Characterization of a cDNA Encoding a Manganese Peroxidase, from the Lignin-degrading Basidiomycete *Phanerochaete chrysosporium*

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A cDNA clone of a manganese peroxidase (MnP) from *Phanerochaete chrysosporium* was isolated and characterized. The cDNA contains 1314 nucleotides excluding the poly(A) tail and the coding region has 68% G + C content. The deduced mature MnP protein contains 357 amino acids and is preceded by a 21-amino acid leader sequence. The experimentally determined N-terminal sequence of the purified MnP-1 protein, pl = 4.9, corresponds to the deduced N-terminal sequence of the gene. The Mr of the mature MnP-1 deduced from the cDNA is 37,439, which is ~81.4% of the experimentally determined molecular weight. The difference is due to glycosylation and a single potential N-glycosylation site with the general sequence Asn-X-Thr/Ser is present in the deduced MnP-1 sequence. Consistent with the peroxidase mechanism of MnP, the proximal histidine, the distal histidine, and the distal arginine are all conserved and regions flanking these residues display homology with other peroxidases. Northern blot analysis indicates that MnP expression is controlled by nutrient nitrogen at the level of transcription. Southern blot hybridization analysis suggests that MnP-1 is a member of a family of MnP genes.

Lignin, the second most abundant natural polymer, is a complex optically inactive and random phenylpropanoid matrix that comprises 20-30% of woody plants (1, 2). Since the biodegradation of cellulose is retarded by the presence of lignin (2-4), the catabolism and potential utilization of this polymer are of enormous importance. White rot Basidiomycetes are primarily responsible for the initiation of the decomposition of lignin in wood (2-4). When cultured under lignolytic conditions the white rot fungus *Phanerochaete chrysosporium* secretes two extracellular heme peroxidases, lignin peroxidase; bp, base pair.

EXPERIMENTAL PROCEDURES

Organisms—*P. chrysosporium* strain OGC101, a derivative of ME446 (18), was used throughout this study. Escherichia coli strains JM109 and JM101 (19) were used for transfections and growth of phage M13 derivatives. Strains Y1088 and Y1090 were used as hosts for λgt11 (20).

Enzymes and Nucleotides—Enzymes were obtained from Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs, and Amersham Scientific. Assay conditions were as recommended by the suppliers; otherwise they were standard (21). λgt11 phage DNA and the adapter molecules (16-mer and 20-mer) used in cDNA cloning were provided by J. Adelman (Oregon Health Sciences University, Portland, OR). Oligo(dT)-cellulose and random hexanucleotide primer were obtained from Pharmacia LKB Biotechnology Inc. The cDNA synthesis kit and oligo(dT) primer were obtained from Boehringer Mannheim. [α-thio-32P]dATP and [α-32P]dCTP were obtained from Du Pont-New England Nuclear.

Amino-terminal Sequence Analysis of MnP-1—MnP was purified from acetate-buffered agitated cultures as previously described (12, 22). Fast protein liquid chromatography (23) was used in the final step to separate several MnP isozymes. The apoprotein of MnP-1, the isozyme eluting at 26% Na acetate, was prepared (24), reduced, and carboxymethylated with iodoacetic acid as described previously (25). Amino-terminal sequence analysis was carried out by A. Smith at the Protein Structure Laboratory, University of California, Davis.

cDNA Library—RNA was isolated from 5- and 6-day-old low nitrogen (1.2 mM Na tartrate) and high nitrogen (12 mM Na tartrate) shaking cultures of *P. chrysosporium* (25, 26). Frozen mycelia were blended to a powder under liquid N and then ground with sand in tris-(2-aminoethyl)amine hydrochloride/50 mM sodium acetate/50 mM sodium phosphate/0.1 M NaCl buffer (27, 28). The RNA was purified from the aqueous phase by pelleting it through CCl4 (29). Approximately 0.3 mg of total RNA was recovered per gram of mycelium. Poly(A) RNA was isolated by two passages of total RNA over oligo(dt)-cellulose (30).
cDNA was synthesized by the method of Gubler and Hoffman (31). EcoRI adapter molecules were ligated to cDNA products (32) and molecules greater than ~500 bp were recovered from a 5% polyacrylamide gel and ligated to EcoRI cut λgt11 arms (33). Ligation products were packaged in *E. coli* and amplified as described (20).

