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Molecular Biology of the Lignin-Degrading Basidiomycete *Phanerochaete chrysosporium*

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INTRODUCTION

Plant biomass, consisting primarily of lignin, cellulose and hemicellulose, constitutes the major end product of photosynthetically fixed carbon. Lignin is the second most abundant natural polymer, making up 15 to 30% of the woody cells walls of gymnosperms (softwood) and angiosperms (hardwood). Lignin forms a matrix surrounding the cellulose, the most abundant natural polymer. Since this encrusting matrix significantly retards the microbial depolymerization of cellulose, the degradation of lignin is a significant step in the global carbon cycle (41, 59, 168). Furthermore, the presence of this intractable polymer is an obstacle to the efficient utilization of cellulose in a wide range of industrial processes (52).

Lignin is a phenylpropanoid polymer synthesized from the phenolic precursors coniferyl, synapyl, and *p*-coumaryl alcohols (59, 168). Free-radical condensation of these precursors, initiated by plant cell wall peroxidases, results in the formation of a heterogeneous, amorphous, optically inactive, random, and highly branched polymer with at least 12 different types of linkages such as aryl ether and carbon-carbon bonds connecting the aromatic nuclei. These linkages are not subject to enzymatic hydrolysis. This unique structure requiring depolymerization by extracellular oxidative mechanisms accounts for the recalcitrance of lignin toward degradation by most microorganisms (41, 52, 81, 103).

The following is a brief review of the microbiology and biochemistry of lignin degradation which serves as an introduction to a more extensive review of the molecular biology of this process. More complete reviews of various aspects of the biochemistry of lignin degradation can be found elsewhere (25, 38, 52, 81, 88, 103, 170).

Metabolic Studies

White rot basidiomycete fungi are the only known organisms which are capable of degrading lignin extensively to CO_2 and H_2O in pure culture. Indeed, these organisms are able to degrade all of the major polymers in wood: cellulose, hemicellulose, and lignin. Early work indicated that lignin degradation by the best-studied lignin-degrading fungus, *Phanerochaete chrysosporium*, is both oxidative and nonspecific (42, 70, 104). Chemical analysis of white-rotted residual lignin and of lignin model compounds indicated that side-chain cleavage and aromatic-ring-opening reactions occur simultaneously (81, 88, 103, 128). Since lignin is such a complicated and insoluble polymer, it is difficult to elucidate the mechanism of its oxidation by the analysis of degradation products. Therefore, early research focused on the metabolism of dimeric lignin model substrates and demonstrated that a multiplicity of oxidative reactions were catalyzed by the lignin-degradative system of *P. chrysosporium* (38, 81, 88, 103, 128).

Physiological Studies

The culture parameters for lignin degradation by *P. chrysosporium* have been investigated by using ^{14}C-labeled synthetic lignins as substrates (reviewed in references 38, 52, and 103). Lignin is degraded by this organism only during secondary (idiophasic) metabolism, whose onset is triggered by depleting cultures for nutrient nitrogen, carbon, or sulfur. Nitrogen limitation is most commonly used in experiments with *P. chrysosporium*. The effect of nitrogen limitation is not surprising given the low levels of nitrogen found in wood (38, 103). However, lignin degradation by several other white rot fungi apparently is not stimulated by N limitation (38, 103, 144). In addition to lignin degradation, several other features of secondary metabolism in *P. chrysosporium* triggered by N limitation have been studied. The formation of an extracellular glucan and synthesis of veratryl (3,4-dimethoxybenzyl) alcohol also occur as secondary metabolic events (80, 103, 117, 174). The onset of secondary metabolism is associated with an increase in intracellular cyclic AMP (cAMP) levels (26, 122); however, the role of this metabolite in regulating lignin degradation remains to be clarified.

Lignin degradation is dependent on the presence of a readily metabolizable cosubstrate such as glucose; in addition, increasing the O_2 levels in culture has a strong activating effect on the rate of lignin degradation (38, 103). Evidence also suggests that manganese is important in lignin degradation. MnO_2 precipitates accumulate in wood after decay by several white rot fungi (20), and lignin degradation by several white rot fungi is strongly dependent on the presence of manganese (116, 144). Furthermore, an extracellular manganese peroxidase, first discovered in *P. chrysosporium* (114), has been identified in cultures of numerous white rot fungi (144).

Degradation of Aromatic Pollutants

White rot fungi are also the focus of considerable attention because of their ability to degrade toxic aromatic pollutants. Since lignin contains a variety of bonds that are commonly present in aromatic pollutants and since the lignin-degradative system of these fungi is nonspecific and oxidative, several laboratories have examined such fungi as potential bioremediation agents (33, 84). Over the past few years, the degradation of a large spectrum of environmental pollutants by *P. chrysosporium* has been reported (33, 48, 84, 85, 92, 190–192). In most of these studies, ^{14}C-labeled compounds were used to demonstrate mineralization of the pollutant to CO_2. However, several recent reports have described in detail the pathways for the degradation of the persistent pollutants dichlorophenol (191), dinitrotoluene (190), dichlorodibenzodioxin (192), and anthracene (85). In each case, the evidence suggests that the lignin-degradative system of *P. chrysosporium* is involved in the degradation of these pollutants and that the pollutants are metabolized by cycles of oxidation and subsequent quinone reduction reactions, leading to intermediates which undergo aromatic-ring cleavage.

Biochemistry of Lignin Degradation

In 1983 and 1984, two extracellular enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP), were discovered in *P. chrysosporium* (72, 114, 183). These enzymes have been demonstrated to be major components of the lignin degradation system of this organism. Since their discovery, these two enzymes have been purified and exten-

sively characterized biochemically. The following discussion summarizes the properties of LiP and MnP.

General Properties of Lignin Peroxidase and Manganese Peroxidase

LiP has been purified by a combination of anion-exchange chromatography, gel filtration, fast protein liquid chromatography, and isoelectric focusing (81, 102, 103, 118). The enzyme is present as a series of glycosylated isozymes with pIs ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDa. Each isozyme contains 1 mol of iron heme per mol of protein (81, 102, 118, 161). Although LiP has been detected in several white rot fungi (38, 103), it has not been detected in others (65, 144).

MnP has also been purified to electrophoretic homogeneity (71, 81). The enzyme exists as a series of glycosylated isozymes with pIs ranging from 4.2 to 4.9 and with molecular masses ranging from 45 to 47 kDa. Each isozyme also contains 1 mol of iron heme per mol of protein (71, 118, 139).

Biophysical Studies

Detailed electroparamagnetic resonance, resonance Raman, electronic absorption, and nuclear magnetic resonance spectral studies of LiP and MnP (9, 15, 46, 81, 103, 111, 130) demonstrate that the native forms of these enzymes, like that of horseradish peroxidase (HRP), exist as ferric, high-spin, pentacoordinate heme proteins with the protein ligated to the heme iron via a histidine (proximal histidine). The location of the proximal histidine has been confirmed by cDNA sequences of LiP and MnP (see below) and by the recent elucidation of the X-ray crystal structure of LiP (50). Indeed, the X-ray crystal structure of LiP (50) demonstrates that the distal histidine and arginine, which have been proposed to play key roles in the catalytic cycle of cytochrome *c* peroxidase (CcP) (145), are also conserved in the active site of LiP. In addition, a hydrogen bond linking Asp-235 to the proximal His of CcP is evident in the crystal structure of both CcP and LiP. This hydrogen bond also has been proposed to play a key role in the peroxidase mechanism (58). The conservation of these residues in LiP and MnP has been confirmed by comparison of cDNA sequences (see below).

Catalytic Cycles of Lignin and Manganese Peroxidases

The catalytic cycles of both LiP and MnP are similar to that of HRP (81, 160, 184, 197). The primary reaction product of LiP with H_2O_2 is the two-electron oxidized state compound I, LiPI. As with HRP, LiPI is reduced back to the native enzyme via two single-electron steps with compound II, LiPII, as an intermediate (scheme 1). In the process, the aromatic reducing substrate is oxidized to an aryl cation radical ($Ar^{+}\cdot$) (see below).

$$\begin{aligned} \text{LiP} + H_2O_2 &\rightarrow \text{LiPI} + H_2O \\ \text{LiPI} + \text{Ar} &\rightarrow \text{LiPII} + Ar^{+}\cdot \\ \text{LiPII} + \text{Ar} &\rightarrow \text{LiP} + Ar^{+}\cdot + H_2O \end{aligned} \qquad \text{Scheme 1}$$

The important difference between these peroxidases is in the nature of the reducing substrate (81, 103, 170). LiP catalyzes the oxidation of nonphenolic lignin model compounds such as veratryl alcohol to veratryl aldehyde. Therefore the unique feature of this enzyme is that it is able to oxidize aromatic compounds with redox potentials beyond the reach of HRP and many other peroxidases. Kinetic results also indicate a "Ping-Pong" mechanism in which H_2O_2 first oxidizes the enzyme and the oxidized enzyme intermediate (compound I) reacts with veratryl alcohol (124, 184). The enzyme has an extremely low pH optimum (~pH 2.5) for a peroxidase, and its pH dependence apparently is controlled by the pH dependence of the reduction steps in the catalytic cycle (124).

