatly diminished electron density at the proposed Mn$^{II}$ site. The electron density returns upon co-crystallizing MnP$^*$ in excess of Asp-179, Glu-35, and Glu-39 do not change significantly in the mutants compared with that for the wild-type MnP. This higher redox potential would negatively affect the electron transfer rate. There is some support for this idea since mutagenesis results with other peroxidases show that decreasing the electrostatic character of the proximal His heme ligand results in an increase in heme redox potential (53).

The mutations also alter the electrostatic environment at the binding site. In the wild-type protein, the Mn$^{III}$ is surrounded by four carboxylates, one of which pairs with Arg-177, yielding a net charge of −3. In the single mutant one of these negative charges is removed, and in the double mutant two negative charges are removed. The excess negative charge in the wild-type protein may promote oxidation of Mn$^{III}$ to Mn$^{IV}$. The loss of this electrostatic energetic incentive in the mutants may also partially explain the decrease in the electron transfer rate. Previous work shows that the Mn$^{III}$ produced by the enzyme is released as a Mn$^{III}$-chelator complex. The latter forms a stable diffusible oxidant (23). The wild-type and mutant structures may help to elucidate this part of the catalytic cycle. Unlike other peroxidases, the heme propionate side chains of MnP are solvent-exposed, allowing access for Mn$^{III}$ binding (Fig. 5A). The metal ligand distances of the Mn$^{III}$ ligands increase in the following order: OD1 of heme propionate (2.34 Å), OD1 of Asp-179 (2.57 Å), OE1 of Glu-35 (2.69 Å), and OE1 of Glu-39 (2.82 Å). The B factor or temperature factor for these ligands increases in the same order. These subtle differences suggest that, although still required for Mn$^{III}$ binding, Glu-35 and Glu-39 are weaker ligands than the heme propionate or Asp-179. Comparison of the native and mutant MnP structures also suggests that the Glu-35 and Glu-39 side chains assume different conformations depending upon whether or not Mn$^{III}$ is bound. When the Mn$^{III}$ is bound, the ligands are oriented toward the metal. In the absence of manganese, the side chains of Glu-35 and Glu-39 swing away to disperse the negative charge, resulting in the formation of an open cavity. This suggests that
these two ligands may act as a gate for Mn\textsuperscript{II}, binding the incoming Mn\textsuperscript{II} in their closed conformations and releasing the oxidized Mn\textsuperscript{III} in their open conformations. Fig. 5, B and C, shows that the propionates in the mutant structures are more solvent-exposed when Glu/Gln-35 and Glu-39 are in their open conformations. Such a gate could facilitate productive catalysis, particularly since Mn\textsuperscript{III} must bind to a dicarboxylic acid to serve as a diffusible oxidant. Glu-35 and Glu-39 may facilitate the release of the Mn\textsuperscript{III} to an incoming dicarboxylic acid.

It also is possible that the open nature of the Mn\textsuperscript{II} binding site in the manganese-free protein might facilitate the binding of a Mn\textsuperscript{II}-oxalate complex in the manganese-saturated MnP. When the amino acid ligands form a closed site, the Mn\textsuperscript{II}-chelator complex may not be able to enter. Although free Mn\textsuperscript{II} can bind to the enzyme, as demonstrated here and in our previous work (23, 39), a range of kinetic experiments suggest that a Mn\textsuperscript{II}-chelator complex is the best substrate for the enzyme (17, 33, 34). If the Mn\textsuperscript{II}-chelator complex is the real MnP substrate, the two water molecules in the MnP crystal structure (39) would be replaced by the chelator. To date, we have not been able to obtain a co-crystal of MnP and a Mn\textsuperscript{II}-chelator complex.

Despite the presence of this unique Mn\textsuperscript{II} binding site, the overall structure of MnP is very similar to all other non-mammalian heme peroxidases for which structures are available. Apparently the localized structural alterations near the surface of the protein required to form the Mn\textsuperscript{II} site do not induce significant changes in the core peroxidase structure. For example, the structure of P. chrysosporium LiP is very similar to that of MnP but lacks the Mn\textsuperscript{II} site. LiP has only one of the three acidic residues, Glu-40 in LiP (6) (Fig. 6). In place of Glu-35 and Asp-179, LiP contains alanine (Ala-36) and asparagine (Asn-182), respectively (39). Although it is possible to accommodate an aspartic acid in place of asparagine (Asn-182) in the LiP structure, the space occupied by the side chain of Glu-35 in MnP is filled by the backbone structure of the C terminus in LiP. MnP has a longer C terminus, which deviates considerably in its course from that of LiP. In addition, Arg-177 pushes the polypeptide chain out and away from the main body of the protein to form the Mn\textsuperscript{II} site in MnP. The corresponding residue in LiP is an alanine (Ala-180). Finally, MnP has an extra disulfide that helps to force the polypeptide chain away from the body of the protein. These differences result in the formation of space for Glu-35 near the cation binding site. These comparisons suggest that constructing a productive Mn\textsuperscript{II} binding site in LiP by protein engineering may require more than a few simple amino acid substitutions, although it should be possible by a combination of additional genetic, kinetic, and structural studies to more precisely elucidate the electron transfer pathway in the MnP enzyme system.

REFERENCES

1. Buswell, J. A., and Oder, E. (1987) CRC Crit. Rev. Biotechnol. 6, 1–60
2. Kirk, T. K., and Farrell, R. L. (1987) Annu. Rev. Microbiol. 41, 465–505
3. Gold, M. H., Wariishi, H., and Valli, K. (1989) ACS Symp. Ser. 389, 127–140
4. Gold, M. H., and Alic, M. (1996) Microbiol. Rev. 57, 605–622
5. Bumpus, J. A., and Aust, S. D. (1987) BioEssays 6, 166–170
6. Hammel, K. E. (1989) Enzyme Microb. Technol. 11, 776–777
7. Valli, K., Breck, B. J., Joshi, D. K., and Gold, M. H. (1990) Appl. Environ.

* LiP residue numbers are shown in parentheses throughout.