**Plaque Screening**—Rabbit polyclonal antibody was prepared against purified MnP. Plaque screening was performed as described previously (20) using a goat anti-rabbit antibody conjugated with alkaline phosphatase. Approximately 0.3% of all cDNA clones were positive by this method. Positive plaques were purified prior to preparation of phage stocks.

**Restriction Analysis and Subcloning**—λgt11 recombinant DNA was purified from plate lysates by standard methods (21), with modifications (34). The phage DNA was digested with EcoRI to release the cDNA insert; none of the cloned inserts contained an internal EcoRI cleavage site. The cDNAs were then subcloned directly from low melting agarose (FMC Sea Plaque) (35) into EcoRI-cut M13mp18 (19) and transfected into JM101 (36). C-tests (36) were used to identify both orientations of each cDNA insert. Single-stranded templates were prepared (36) and clones were screened by limited sequence analysis and comparison with the MnP-1 N-terminal sequence. A restriction map for replicative form DNA from the MnP-1 clone was produced by standard procedures (21, 37) and restriction fragments of the MnP-1 cDNA were subcloned directly from gels into mp18 or mp19 replicative form. Both orientations of one 260-bp PstI fragment from the MnP-1 coding region gave blue rather than clear plaques (37). This fragment was detected by plaque hybridizations using an MnP-1-specific probe.

**Sequence Analysis**—Dideoxy sequence analysis (38) was carried out as described (38) except that 7-deaza-dGTP was substituted for GTP to avoid compression artifacts (39) in the G + C-rich MnP-1 coding region. Chain extension products were labeled with 32P. Gels were prepared, run, and treated as described (40).

**Southern and Northern Blot Hybridizations**—Fungal DNA was prepared as described (41) except that ethidium bromide was used in the density gradient. DNA from restriction digests was electrophoresed and transferred to Biotrace RP membranes, and hybridized at 42°C (21). RNA was electrophoresed out as described (36) except that 7-deaza-dGTP was substituted for GTP.

**Results and Discussion**

**Nucleotide Sequence of the cDNA**—A set of overlapping restriction fragments of the MnP-1 cDNA subcloned into M13mp18 (19) was sequenced according to the scheme in Fig. 1. All but 162 bp (12%) of the sequence were obtained from both strands of the cDNA; the remainder was sequenced (5 ×) without apparent ambiguity. A spontaneous deletion (indicated by Δ in Fig. 1) of 321 bp at the 5’ end occurred during subcloning and was utilized in the sequence analysis. The cDNA sequence of MnP-1 and the predicted translation product is shown in Fig. 2. The cDNA sequence comprises 1314 nucleotides plus a poly(A) tail and contains an open reading frame coding for a protein of 378 amino acids. The 1154-bp coding region is flanked by 42-bp 5’- and 138-bp 3’-noncoding regions. The experimentally determined N-terminal sequence of the first 22 amino acids of MnP-1 isozyme 1 beginning with the position 1 matches the predicted cDNA translation product, indicating that this cDNA encodes the MnP-1 isozyme. This same MnP-1 protein was used to raise the polyclonal antibodies for this study. The sequence AATACA, 16 bp upstream of the poly(A) tail, is similar to the canonical eukaryotic polyadenylation signal AATAAA (46). The sequence found in MnP-1 is identical to that found in the LiP cDNA CLG5 (9). Polyadenylation signals from filamentous fungi do not always exactly match the canonical sequence (47).