The oxidation of lignin and other phenols by MnP is dependent on free manganous ion (71, 81, 139). As shown in scheme 2, the primary reducing substrate in the MnP catalytic cycle is Mn(II), which efficiently reduces both compound I (MnPI) and compound II (MnPII), generating Mn(III), which subsequently oxidizes the organic substrate. Organic acids such as oxalate and malonate, which are secreted by *P. chrysosporium* (202), stimulate the MnP reaction by stabilizing the Mn(III) so that it can diffuse from the surface of the enzyme and oxidize the insoluble terminal substrate, lignin (69, 71, 81, 198, 202).

$$\begin{aligned} \text{MnP} + H_2O_2 &\rightarrow \text{MnPI} + H_2O \\ \text{MnPI} + \text{Mn(II)} &\rightarrow \text{MnPII} + \text{Mn(III)} \\ \text{MnPII} + \text{Mn(II)} &\rightarrow \text{MnP} + \text{Mn(III)} + H_2O \end{aligned} \qquad \text{Scheme 2}$$

Reactions Catalyzed by Lignin and Manganese Peroxidases

Lignin peroxidase catalyzes the H_2O_2-dependent oxidation of a wide variety of nonphenolic lignin model compounds and aromatic pollutants (38, 81, 88, 103, 128, 170, 191, 192), including synthetic lignin (86). These reactions include benzylic alcohol oxidations, side-chain cleavages, ring-opening reactions, demethoxylations, and oxidative dechlorinations. All of these reactions are consistent with a mechanism involving the initial one-electron oxidation of susceptible aromatic nuclei by an oxidized enzyme intermediate to form a substrate aryl cation radical (103, 124, 160, 170). The latter can undergo a variety of nonenzymatic reactions to yield a wide range of final products. Redox potential, in part, determines whether an aromatic nucleus is a substrate for LiP. Strong electron-withdrawing groups such as an α-carbonyl group tend to deactivate aromatic nuclei, whereas alkoxy groups such as are found in lignin tend to activate them (81, 88, 103, 170). The ability of LiP to oxidize lignin nonspecifically to generate cation radicals which undergo a variety of nonenzymatic reactions accounts for the variety of metabolic products observed.

Manganese peroxidase catalyzes the H_2O_2-dependent oxidation of lignin (201) and lignin derivatives (115) and a variety of phenolic lignin model compounds (81, 189, 200). It has been demonstrated that Mn(II) is the preferred substrate for MnP (71, 81, 197, 198, 202). The enzyme oxidizes Mn(II) to Mn(III), which diffuses from the enzyme surface and in turn oxidizes the phenolic substrate. Organic acids, such as oxalate and malonate, which are produced by *P. chrysosporium*, activate the MnP system by chelating Mn(III) to form stable complexes with high redox potentials (200) and by facilitating the dissociation of Mn(III) from the enzyme (198). Thus Mn ion participates in the reaction as a diffusible redox couple rather than as an enzyme-binding activator. This is supported by the demonstration that chemically prepared Mn(III) complexed with an organic acid such as malonate mimics the MnP reactions (189, 200). The initial reaction of Mn(III) with a phenol is a one-electron oxidation to form a phenoxy radical intermediate (189, 200). Subsequently, alkyl-phenyl cleavage, C_α-C_β cleavage, or benzylic carbinol oxidation yields the variety of products observed (189, 200, 201).

Laccase

Many white rot fungi, with the notable exception of *P. chrysosporium*, produce an extracellular laccase (38, 52, 88, 103, 144). Laccase is a blue copper protein which catalyzes the one-electron oxidation of phenols to phenoxy radicals. Like MnP, laccase can catalyze the alkyl-phenyl and C_α-C_β cleavage of phenolic lignin dimers (88). It also catalyzes the demethoxylation of several lignin model compounds (52, 103). Several white rot fungi produce laccase and MnP but apparently not LiP (65, 144), suggesting that they degrade lignin by an oxidative mechanism somewhat different from that of *P. chrysosporium*.

H_2O_2-Producing Enzymes

In addition to peroxidases and laccases, white rot fungi produce a variety of oxidases that are capable of generating H_2O_2, presumably for utilization by extracellular peroxidases during the degradation of lignin. These oxidases include glyoxal oxidase, an extracellular, idiophasic, copper-containing enzyme (99); glucose oxidase (53, 98); veratryl alcohol oxidase (27); and methanol oxidase (137). The exact role of each of these enzymes in lignin degradation remains to be elucidated.

The discovery of the enzymes involved in lignin degradation by *P. chrysosporium* has led to their biochemical, biophysical, and physiological characterization. This work has served as an indispensable background for studies on the molecular biology of lignin degradation by this organism.

MOLECULAR BIOLOGY OF *P. CHRYSOSPORIUM*

P. chrysosporium Genome

There are three commonly used wild-type strains of *P. chrysosporium*, i.e., ME-446 (36), BKM-F-1767 (34), and OGC101, a conidial derivative of ME-446 (7). The mycelia of these and other *P. chrysosporium* strains are coenocytic, with few septa (35, 180) and as many as 15 randomly dispersed nuclei per cell (7). The septa lack clamp connections (36, 180). OGC101, ME-446, BKM-F-1767, and at least two other strains (ML-21 and P-1271) are heterokaryotic, consisting of two or more genetically distinct nuclei. Although the multinucleate conidia are generally heterokaryotic, the basidiospores contain two identical nuclei (4, 7, 149) arising from a postmeiotic mitotic division (173). Thus, single basidiospore isolates give rise to homokaryons which can differ from each other and from the parental wild-type heterokaryon in a variety of characteristics (7, 149). Evidence has been presented for both homothallic (1, 5, 7) and heterothallic (152, 182) mating systems in various strains of *P. chrysosporium*. However, nutritionally forced heterokaryons can be formed between any two auxotrophs from different complementation groups, and genetic recombination has been demonstrated in most of these heterokaryons (1, 4, 108, 109), indicating the absence of mating-type incompatibilities among these specific strains (54).

The haploid genome size of strain ME-446 has been estimated to be 4.4×10^7 bp, of which 20 to 30% is mitochondrial and ribosomal DNA (148, 150). Strains ME-446 and BKM-F-1767 have chromosomal G+C contents of 59% (148). These values are similar to those reported for other basidiomycetes (112).

Raeder and Broda (149) demonstrated the segregation of restriction fragment length polymorphisms in single basidiospore progeny of strain ME-446 by probing with genomic clones expressed only during secondary metabolism (28, 151). Using additional probes including *lip* clones and a cellulase gene, Raeder et al. (153) compiled a preliminary genetic map consisting of six linkage groups plus four unlinked markers, the latter including a *lip* gene cluster and a single marker exhibiting unequal segregation.

Using contour-clamped homogeneous electric field gel analysis of *P. chrysosporium* ME-446 and BKM-F-1767, Gaskell et al. (66) resolved at least seven chromosomes, two of which may have been doublets. However, basidiospore isolates from these strains exhibited chromosome length polymorphisms.

Gene Families Encoding Extracellular Peroxidases

Lignin peroxidase. (i) *lip* sequences. The LiP isozymes of *P. chrysosporium* are encoded by a family of closely related genes, all of which cross-hybridize to various extents (29, 209). The first cDNA clones for lignin peroxidase were isolated from strain BKM-F-1767 by using synthetic oligonucleotide probes based on partial amino acid sequences of LiP isozyme H8 (207, 209). Shortly thereafter, the first cDNA (186) and genomic (178) clones for this isozyme were sequenced. To date, two cDNA (10, 186) and approximately 10 genomic (10, 66, 89, 91, 133, 169, 178, 196) clones of LiP H8 or related isozymes from BKM-F-1767 have been sequenced. cDNA and genomic sequences are also available for BKM-F-1767 isozymes H2 (45, 134) and H10 (45, 66, 208), as well as one other cDNA sequence that may encode H6 (10) and a complete genomic sequence (135) encoding an undetermined LiP isozyme. In addition, two H8-related genes (12, 29) and a cDNA (95) identical to the H2-encoding sequence from BKM-F-1767 have been isolated from strain ME446 and sequenced. cDNA (164) and genomic clones (162) encoding H8 from strain OGC101 also have been sequenced. Nucleotide identities within the coding regions of all these *lip* sequences are from 71.5 to 99.5%. Amino acid identities are from 68.5 to 99.5%.

Since wild-type strains of *P. chrysosporium* are heterokaryotic, it might be expected that some of the sequenced *lip* genes would be allelic. Although a high degree of homology at the amino acid and nucleotide levels and identical intron/exon structure (see below) may suggest that two genes are allelic, gene duplications could also account for these similarities. Therefore, it is necessary to examine the segregation of specific sequences in homokaryotic basidiospore progeny obtained from the wild-type strain. This can be accomplished by probing DNA obtained from single basidiospore progeny with a clone-specific fragment or oligonucleotide. This method has been used to demonstrate that three very similar *lip* clones from BKM-F-1767 are not allelic (169), whereas several other sequenced genes are alleles (66). A recent report describes a method for identifying alleles of the gene encoding H8 by using the polymerase chain reaction to amplify a variable 3′ region of the gene (67). However, chromosomal polymorphisms among basidiospore progeny (66) may complicate this analysis. Although the exact number, allelic relationships, and relative expression of *lip* genes remain to be determined, the isolation of several nonallelic cDNAs from strain BKM-F-1767, as well as Northern (RNA) blot analyses with gene-specific probes, demonstrates that multiple *lip* genes are expressed in *P. chrysosporium*.

(ii) Coding region. The *lip* genes each encode a mature protein product of 343 to 345 amino acids preceded by a 27- or 28-residue leader sequence. The encoded mature proteins

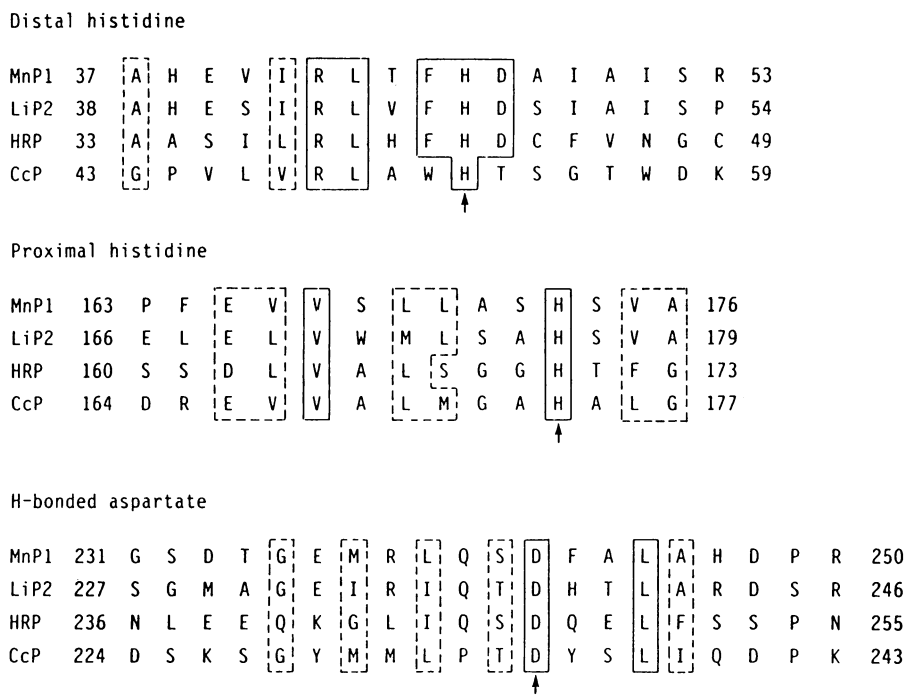

FIG. 1. Conserved peroxidase sequences surrounding active-site residues. Identical amino acids are enclosed in solid boxes, and similar amino acids are enclosed in dashed boxes. The sequences are MnP1 (146), LiP2 (164), HRP (204), and CcP (97). Numbering of amino acids begins with the first amino acid of the mature protein. Arrows indicate the distal and proximal histidines and the H-bonded aspartate.

have molecular weights of 36,360 to 36,607, compared with apparent molecular weights of approximately 38,000 to 43,000 (56, 118). The remaining 6 to 13% of the apparent molecular weight presumably are accounted for by glycosylation. All of the LiP isozymes examined appear to contain approximately 2.5 to 3 kDa of asparagine-N-linked carbohydrate (56, 73) which includes mannose 6-phosphate (110), and are likely to contain O-linked carbohydrate as well (56). All of the sequenced *lip* genes, except those encoding the H10 isozyme, have one potential N-glycosylation site with the sequence Asn-X-Thr/Ser (106). The H10-encoding sequences both have two potential N-glycosylation sites (66, 208). All of the sequences have numerous potential sites for O glycosylation. It is possible that some LiP isozymes are encoded by a single gene but differ in posttranslational modifications, including the amount and type of glycosylation or phosphorylation.

Sequences surrounding the residues thought to be essential for peroxidase activity (distal and proximal histidines and the distal arginine) are not only conserved among the *lip* genes but also are conserved between LiPs and other peroxidases (Fig. 1) (146, 163, 186). It has been proposed that an aspartic acid that is hydrogen bonded to the proximal His affects compound I formation in CcP (49, 58). Progressive sequence analysis has demonstrated that this aspartic acid is also conserved in LiP and MnP (163), and hydrophobic cluster analysis has identified an Asp in various plant peroxidases that align with this Asp in CcP (87) (Fig. 1). The conservation of these amino acids among fungal and plant peroxidases is reflected in the similarity of their catalytic cycles (see above).

The C termini of all sequenced LiPs, except the H8-encoding gene from OGC101 (*LG2*) and the H2-encoding sequence from BKM-F-1767 (*CLG4*), are identical, consisting of the amino acid sequence IPPPPGA. The terminal *LG2* sequence is IPPHKA (162), and the *CLG4* sequence is IPPPPSPN (45). The significance of this proline-rich C terminus is not known.

The coding regions of the *lip* genes contain approximately 60 to 65% G+C, whereas the 3′ noncoding regions are approximately 44 to 49% G+C. The gene *LG2*, encoding the H8 isozyme from strain OGC101, has the highest G+C codon bias of any sequenced *P. chrysosporium* gene (162). Since this isozyme accounts for the major fraction of the LiP produced by this strain (161) and since this sequence is present as a single-copy gene, a correlation between its high codon bias and high gene expression is likely (162).

(iii) Leader sequence. The mature LiP protein is preceded by a 27- or 28-amino-acid leader sequence. Prediction of the signal peptidase cleavage site and S-factor analysis (195) led to the suggestion that LiP is synthesized as a preproenzyme composed of a 21-amino-acid prepeptide followed by a 7-amino-acid propeptide (45, 164). In vitro transcription and translation of the cDNA encoding isozyme H8 from strain OGC101 (*L18*) followed by processing with signal peptidase from canine pancreatic microsomes, has confirmed that the signal peptide is cleaved following Ala-21 (164). The 21-amino-acid LiP signal peptide consists of N-terminal, hydrophobic, and C-terminal segments that are typical of secreted proteins (164, 194). The 7-amino-acid propeptide has a cleavage site following the sequence -Lys-Arg- (45, 164) (Fig. 2). This suggests that its removal involves an endoprotease of the subtilisin family of serine proteases (16), similar to the KEX2 endopeptidase that cleaves the α-factor and killer toxin precursors in *Saccharomyces cerevisiae* (63). The Lys residue at the −4 position relative to the mature protein, found in many although not all LiP sequences (Fig. 2), is additional evidence that this constitutes a serine protease cleavage site (16). Many secreted and compartmentalized proteins and peptide hormones contain similar

LiP Subfamily	Signal Peptide			Propeptide	Mature Protein
	N	H	C		
I	MAFKQ	LFAAISLALSL V TV LI	SAANA TTVQG	AAVKEKR VV I	ATCSNGKT A A
II	MAFKK	LLAVLTAALSL	RAAQG	AAV-EKR	ATCSNGKV
III	MALKQ	LAAAVALALSI	QAAQG	AAVKEKR	ATCSNGAT
IV	MAFKQ	LLAALSVALTL	QVTQA	APNLDKR	VACPDGVH

FIG. 2. N-terminal regions of LiP proteins. Subfamily I consists of eight sequences either known to encode isozyme H8 or having the same intron structure as the H8-encoding isozymes (Fig. 3). The top sequence is composed of the amino acids occuring most frequently at each position. Position 4 of the propeptide is equally likely to be K or I. The sequences making up subfamily I are ML1 (186), LG1 (12), LIG1 (29), ML4 (10), 0282 (169), GLG3 (133), LPOB (91), and LG2 (162). Subfamily II is composed of two identical amino acid sequences: GLG2 (208) and CLG5 (45). Subfamily III is the sequence GLG6 (135), and subfamily IV is GLG1 (134). Intron 1 of subfamilies I, II, and III (Fig. 3) is located within the codon for the underlined amino acid. N, H, and C denote the N-terminal, hydrophobic, and C-terminal segments, respectively, of the signal peptide.

propeptides, which may be important for accurate cleavage by signal peptidase (206). Other possible roles for propeptides include involvement in protein targeting, polypeptide folding (105), or maintaining the enzyme in an inactive state during translocation (136). Presumably the LiP signal peptide is cleaved during transit into the endoplasmic reticulum, whereas propeptide cleavage may occur in the trans-Golgi complex (63).