**Signal Peptide and Initiation Codon**—The amino acid sequence of the mature protein (357 amino acids) is preceded by a 21-amino acid leader sequence, consisting of N-terminal, hydrophobic, and C-terminal domains, which are characteristic of signal peptides (48–50). Threonine is common at the signal peptide carboxyl terminus (50, 51); however, the proline occurring 1 amino acid before the peptide cleavage site in the MnP-1 precursor is unusual (48, 50). The ATG codon at the start of the signal peptide initiates the longest open reading frame of the cDNA. This start codon is positioned correctly when the MnP-1 cDNA is aligned with the sequences of three related LiP genes (8–10). Genomic DNA sequence analysis (in progress) and mRNA start site determinations will verify the assignment of the initiation codon. The sequence surrounding the ATG initiation codon (Fig. 2) is TCCAGATGG. The optimal context for a higher eukaryote initiation codon is CCA/GNNATGG (most conserved underlined) with a purine at position −3 (52). All of the *P. chrysosporium* LiP genes examined to date (8–10), except the recently published *Lig 4* sequence (53), have a Guo at position −3. Like MnP-1, *Lig 4* has a Cyl at −3.

**Codon Usage**—The codon usage of the coding region of MnP-1 has an extreme G + C (68%) bias as compared with the 59% G + C content for the total *P. chrysosporium* genomic DNA (54). In contrast, the 3’-noncoding region has a 45% G + C content. The proportion of G + C in degenerate codon positions is very high. Examples of extreme selectivity of codons include: 1) Leu, 30 CTG/C versus 0 CTA/T or TTA/G and 2) Pro, 28 CCG/C versus 3 CCT/A.

During the onset of secondary metabolism, MnP accounts for at least 2% of the total protein, a conservative estimate since MnP turns over at a rapid rate. The extreme codon bias for MnP-1 reflects its high rate of synthesis and is similar to that found for highly expressed yeast genes (55) as well as for those of other filamentous fungi (9, 47).

**Characterization of the Deduced Protein**—Translation of the sequence encoding the mature MnP yields a protein of 357 amino acids. The calculated molecular weight of 37,493 is 81% of the reported *M,. of ~46,000 for the mature protein (12, 13). The difference can be accounted for by post-translational glycosylation; at least one isozyme of MnP contains ~17% carbohydrate (13). A similar difference in the deduced and observed molecular weights has been reported for LiP (8, 9). A single potential N-glycosylation site conforming to the general rule Asn-X-Thr/Ser (65) is located at Asn position 217 (boxed) (Fig. 2). An additional 49 Thr/Ser residues are potential sites for O-glycosylation.

Table I shows the amino acid composition of the mature protein. Ala (12%) and Pro (8.4%) are particularly abundant. The preponderance of Asp + Glu (10.6%) over Lys + Arg (5.6%) is consistent with the acidic pl of MnP-1, 4.9.

![Fig. 1. Strategy for determining the nucleotide sequence of the MnP-1 cDNA. Arrows indicate the strand sequenced and the extent of the sequence determined from various restriction sites. Restriction enzymes, S/S, and PstI (P) were used to generate fragments for subcloning. Δ indicates the beginning of a deletion clone as described in the text.](image-url)
cDNA for a Manganese Peroxidase