(iv) Intron structure. All of the sequenced *P. chrysosporium lip* genes contain either eight or nine introns ranging in size from 49 to 78 bp. The intron/exon junction sequences conform to those in other filamentous fungi, with PuPy (almost always GT) at the 5′ end of the intron and AG at the 3′ end. The internal lariat formation sequences are not as strictly conserved (14, 196). Six of the introns are in identical positions in all of the *lip* genes (Fig. 3), and the sequences surrounding the intron/exon junctions are also conserved (163). In all of the sequences except *GLG1*, encoding the H2 isozyme from BKM-F-1767 (134), intron 1 splits the signal sequence from the propeptide (Fig. 3). In all of the *lip* genes, the distal His and distal Arg, which are separated by only two amino acids in the protein sequence, are separated by an intron (intron 2 in Fig. 3) and a 3′ intron/exon junction is adjacent to the proximal His codon (intron 6 in Fig. 3). Likewise, the last intron separates the C-terminal proline-rich exon from the remainder of the coding sequence in all sequenced *lip* genes.

We have proposed that the *P. chrysosporium lip* gene family can be divided into four subfamilies on the basis of intron/exon structure (162) (Fig. 3). Subfamily I includes all of the H8 and additional related sequences. Subfamily II consists of the H10 gene *GLG2* (208) and its allelic variant *GLG5* (66), and subfamily III consists of *GLG6* (135). Subfamilies II and III are identical to subfamily I, except for the addition of an intron into the largest exon (exon 7). Subfamily IV, consisting of the H2 sequence *GLG1* (134), has both of the extra introns of subfamilies II and III but lacks introns 1 and 4 from all other groups (Fig. 3). Verification of this proposed subfamily classification will require identification of the protein isozymes encoded by several of the sequenced genes, as well as sequencing of the genes encoding additional isozymes. This classification is not incompatible with one proposed previously on the basis of peptide fragmentation patterns and slight differences in reactivity (56). However, the number and position of introns in the *lip* genes constitute more fundamental and clear-cut criteria for classification of subfamilies than does the chromatographic and kinetic behavior or peptide analysis of the proteins. Recent evidence suggests that *lip* subfamilies may be differentially regulated (181).

(v) Regulatory sequences. The 5′ noncoding regions of the sequenced *lip* genes contain a TATA box positioned 66 to 81 bp upstream of the translation initiation codon and a CAAT sequence located between −107 and −228. S1 nuclease mapping has confirmed that transcription initiates downstream of these sequences (89, 208). Sequence elements similar or identical to those which bind the cAMP response element-binding protein (120) and the cAMP-responsive activating protein 2 (93) have been found in the promoter region of many but not all sequenced *lip* genes; in the *LG2*

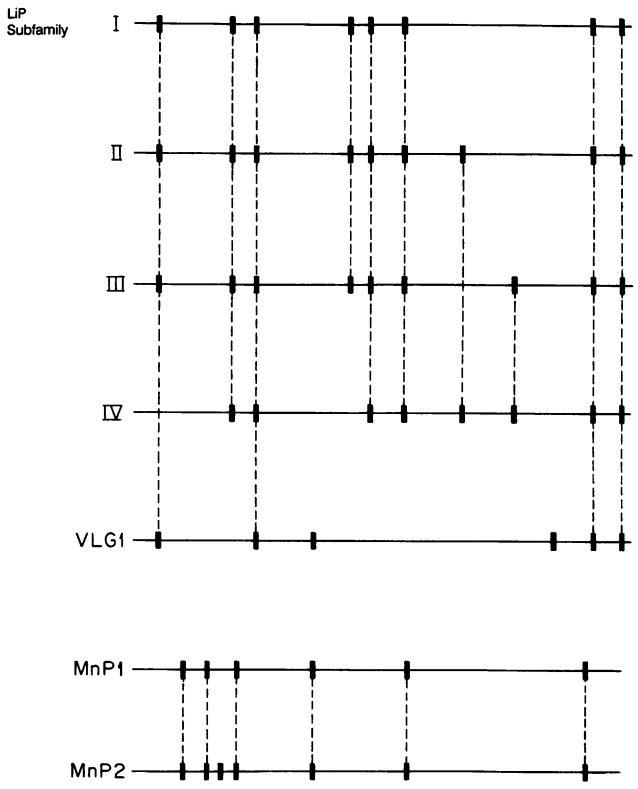

FIG. 3. Intron positions within *lip* and *mnp* genes. The sequences that make up each subfamily of *lip* genes are listed in the legend to Fig. 2. *VLG1* is a *lip* gene from *Trametes versicolor* (19). The *mnp1* and *mnp2* sequences are from references 75 and 126, respectively.

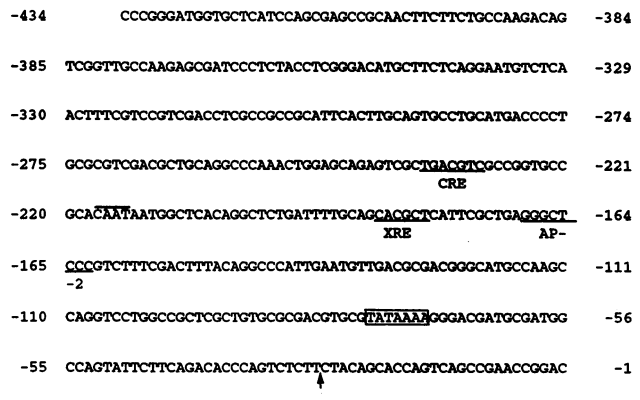

FIG. 4. Promoter region of the *LG2* gene encoding LiP isozyme H8 (162). The TATAA sequence is boxed, and the CAAT sequence is overlined. A cAMP response element (CRE), a xenobiotic response element (XRE), and an AP-2-binding site are underlined (162). The arrow denotes the start of the corresponding cDNA (164).

gene encoding the H8 isozyme from OGC101, they are located at −228 and −161, respectively, relative to the translation initiation codon (162) (Fig. 4). Sequences identical to mammalian xenobiotic response elements, which activate gene transcription in response to aromatic hydrocarbons (51, 62), are also found within the promoter regions of some *lip* genes (at −179 relative to the initiation ATG of *LG2*) (162) (Fig. 4). Analysis of the significance of these putative promoter sequences may increase our understanding of the regulation of these important genes. Almost all of the *lip* gene translation initiation codons are embedded within the eukaryotic consensus sequence GNN*ATG*G (107), and most have the sequence GAC*ATG*G. The putative 3′ polyadenylation signals are more variable, as is typical for fungal genes (14). The *lip* cDNA corresponding to the *LG2* gene has the sequence AATCAA 24 bp upstream of the poly(A) tail (164). Two other *lip* cDNAs have the sequence AATAT/CA 14 bp upstream of the poly(A) tail (45).

(vi) Clustering of *lip* genes. There is evidence for clustering of *lip* genes in the *P. chrysosporium* genome. Two clones, each carrying alleles of two convergently transcribed *lip* genes, have been isolated (66, 91). The genes are separated by 1.3 kb of flanking DNA, and a third *lip* gene is located about 15 kb distant (66). Restriction fragment length polymorphism mapping of strain ME-446 localized the *lip* genes to two unlinked clusters (153), one of which contained the H2-encoding gene (95). Contour-clamped homogeneous electric field gels of BKM-F-1767 localized five *lip* genes to one dimorphic chromosome (66) and a sixth gene encoding isozyme H2 to a second dimorphic chromosome (181).

(vii) *lip* genes from other fungi. *P. chrysosporium lip* gene probes have been shown to hybridize to multiple sequences in the DNA of other white rot fungi (91, 101). A cDNA clone encoding a lignin peroxidase gene from *Phlebia radiata* has been sequenced and is approximately 60% identical to nucleotide and amino acid sequences of *P. chrysosporium lip* genes (167). Likewise, a sequenced lignin peroxidase gene from *Trametes versicolor* has 55 to 60% amino acid identity with *P. chrysosporium lip* genes (19). As in *P. chrysosporium*, the *T. versicolor* LiPs are encoded by multiple genes, at least some of which are clustered in the genome (19). The positions of four of the six introns are also conserved between the *T. versicolor VLG1* gene and the *P. chrysosporium lip* genes (Fig. 3) (162).

Manganese peroxidase. (i) *mnp* sequences. Like LiP, the MnP isozymes are encoded by multiple genes (142, 146). The first *mnp* cDNA (*mnp1*), from strain OGC101, was cloned by using polyclonal antibodies raised against purified MnP isozyme 1 (146). cDNA clones corresponding to MnP isozyme H4 from BKM-F-1767 (140) and to two H4-encoding alleles from OGC101 (*mnp2a* and *mnp2b*) (126) also have been characterized. Genes corresponding to the cDNA clones from OGC101 have been sequenced (75, 126).

(ii) Coding region. The *mnp1* gene from OGC101 encodes a mature protein of 357 amino acids preceded by a 21-amino-acid leader sequence (146), whereas the H4-encoding sequences predict a 358-amino-acid protein preceded by a 24-amino-acid leader sequence (126, 140). The coding regions of *mnp1* and *mnp2* have a nucleotide identity of approximately 70% and an amino acid identity of 88%. The *mnp* and *lip* cDNAs exhibit about 50 to 65% identity at the amino acid level and about 60% identity at the nucleotide level. Both enzymes have an abundance of acidic residues, consistent with their low pIs. The MnP leader sequences consist of N-terminal, hydrophobic, and C-terminal domains characteristic of signal peptides (146, 194). However, the MnP leaders lack an obvious propeptide ending in a dibasic amino acid pair, as found in the LiP sequences. The sequences flanking the proximal His, distal His, and distal Arg are similar to those in LiP and other peroxidases (146). Likewise, the sequences surrounding the conserved Asp (Asp-235 in CcP; see above) are similar in the LiP and MnP sequences (163) (Fig. 1). The MnP proteins lack the proline-rich C-terminal region found in the LiPs. Whereas MnP1 contains three potential N-glycosylation sites, the MnP2 sequences contain four potential N-glycosylation sites. Both isozymes have numerous potential O-glycosylation sites. The coding regions of the *mnp* genes have a high G+C content, but their codon bias indices are lower than those of most of the *lip* sequences, with the *mnp2* sequence exhibiting less codon bias than the *mnp1* sequence (162).

(iii) Intron structure. The *mnp1* gene contains six introns ranging in size from 57 to 72 bp (75), and the *mnp2* gene contains seven introns of 50 to 55 bp (126). The positions of six of the introns are identical in the two genes, with the additional *mnp2* intron splitting the distal His codon in exon 3 of *mnp1* (Fig. 3). This suggests that MnP1 and MnP2 may belong to different subfamilies (see above). The intron splice junction sequences of the *mnp* genes all conform to the GT---AG rule, and all of the putative internal lariat formation sites conform to the CTPuAPy rule (14), with the exception of one intron in each gene which has a C in place of a purine. There is little similarity in intron positions between the *mnp* and *lip* genes. For example, the *mnp* genes lack the leader sequence intron found in the *lip* genes. The *mnp* genes also lack the intron adjacent to the C terminus (Fig. 3).

(iv) Regulatory sequences. The 5′ untranslated region of the *mnp* genes contain a TATAA element at position −81 relative to the translation initiation codon, as well as three inverted CCAAT elements (ATTGG). The initiation codon is embedded in the eukaryotic consensus sequence GCA*ATG*G (107). The promoter regions of the *mnp* genes contain putative recognition sites for the general transcription factor SP-1 (GGGCGG), and the *mnp1* gene also contains a putative recognition site for AP-2 (TGGGGA) (205) (Fig. 5). The promoter regions of both *mnp* genes contain several other sequence motifs: Four putative heat shock elements (HSEs) similar to the consensus sequence C--GAA--TTC--G (121) are located within 400 bp upstream of the *mnp1* translation initiation codon (75). The *mnp2* gene contains six HSEs

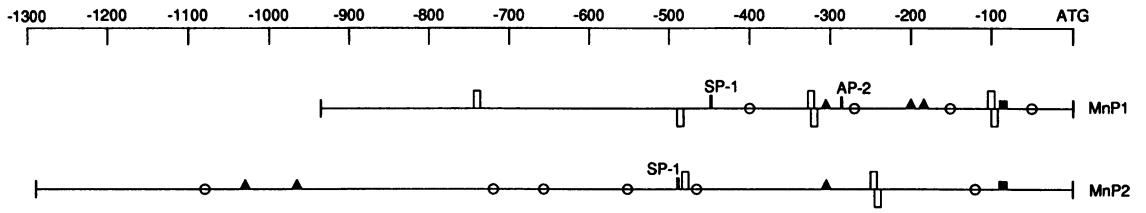

FIG. 5. Comparison of the *mnp1* and *mnp2* promoter regions (75, 126). Symbols: ■, TATAAA; ▲, inverted CCAAT; ○, HSE; □, MRE.

within 1,100 bp of the initiation Met (126). The HSEs match the eukaryotic consensus in six or seven of the eight positions and are interspersed with the inverted CCAAT elements in an arrangement similar to that reported for the human and rodent hsp70 promoters (82) (Fig. 5). The *mnp* promoters also contain putative consensus metal response elements (MREs), identical to the consensus regulatory sequences found in mouse metallothionein genes (43). Four of the five consensus MREs in the *mnp1* promoter and two of the three MREs in the *mnp2* promoter are arranged in pairs with overlaps to form a 4-bp palindrome (5) (Fig. 5). This arrangement is not found in other MREs so far identified. There is evidence to suggest that the MREs and HSEs of the *mnp* promoter have physiological significance (see below).

Structural features. Although the *lip* and *mnp* sequences have less similarity to other peroxidase sequences outside of the residues surrounding the active-site amino acids, hydrophobic cluster analysis has been used to demonstrate other common characteristics and to suggest evolutionary relationships among peroxidases. Dividing the peroxidases into three groups—LiP from *Phlebia radiata* and MnP and LiP from *P. chrysosporium*, HRP and turnip peroxidase, and yeast CcP—Henrissat et al. (87) have obtained hydrophobic cluster analysis scores that are greater than 75% within the groups and 60 to 70% between groups. The amino acid conservation is greater than 40% within the groups and 12 to 19% between groups. Enzymes such as catalase and chloroperoxidase show no homology with these groups by hydrophobic cluster analysis. On the basis of their hydrophobic cluster analysis, these workers suggest that of 13 helical segments in yeast CcP, 10 are conserved in the other peroxidases. They also conclude that the LiPs have a 40- to 50-residue C-terminal extension compared with the other peroxidases (87).

Recently, progressive sequence alignment has been used to align CcP with MnP, LiP, HRP, and peroxidases from turnip, tobacco, tomato, and potato (163). Alignment of CcP with the amino acid sequence of LiP2 from OGC101 indicates 20.8% identity and 47.0% similarity. Results with other LiP sequences are similar. This analysis also demonstrates that MnP is more closely related to CcP (22 to 23% identical) than are the LiPs from *P. chrysosporium*, *T. versicolor*, and *Phlebia radiata* and the plant peroxidases are more distantly related. Of the 30 gaps in the aligned sequences, 22 (73%) occur between elements of CcP secondary structure, in segments expected to form loops at the protein surface. Several of these alignment gaps occur very close to intron positions in MnPs and LiPs (163).

The eight LiP cysteine residues align with 8 of the 10 cysteine residues in MnP (163). Likewise, the positions of the cysteine residues are conserved among the plant peroxidases. However, the positions of the cysteine residues are not conserved between the fungal and plant peroxidases, nor do any of these cysteines align with the single cysteine residue in CcP, the only intracellular enzyme in the analysis (Fig. 6). Since the identity and positions of the active-site residues are conserved among the fungal and plant peroxidases (Fig. 1), these major differences in cysteine positions between the two groups suggest that evolution of the extracellular fungal and plant peroxidases diverged early from a common intracellular ancestor (163).

Regulation. (i) Nitrogen and carbon regulation. Ligninolytic activity in cultures of *P. chrysosporium* is a secondary metabolic function that is triggered by depletion of nutrient nitrogen (100, 104). Addition of NH_4^+ or various nitrogenous compounds to ligninolytic cultures suppresses the degradation of lignin as well as production of the secondary metabolite veratryl alcohol (57, 103). However, there is some evidence that nitrogen regulation may be strain dependent (37) and that it may be affected by the available carbon source (166, 188).

Several selections have been devised for mutants that are nitrogen deregulated for lignin degradation. One such selection utilizes polymeric dyes which can serve as substrates for the ligninolytic system (70, 79). This protocol has been used to isolate mutant strains of *P. chrysosporium* that decolorize the dyes rhemazol brilliant blue (113) and poly R (24) under high-nitrogen, low-oxygen conditions. Tien and Myer (185) mutagenized a lysine auxotroph and selected for mutants that grow on a nitrogen-sufficient medium supplemented with an adduct of lysine and a lignin model compound. Nitrogen-deregulated mutants often appear to produce more extracellular peroxidase than does the comparable wild-type strain (24, 138), probably because of the increase in

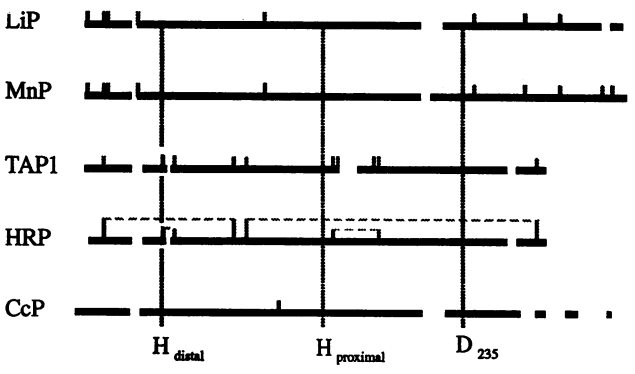

FIG. 6. Cysteine positions in peroxidases. The short vertical lines represent the cysteines. The horizontal lines in HRP represent the disulfide linkages. Positions of the conserved catalytic residues are indicated. LiP sequences are from references 29, 45, 162, 167, and 186. MnP sequences are from references 140 and 146. HRP is from reference 204, and CcP is from reference 97. TAP1 is tomato anionic peroxidase 1 (165). Gaps in the aligned sequences that are longer than four amino acids are indicated by spaces (163).

cell mass under nutrient-rich conditions. Dye degradation has also been used to isolate a LiP^- MnP^+ mutant (23), indicating that regulation of the two enzymes can be uncoupled.