The poly(A) tail, the 3' noncoding region. The initiation codon is followed by a series of amino acids, including Val, Gly, and others, as shown below:

```
1189 Val
799 GCGcAcGWCCGccCACGGCGlGCATCTGcCAGccCTn;On; 840
344 Gly &r
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The nucleotide sequence is deduced from the amino acid sequence. The 5' noncoding region contains potential N-glycosylation sites.

The peroxidase sequences used were cytochrome c peroxidase (CCP) (65), horseradish peroxidase (HRP) (86), and Lip (8). Identical amino acids are enclosed in solid boxes, and similar amino acids are enclosed in dashed boxes.

The proximal histidine may aid in the heterolytic cleavage of H₂O₂ during the proximal histidine ligated to the heme iron of MnP-1.

The amino acid composition of MnP-1 is shown in the table below:

| Amino acid | Number | Percent |
|------------|--------|---------|
| Ala        | 43     | 12      |
| Arg        | 12     | 3.4     |
| Asn        | 15     | 4.2     |
| Asp        | 23     | 6.4     |
| Cys        | 10     | 2.8     |
| Gln        | 18     | 5.0     |
| Glu        | 15     | 4.2     |
| Gly        | 20     | 8.1     |
| His        | 8      | 2.2     |
| Ile        | 15     | 4.2     |
| Leu        | 28     | 7.8     |
| Lys        | 8      | 2.2     |
| Met        | 7      | 2.0     |
| Phe        | 22     | 6.4     |
| Pro        | 30     | 8.4     |
| Ser        | 29     | 8.1     |
| Thr        | 20     | 8.1     |
| Trp        | 1      | 0.3     |
| Tyr        | 2      | 0.7     |
| Val        | 23     | 6.4     |
| Total      | 357    |         |

The table shows the amino acid composition of MnP-1, with Ala being the most abundant, followed by Arg, Asn, and Asp. The proximal histidine is critical for peroxidase activity.

**Figure 3.** Comparison of *P. chrysosporium* MnP-1 and other peroxidases at regions near the proximal and distal histidines.

The peroxidase sequences used were cytochrome c peroxidase (CCP) (65), horseradish peroxidase (HRP) (86), and Lip (8). Identical amino acids are enclosed in solid boxes, and similar amino acids are enclosed in dashed boxes.

The proximal histidine is critical for peroxidase activity, and the amino acid composition is shown in the table above.

**Amino Acid Residues Involved in the Catalytic Mechanism**

Most plant and fungal peroxidases contain 2 histidines and an arginine that are essential for activity (57, 58). Recent EPR and resonance Raman evidence from this laboratory (15) demonstrated the presence of a proximal histidine ligated to the heme iron of MnP-1. Comparative sequence analysis (Fig. 3) indicates that the proximal His has been conserved in a variety of plant and fungal peroxidases (8, 57-59) in a homologous sequence. Our preliminary resonance Raman evidence indicates that the proximal His is strongly H-bonded (15). It has been suggested that the anionic character of the H-bonded proximal His stabilizes the high valent iron in peroxidase-oxidized states, compounds I and II, by releasing electron density into the heme iron (57, 58, 60). It has also been suggested that the anionic character of the proximal histidine may aid in the heterolytic cleavage of H₂O₂ during the formation of compound I (61).

**Fig. 2.** The complete nucleotide sequence for the MnP-1 cDNA. The amino acid sequence of the predicted translation product is shown below the nucleotide sequence. The MnP-1 coding region is flanked by a 42-bp 5' noncoding region and a 138-bp (exclusive of the poly(A) tail) 3' noncoding region. The initiation codon is followed by a sequence of amino acids, including Val, Gly, and others, as shown below:

```
1189 Val
799 GCGcAcGWCCGccCACGGCGlGCATCTGcCAGccCTn;On; 840
344 Gly &r
```

The nucleotide sequence is deduced from the amino acid sequence. The 5' noncoding region contains potential N-glycosylation sites.

The peroxidase sequences used were cytochrome c peroxidase (CCP) (65), horseradish peroxidase (HRP) (86), and Lip (8). Identical amino acids are enclosed in solid boxes, and similar amino acids are enclosed in dashed boxes.

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by a leader sequence (amino acids -22 to -1). This apparent signal peptide is underlined. ● and ▲ denote the distal and proximal histidines, respectively. The potential N-glycosylation site at position 217 in the amino acid sequence is boxed and a possible polyadenylation signal is overlined.
Fig. 3 also shows the amino acid sequences flanking the distal histidine (amino acid residue 46 in mature MnP-1). Both the distal histidine and distal arginine (amino acid residue 42) are conserved in a variety of plant and fungal peroxidases including MnP and LiP (Fig. 5). In addition, little variation is observed for the location of the proximal and distal histidines (amino acid residues 42-52, whereas the proximal histidines are all located in the region of amino acids 42-52, whereas the proximal histidines are all located in the region of amino acids 170-174 in MnP, LiP, horseradish peroxidase, and cytochrome c peroxidase.

A variety of kinetic studies indicate that heme peroxidases possess a distal ionizable group with a pK in the range of 3.0-5.0 which controls the pH dependence of compound I formation (62). Based on the crystal structure of cytochrome c peroxidase (58), it has been proposed that the distal histidine serves as an acid-base catalyst in the heterolytic cleavage of H2O2. Although MnP compound I is similar to other peroxidases in its spectral features (16), the second-order rate constant for MnP compound I formation is dependent on pH over the range 3-8 (67). This same pH independence was observed for LiP compound I formation (63, 64). If, as is likely, the distal histidine serves as the distal ionizable group, then its apparent pK must be significantly shifted in MnP and LiP. This might occur if the histidine were in a hydrophobic pocket, yet still accessible to H2O2.

Similarities between MnP and LiP—The amino acid sequence identity between the translation products of the MnP-1 cDNA and each of the three sequenced LiP cDNAs (8, 9) is nearly 50%. Corresponding nucleotide sequence identity is ~60%. Amino acid similarity between MnP-1 and LiP ML-1 (8) in sequences surrounding the catalytic histidines is >78% (Fig. 3). MnP and LiP have approximately the same number of amino acid residues (357 and 344, respectively) for the mature proteins. Alanine, phenylalanine, proline, serine, and threonine residues occur frequently in both peroxidases. In addition, both proteins have an abundance of acidic residues consistent with the low pI for all isoforms of MnP and LiP (17). Both enzymes are glycoproteins and hydrophathy plots of MnP and LiP (data not shown) also demonstrate strong similarities.

Comparison also indicates differences between the MnP-1 cDNA and the LiP cDNAs. Signal peptides of the sequenced LiP isozymes are 6-8 amino acids longer than the MnP-1 signal peptide. In addition, the C terminus of the LiP signal peptide contains a pair of basic amino acids (8, 9), absent in the MnP-1 signal peptide. At the codon level, MnP-1 has a higher G + C content and a more pronounced codon bias as compared with the LiPs. MnP-1 has one potential N-glycosylation; however, its location within the sequence is different from that found for the three LiP proteins (8, 9).

Regulation and Multiplicity of MnP Genes—Lignin degradation of P. chrysosporium is a secondary metabolic process that is triggered by nitrogen limitation (3, 4) and LiP and MnP activity appear in the extracellular medium only during the secondary metabolic phase of growth (3, 4, 8, 12). Northern blot analysis has demonstrated that the expression of LiP isozymes is controlled at the level of gene transcription by available nutrient nitrogen (8). Fig. 4 shows a Northern blot of equal amounts of total RNA from 5-day-old high and low nitrogen cultures probed with 32P-labeled MnP-1 cDNA. Hybridizable MnP-1 mRNA is only apparent from low N cultures, indicating that MnP gene expression, like LiP, is regulated at the level of transcription by nutrient nitrogen.

Fig. 5 shows a Southern blot of wild-type P. chrysosporium DNA digested with a variety of restriction enzymes and probed with 32P-labeled MnP-1 cDNA. For each different digest, hybridization to more than two different high molecular weight DNA fragments is observed, suggesting the existence of at least two different MnP-1 genes. Recently, we have also isolated another MnP cDNA clone (MnP-2) which only weakly cross-hybridizes with our MnP-1 probe and which gives a distinct hybridization pattern with wild-type DNA. These results demonstrate the presence of at least two MnP genes in P. chrysosporium and suggest that the MnP-1 genes comprise a subfamily. Since strain OGC101 (and other wild-type strains of P. chrysosporium) is a heterokaryon (18, 54), it is possible that one or more restriction fragment length polymorphisms for allelic MnP genes contribute to the complexity of the hybridization patterns. We are characterizing additional cDNA and genomic clones for both MnP and LiP. We intend to study the sequence and detailed organization of these genes and to define upstream sequences involved in their regulation.

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