Tien and Tu (186) used in vitro translation of poly(A) RNAs and Northern blot analysis to demonstrate that LiP is regulated by nutrient nitrogen at the mRNA level. Northern blot analysis indicates that MnP also is regulated by nutrient nitrogen at the transcript level (146).

There is evidence to suggest that the LiP and MnP isozymes may be differentially regulated by carbon and nitrogen. The nitrogen-deregulated mutants appear to express a different complement of LiP isozymes than the wild-type strains do (24, 113, 185). It has been suggested that the LiP isozymes H2 and H8 from strain BKM-F-1767 are both induced under nitrogen limitation, whereas only H2 is induced under carbon limitation (89). It recently has been confirmed (181) that the H2-encoding transcript (*GLG4*) is the most abundant transcript found under carbon limitation and that transcript levels under nitrogen limitation were approximately 1,000-fold lower. The H10-encoding gene (*GLG5*) was detectable only under nitrogen limitation. Another *lip* transcript (*V4*) was also more abundant under nitrogen limitation. H8-encoding and related sequences appeared to be expressed at similar levels in carbon- and nitrogen-limited cultures. *mnp* and glyoxal oxidase transcripts also were present under both carbon and nitrogen limitation (181). A recent study using ME-446 demonstrated that only the gene encoding H2 was expressed in cultures containing 0.1% glucose (low carbon) (95).

Pease and Tien (142) recently showed that in carbon-limited cultures, MnP isozyme H4 appears first, with H3 predominating later. Under these conditions no H5 is produced, whereas in nitrogen-limited cultures all three isozymes are present from an early time point.

However, both strain differences and other culture conditions including agitating versus stationary cultures, buffer composition (44, 103), and concentrations of various trace metals (102) can affect isozyme profiles. Furthermore, very little is known about the relative stabilities of MnP and LiP mRNAs and proteins. There is some evidence that MnP proteins are more stable in carbon-limited than in nitrogen-limited cultures, presumably because of the extracellular proteases synthesized under different conditions. LiP may be less stable than MnP under both carbon and nitrogen limitation (142), and a decline in LiP activity has been correlated with the appearance of an extracellular protease activity (47). The mechanisms for the regulation of the *P. chrysosporium* ligninolytic system by carbon and nitrogen levels, as well as by oxygen levels (100), remain to be elucidated.

(ii) Regulation by cAMP. The onset of ligninolytic activity in *P. chrysosporium* has been correlated with increased levels of cAMP (26, 123). Recent studies with cAMP inhibitors suggest that LiP is inhibited and MnP production is delayed or inhibited when intracellular cAMP levels are decreased (26).

(iii) Regulation of LiP by aromatic compounds. The addition of veratryl alcohol, a secondary metabolite of *P. chrysosporium* and a LiP substrate, or benzyl alcohol to fungal cultures results in increased LiP activity (55, 102, 103, 119, 187). Although veratryl alcohol has been shown to protect LiP from inactivation by H_2O_2, it is less likely that benzyl alcohol is protecting the enzyme in this way (193, 199). It is conceivable that these or other aromatic compounds, including possible LiP substrates or reaction products, induce LiP expression by mechanisms that remain to be determined.

(iv) Regulation of MnP by manganese. Accumulation of MnP activity in the extracellular medium of nitrogen-limited cultures depends on the presence of Mn(II), the substrate for the enzyme (22, 32, 68, 76). Western immunoblots of intracellular and extracellular *P. chrysosporium* protein demonstrate that MnP protein is present only under nitrogen-limiting conditions and only in the presence of Mn. This indicates that Mn is necessary for MnP synthesis rather than for the activation of a preexisting protein (32).

mnp mRNA is detectable on Northern blots of RNA isolated from cultures grown in the presence, but not in the absence, of Mn. The *mnp* mRNA is first detectable in 4-day-old cultures and reaches a maximum level in 5-day-old nitrogen-limited cultures grown in the presence of 180 μM Mn (32). Only Mn-supplemented cultures yield MnP protein on in vitro translation of poly(A) RNAs. Addition of Mn to 5-day-old nitrogen-limited, Mn-deficient cultures results in detectable *mnp* mRNA within 40 min, and the amount of *mnp* mRNA detected is a function of the amount of Mn added, up to a maximum of 180 μM. Actinomycin D, but not cycloheximide, inhibits *mnp* mRNA accumulation, indicating that Mn acts at the level of gene transcription, even in the absence of ongoing protein synthesis (30). Various other metals cannot replace Mn as inducers of either MnP activity (32) or *mnp* mRNA (30).

Although these initial studies were performed with *mnp1* probes, it has now been confirmed that *mnp2* gene expression is likewise dependent on the presence of Mn (126). High Mn concentrations have been shown to increase MnP activity in several other white rot fungi as well (22). The white rot basidiomycete *Dichomitus squalens* expresses MnP and degrades lignin only in the presence of Mn, suggesting that MnP is essential for lignin degradation by this organism (144).

In contrast to its effect on *mnp* gene transcription, Mn has no significant effect on mycelial dry weights (22, 32), on rates of carbon and nitrogen consumption, on the amount of extracellular protein (22), or on synthesis of the secondary metabolite veratryl alcohol (32). However, several additional protein bands are detectable among the in vitro translation products of poly(A)RNAs isolated from Mn-supplemented cultures (30), suggesting that other *P. chrysosporium* proteins may be regulated by Mn. Orth et al. (138) recently showed that glyoxal oxidase production in a nitrogen-deregulated lysine auxotroph is entirely dependent on the presence of Mn and is undetectable at Mn concentrations as low as 6 μM. The adenylate cyclase of *P. chrysosporium* apparently requires Mn for maximal activity (122), and there is evidence that intracellular cAMP may be involved in the regulation of the lignin degradation system of *P. chrysosporium* (26, 28, 123).

Currently we are examining whether the putative MREs of the *mnp* promoter regions (Fig. 5) are involved in the Mn regulation of *mnp* gene transcription (see below). Several metal ion-regulated gene transcription systems have been studied in detail (94, 154). Most of these are single-component systems, wherein a single intracellular metalloregulatory protein functions as both the metal receptor and the *trans*-acting transcription factor (154). Although the possibility that Mn is stabilizing *mnp* mRNA or is affecting *mnp* gene transcription via a multicomponent system has not been ruled out, the existence of an Mn-binding transcription factor similar to the Cu-binding Ace1 protein that activates transcription of the *S. cerevisiae* metallothionein gene (64) is an attractive model for the *mnp* system. Although Mn is known to be involved in the synthesis of some secondary

metabolites in other fungi (172, 203), this is, to our knowledge, the first instance of Mn regulation of gene transcription to be examined at the molecular level.

(v) **Heat shock.** Northern blot analysis demonstrates that a 45°C heat shock for 1 h results in the accumulation of *mnp1* mRNA even in cells grown in the absence of Mn (31, 75, 76). *mnp* mRNA can be detected within 10 min following transfer of the cultures from 37 to 45°C. As with Mn induction of *mnp* gene transcription, heat shock is an effective inducer only under nitrogen depletion and the effect is inhibited by actinomycin D. Heat shock-induced *mnp* mRNA is no longer detectable 1 h after the temperature has returned 37°C, and the effects of heat shock and Mn on *mnp* gene transcription appear to be additive (31).

These results suggest that the putative HSEs found in the promoter region of the *mnp* genes (Fig. 5) are physiologically functional. However, induction of *mnp* gene transcription by heat shock in the absence of Mn does not result in detectable MnP activity or in either intracellular or extracellular MnP protein as detectable by Western blots. In the presence of both Mn and heat shock, active MnP protein is produced (31). It is not known why heat shock does not result in MnP production. It is possible that Mn is required for processing of the primary transcript, for stabilization of the *mnp* mRNA, or for some later posttranscriptional step. It is also possible that the Mn-induced and heat shock-induced transcripts are not identical. The *mnp* genes are not typical heat shock genes in that they have introns and lack the long 5′ untranslated leader sequences that are characteristic of such genes (75, 121).

The significance of heat shock regulation of *mnp* genes is not known. No HSEs have been identified in the promoter regions of *lip* genes, nor is there any evidence that these genes are regulated by heat shock. However, lignin degradation is an oxidative process, and *P. chrysosporium* produces H_2O_2 as part of its lignin-degrading system. Therefore it is conceivable that oxidative stress, acting through the heat shock system, is a factor in the regulation of MnP expression (75).

Possible interrelationships between heat shock and metal regulation of *mnp* gene expression are also of interest. To our knowledge, the rat heme oxygenase gene contains the only other promoter in which both HSEs and MREs have been identified. This gene is known to be regulated by heat shock and heavy metals, as well as by heme, bromobenzene, and endotoxin (131). It also has been shown that the *S. cerevisiae* metallothionein gene (*CUP1*) is regulated by heat shock via the heat shock transcription factor and HSEs (175), as well as by copper and silver via the Ace1 protein (90).

Currently our laboratory is studying the mechanisms of Mn and heat shock regulation of *mnp* gene transcription by using a *mnp1* promoter-reporter gene construct (see below).

Heterologous expression. A *lip* cDNA lacking the leader sequence has been expressed in *Escherichia coli* strains. The apoprotein is present in inclusion bodies and lacks glycosylation (11). However, since successful reconstitution of the holoenzyme has not been described, this system is not currently suitable for producing active LiP.

Recently, *P. chrysosporium lip* and *mnp* cDNAs both have been expressed in the baculovirus system. A cDNA clone encoding LiP isozyme H8 was used to express glycosylated extracellular protein containing Fe protoporphyrin IX and capable of oxidizing iodide and veratryl alcohol. However, the recombinant protein is only about 50% active against veratryl alcohol compared with native LiP. Two recombinant proteins, differing slightly in molecular weight, were obtained, both of which react with anti-H8 monoclonal antibodies. Smaller intracellular immunoreactive proteins were also detected (96). Likewise, a cDNA encoding MnP isozyme H4 has been expressed in the same system and produces a MnP antibody-reactive extracellular protein of the correct molecular weight. The enzyme is active against the usual MnP substrates and is dependent on both Mn(II) and H_2O_2 for activity, although it has a slightly lower pH optimum than the fungal H4 does (141).

Other *P. chrysosporium* Genes

Cellulases. Cellobiohydrolase genes from *P. chrysosporium* have been cloned by using a fragment of the exocellobiohydrolase I gene from *Trichoderma reesei* as a probe. One of the *P. chrysosporium* genes (*cbh1-3*), encoding a deduced protein of 516 residues and containing two introns (177), exhibits 99.8% nucleotide sequence identity between strains ME-446 and BKM-F-1767 (40). Two additional genes (*cbh1-1*, *cbh1-2*), encoding deduced proteins of 45 and 50 kDa, are located 14 kb upstream of *cbh1-3*. These genes are encoded on the same strand and separated by 750 bp (40). Thus the three sequenced *P. chrysosporium cbh1* genes are clustered in an arrangement quite similar to three of the *lip* genes (66). There is some conservation in intron positions among the three *cbh1* genes, and there appear to be additional *cbh1*-like genes in *P. chrysosporium* (40).

Transcription of *cbh1-3* is induced by cellulose and apparently repressed by glucose (40, 177). *cbh1-1* and *cbh1-2* are not induced by cellulose; nor do they appear to be glucose repressed. The low-level constitutive expression of these genes correlates with their relatively low codon bias. In contrast, *cbh1-3*, which is expressed at levels 10- to 1,000-fold higher than those of *cbh1-1* or *-2* in cellulose or under glucose-nonrepressing conditions (40), has a codon bias similar to many *lip* genes (162).

Biosynthetic genes. The *P. chrysosporium trpC* gene, encoding a 788-residue tryptophan biosynthetic enzyme, has been isolated by complementation of an *E. coli trpC* mutant with a *P. chrysosporium* λEMBL3 genomic library (176). As in other filamentous fungi, the sequenced gene encodes a single protein with three enzymatic activities, glutamine amidotransferase (GAT), indoleglycerolphosphate synthase (IGPS), and phosphoribosyl anthranilate isomerase (PRAI), in the order NH_2-GAT-IGPS-PRAI-COOH. This is also the gene order within the *E. coli* Trp operon. The amino acid sequence is highly conserved among all of the sequenced fungal genes; however, RNA-primed polymerase chain reaction has demonstrated that the *P. chrysosporium* gene contains a single 50-bp intron, the first intron to be found among the fungal *trpC* genes. This intron apparently does not affect expression of the gene in *E. coli* since it is located within the GAT coding region, which is nonessential in that organism. The *P. chrysosporium* gene apparently also contains an insertion of approximately 30 amino acids compared with the other known fungal sequences. Northern blot analysis demonstrates that the gene is expressed in the presence or absence of tryptophan, suggesting that it is constitutive (171). This gene has been used to complement a *Coprinus cinereus* Trp auxotroph (39).

The *ade1* gene, encoding phosphoribosylaminoimidazole synthetase from *P. chrysosporium*, has been cloned from a λEMBL3 library of strain OGC101 by using the *Schizophyllum commune ade5* gene as a probe (8). The identity of the gene has been determined by transformation of the *Neuros-*

pora crassa ade2 auxotroph with the *S. commune* gene (3). The plasmid pADE1, containing a 6-kb insert in pUC18, has been mapped for restriction sites, and the *ade1* gene has been localized to a 2.2-kb fragment by transformation of the *P. chrysosporium ade1* auxotroph with various restriction digests (see below). It is possible that, like the *trpC* gene, the *ade1* gene encodes multiple enzyme functions (8).

The *ura3* gene, encoding orotidylate decarboxylase (ODase), also has been cloned from a λEMBL3 library of strain OGC101 by using the *S. commune ura1* gene as a probe. The plasmid pURA3, containing an 11-kb insert in pUC18, and a subclone pURA3.1, containing a 6.6-kb insert in the vector Bluescript SK+, have been mapped for restriction sites. The *ura3* gene has been localized to a 2-kb fragment by transforming *P. chrysosporium ura3* auxotrophic strains with restriction digests and electroeluted fragments (1).

Recently, we cloned and sequenced the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene from *P. chrysosporium* by using the *Aspergillus nidulans gpdA* gene (147) to probe a λEMBL3 library of strain OGC101. The amino acid sequence of the *P. chrysosporium* glyceraldehyde-3-phosphate dehydrogenase is very similar to those of other fungi (86a, 125). We intend to use the promoter from this gene to attempt to obtain high-level expression of our cloned *mnp* and *lip* genes in *P. chrysosporium* during primary metabolism.

DNA Transformation of *P. chrysosporium*

Heterologous transformation. The first transformations of *P. chrysosporium* (3, 6) were performed by using adenine biosynthetic genes from *S. commune*, pADE2 and pADE5 (60), to complement two adenine-requiring *P. chrysosporium* auxotrophs from different complementation groups, *ade2* and *ade1*, respectively (78). Swollen basidiospores are protoplasted with Novozym 234 and Cellulase CP (77) and transformed by a method similar to that developed for *S. commune* (132, 179). Fragments containing the *ade2* and *ade5* genes from *S. commune* have been subcloned into pUC18 to create the plasmids pADE2-3b and pADE5-2g. These subclones have been mapped for restriction sites, and the approximate locations of the complementing genes within the inserts have been determined by transformation experiments with plasmid digests (3).

Frequencies of approximately 100 to 200 transformants per μg of DNA are obtained with these plasmids, and between 0.05 and 0.15% of viable *P. chrysosporium* protoplasts are transformed. Higher transformation frequencies are usually obtained with linearized than with circular DNA. Southern blot analysis demonstrates that the plasmid DNA is integrated into the chromosomal DNA, often in multiple tandem copies, at various locations in the genome. Occasionally there are indications on Southern blots of plasmid rearrangements or deletions. Since neither of the *S. commune* adenine genes probe the *P. chrysosporium* genes on Southern blots (3, 6), genetic crosses between double mutants transformed with the *ade2* gene and other auxotrophic strains were used to demonstrate that the transforming DNA integrates at sites other than the resident adenine biosynthetic gene (6).

The transforming DNA is mitotically and meiotically stable on both selective and nonselective media. However, it is often necessary to purify transformants, since protoplasts can undergo fusion in the presence of polyethylene glycol (77). In addition, the protoplasts are plated in high concentrations and there is sometimes background regeneration of untransformed protoplasts. Thus transformants are sometimes heterokaryotic, consisting of both transformed and untransformed nuclei, and it is necessary to purify them by fruiting and selecting single basidiospore progeny which are homokaryotic (7). Purified transformants give rise only to transformed progeny, demonstrating that they are meiotically stable.

To develop a transformation system based on complementation of uracil auxotrophs, *P. chrysosporium ura3* auxotrophs deficient in ODase and *ura5* auxotrophs deficient in orotate phosphoribosyltransferase have been selected by 5-fluoroorotate resistance (21). These auxotrophs have been transformed (1) with plasmids containing the complementary pyrimidine nucleotide biosynthetic enzymes from *S. commune* (61) and the ascomycete *Podospora anserina* (17), respectively.

The results from transformations of the *ura3* auxotrophs with the *S. commune* gene are similar to those obtained with the adenine biosynthetic genes from *S. commune*. Transformation frequencies range from approximately 100 to 600 transformants per μg of DNA, and approximately 0.3% of viable protoplasts are transformed. In contrast, *ura5* auxotrophs are transformed with the *Podospora anserina* gene at frequencies of only 1 to 25 transformants per μg of DNA, although the protoplasts regenerate at approximately the same frequency as other auxotrophic protoplasts do. The Ura^+ transformants are mitotically and meiotically stable, and the transforming DNA integrates at various chromosomal locations in single or multiple copies (1).

Homologous transformation. The *P. chrysosporium ade1* and *ura3* genes (see above) have been used to transform the corresponding auxotrophs to prototrophy. The transformation frequencies obtained with plasmids containing these genes (pADE1 and pURA3.1) are approximately the same as those obtained with the corresponding *S. commune* genes. However, in general the transformants appear earlier with the *P. chrysosporium* genes than with the *S. commune* genes (3 to 5 days after plating, compared with 4 to 7 days). After purification, the transformants are mitotically and meiotically stable (1, 8).

Southern blot analyses of transformants obtained with pADE1 and pURA3.1, as well as with the original clones containing 11 to 12 kb of homologous DNA, demonstrate that the DNA is integrated into the chromosome ectopically in single or multiple copies (1, 8). Although this does not rule out the possibility that homologous integration has occurred via a double-crossover or gene conversion event, in all cases the presence of additional bands corresponding to the plasmid DNA suggests that the transforming event involved ectopic integration. However, in the basidiomycete *C. cinereus*, integration at the homologous locus occurs in only about 5% of the transformants (18). If the frequency is similar in *P. chrysosporium*, it is unlikely that homologous integration resulting in disruption of the resident gene would be detected by Southern analysis of a limited number of transformants (see below).

Transformation to drug resistance. In fungi such as *P. chrysosporium*, in which auxotrophic genetic markers are available, transformation systems based on complementation can take advantage of the variety of metabolic genes that have been cloned in other organisms (3, 6), as well as providing a method for cloning homologous genes from a genomic library (60). However, dominant selectable markers are useful for transformation of wild-type strains. Low-frequency transformation of *P. chrysosporium* to G418 re-

sistance has been reported (155, 157), with a vector containing the bacterial kanamycin resistance determinant of Tn*903* (83) and an endogenous extrachromosomal *P. chrysosporium* sequence that apparently supports autonomous replication in *S. cerevisiae* (159) and in *P. chrysosporium*. The vector is maintained in *P. chrysosporium* transformants as an extrachromosomal circular plasmid at a very low copy number under nonselective as well as selective conditions. It is recoverable from the total DNA via high-frequency *E. coli* transformations (158). Likewise, a plasmid containing the kanamycin resistance determinant inserted into the coding region of a *lip* gene encoding isozyme H2 (208) was found to be maintained extrachromosomally (156). Recently, a smaller plasmid was isolated from *P. chrysosporium* transformants that also conferred G418 resistance and apparently was maintained extrachromosomally at a higher copy number (157). A similar example of extrachromosomal maintenance of a recombinant plasmid containing the bacterial hygromycin resistance gene and fungal chromosomal sequences has been reported for drug-resistant transformants of the basidiomycete *Pleurotus ostreatus* (143).

Utilization of *P. chrysosporium* transformation. (i) Studies on gene regulation. Our *P. chrysosporium* transformation system is being used to study the regulation of *mnp* gene transcription by Mn. Transcriptional fusions of the *mnp1* promoter with a reporter gene have been made (74). The *S. commune ura1* gene encoding ODase has been chosen as a reporter since this gene is known to be expressed in *P. chrysosporium* (see above). The coding region of this gene has been ligated to the *mnp1* promoter, immediately upstream of the translation initiation codon, and the resulting gene fusion has been ligated into the multiple cloning site of pUC18. By using the *ade5* gene from *S. commune* as a selectable marker (see above), the *mnp1* promoter-*ura1* reporter gene has been transformed into an *ade1 ura3* double auxotrophic strain. ODase activity in the Ade^+ transformants is detected under the same physiological conditions as MnP activity and only in the presence of Mn, demonstrating that 1,500 bp of the *mnp1* promoter is sufficient to confer Mn regulation on a heterologous reporter (74). This system is being used to define the specific sequences required for Mn regulation of *mnp* gene transcription.

(ii) Gene targeting and disruptions. The ability to target transforming DNA to specific sites in the *P. chrysosporium* genome would facilitate studies such as those described above involving promoter-reporter constructs. Since the site of integration can affect expression of the transforming DNA (13, 127, 129), the ability to target the DNA to a specific genomic location would control for position effects among different transformants.

The isolation of *P. chrysosporium* mutants would be greatly facilitated by the development of a gene disruption or gene replacement system for this organism. An examination of the roles of individual gene products involved in lignin degradation by *P. chrysosporium* has not been possible because of the lack of a selection for nonpleiotropic mutants. Gene disruptions or replacements involving cloned *mnp* or *lip* sequences would lead to mutants deficient in specific isozymes which could be used for analysis of the roles of individual isozymes in lignin degradation by *P. chrysosporium*. Such a system of reverse genetics also could be used to construct additional mutants by using genes isolated from genomic libraries by heterologous probes or from expression libraries by antibodies.

We have constructed a vector that contains the *S. commune ade2* gene (3) ligated into the coding region of the *P. chrysosporium ura3* gene (1) (pUAU). Whereas this plasmid does not complement the *ura3* auxotrophic strain, it is able to transform the *ade2* strain to prototrophy. Ade^+ Ura^- transformants of the *ade2* strain are selected by their ability to regenerate on medium containing uracil and 5-fluoroorotate (see above). In preliminary experiments we have found that 5 to 10% of the Ade^+ transformants obtained with pUAU are also Ura^-. Complementation tests show that these strains are auxotrophic for *ura3*, and preliminary Southern analysis indicates that pUAU has replaced the resident *ura3* gene in these transformants (2). We are continuing these studies with the goal of developing a site-specific integration vector for *P. chrysosporium*. We also intend to develop a vector for disrupting or replacing individual *lip* and *mnp* genes.

CONCLUSIONS

1. Wild-type strains of *P. chrysosporium* are coenocytic and heterokaryotic. However, single basidiospores give rise to homokaryotic strains, and auxotrophic mutants are presumed to be homokaryotic. The genome content of *P. chrysosporium* is similar to that of other filamentous fungi. Restriction fragment length polymorphism and contour-clamped homogeneous electric field gel analyses can be used to map the *P. chrysosporium* genome.

2. LiP isozymes are encoded by multiple related genes which appear to be clustered in the *P. chrysosporium* genome. These genes can be classified into subfamilies on the basis of the number and positions of the introns. The coding regions of the *lip* genes are very G+C rich, which correlates with relative gene expression. The LiP isozymes are regulated at the level of gene transcription by nitrogen limitation, possibly via putative regulatory sequences present in the promoter regions of some *lip* genes. LiP is synthesized as a preproenzyme, and approximately 10 to 15% of its molecular weight is due to glycosylation. *lip* genes from other white rot fungi appear to be similar to those from *P. chrysosporium*.

3. MnP isozymes are also encoded by multiple related genes and alleles. MnP is regulated by nitrogen at the level of gene transcription. Putative MREs and HSEs found in the promoter regions of *mnp* genes presumably are involved in the regulation of *mnp* gene transcription by Mn, the substrate for the enzyme, and by heat shock. A 1,500-bp length of the *mnp* promoter appears to be sufficient to confer Mn regulation on a heterologous reporter gene. Although heat shock, oxidative stress, and other stresses induce gene transcription in the absence of Mn, no MnP protein is synthesized in the absence of Mn. *D. squalens*, another white rot fungus, produces MnP and degrades lignin only in the presence of Mn.

4. The sequences surrounding the active-site residues and various other aspects of protein structure are conserved in LiP, MnP, CcP, and plant peroxidases. The 8 Cys residues in LiP align with 8 of the 10 Cys residues in MnP. Likewise, the positions of the Cys residues are conserved among the plant peroxidases. However, the LiP and MnP Cys residues do not align with those in plant peroxidases, suggesting that the two peroxidase families underwent divergent evolution from a common intracellular precursor.

5. DNA transformation of *P. chrysosporium* auxotrophic strains with both homologous and heterologous biosynthetic genes results in transformation frequencies of up to 500 transformants per μg of DNA. The transforming DNA is mitotically and meiotically stable and is integrated into the

P. chrysosporium genome in single or multiple copies at various ectopic sites. Low-frequency homologous recombination resulting in gene replacement can be selected for. Low-frequency transformation to G418 resistance apparently occurs via low-copy-number maintenance of an autonomously replicating plasmid.

FUTURE DIRECTIONS

Understanding lignin and toxic-compound degradation by *P. chrysosporium* and other white rot fungi at a molecular level will require much additional research. The utilization of *P. chrysosporium* for bioremediation and biotechnological applications will require the identification, characterization, and cloning of additional components of the lignin-degradative system of this organism. Although it may be possible to efficiently express individual components of this system in other hosts, many applications will require the manipulation of the entire lignin-degrading system of *P. chrysosporium*. This, in turn, will require a detailed understanding of the regulatory mechanisms controlling individual components of the system. Mn regulation of MnP is of particular interest, because this is the first example of Mn regulation of gene transcription to be examined at the molecular level. Identification of Mn-responsive transcription factors and other genes that may be regulated by Mn, as well as the examination of possible relationships between regulation by Mn and regulation by heat shock, oxidative stress, or aspects of secondary metabolism such as nitrogen limitation, is an important area for future research. Although secondary metabolites in microorganisms have been extensively studied, little is known about gene regulation in secondary metabolism. The secondary metabolic enzymes of *P. chrysosporium* may prove to be a good model system for understanding the regulatory mechanisms of this important aspect of microbial physiology.

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