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Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*

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We have isolated and characterized a gene coding for the laccase of the lignin-degrading fungus *Phlebia radiata*. The gene has nine introns and recognizable fungal promoter elements. Sequences homologous to the consensus eukaryotic heat-shock regulatory element can be found in the promoter. RNA hybridization results indicate that this gene is regulated at the transcriptional level. The derived laccase amino acid sequence shows homology to plant ascorbate oxidases, suggesting that the basic structure of the laccase is similar to the three-fold repeated β-barrel of the ascorbate oxidases. Potential copper ligands and a residue carrying the prosthetic group pyrroloquinoline quinone (PQQ) in the laccase protein can be identified by homology. The intron/exon structure of the laccase gene suggests that this protein could have evolved by exon shuffling.

Introduction

Lignin degradation mediated by white-rot fungi is thought to be a non-specific oxidative process (for a review see Kirk & Farrel, 1987). The initial step in this process is the removal of an electron from a subunit of lignin by a ligninolytic enzyme. These fungal enzymes can be either peroxidases that use H_2O_2 as their electron acceptor or oxidases (laccases) that use O_2 for this purpose. The ability of laccases to oxidize lignin model compounds with phenolic hydroxyl groups suggests that these enzymes have a role in lignin degradation (Ishihara, 1980). The molecular genetics of the ligninolytic peroxidases has been widely studied (e.g. Tien & Tu, 1987; Brown *et al.*, 1988; Pribnow *et al.*, 1989; Saloheimo *et al.*, 1989) and one report on laccase sequences from a ligninolytic fungus, *Coriolus hirsutus*, has been published (Kojima *et al.*, 1990).

A number of different lignin-degrading enzymes of the white-rot fungus *Phlebia radiata* have been isolated and characterized (Niku-Paavola *et al.*, 1988; Karhunen *et al.*, 1990*a*). One of these is a laccase (EC 1.10.3.2) with a molecular mass of 64 kDa. Recently this enzyme has been suggested to contain two copper atoms, one in type 1 and one in type 2 configurations and the prosthetic group pyrroloquinoline quinone (PQQ) (Karhunen *et al.*, 1990*b*). Most of the laccases described so far have four copper atoms, including also the type 3 binuclear copper pair (Malkin & Malmström, 1970). Moreover, this *Phlebia* enzyme is the first laccase for which the presence of PQQ has been suggested.

In this work we describe the isolation and characterization of the chromosomal gene and cDNA coding for the laccase of *P. radiata*. The regulation of the gene was studied by RNA hybridization. The sequences of the gene and the putative laccase protein are compared with other known multicopper oxidase sequences and the structure and evolution of the enzyme are discussed.

Methods

Strains and vectors. Phlebia radiata ATCC 64658 was grown as described by Saloheimo *et al.* (1989) for RNA and DNA isolations. A chromosomal gene library from *P. radiata* was constructed in the λ EMBL3 vector using the host strain *Escherichia coli* NM 538 (Frischauf *et al.*, 1983). A *P. radiata* cDNA library was constructed in λ gt11 and was grown in the *E. coli* host Y1090 (Young & Davis, 1983). Bluescript M13+ (Vector Cloning Systems) and the host strain *E. coli* DH5 alpha (BRL) were used in plasmid constructions.

Isolation and sequencing of the laccase gene. P. radiata DNA was isolated by the method of Raeder & Broda (1985) and used in construction of a chromosomal gene library in λ EMBL3 (Kaiser & Murray, 1983). *Phlebia* laccase protein was purified and antibodies

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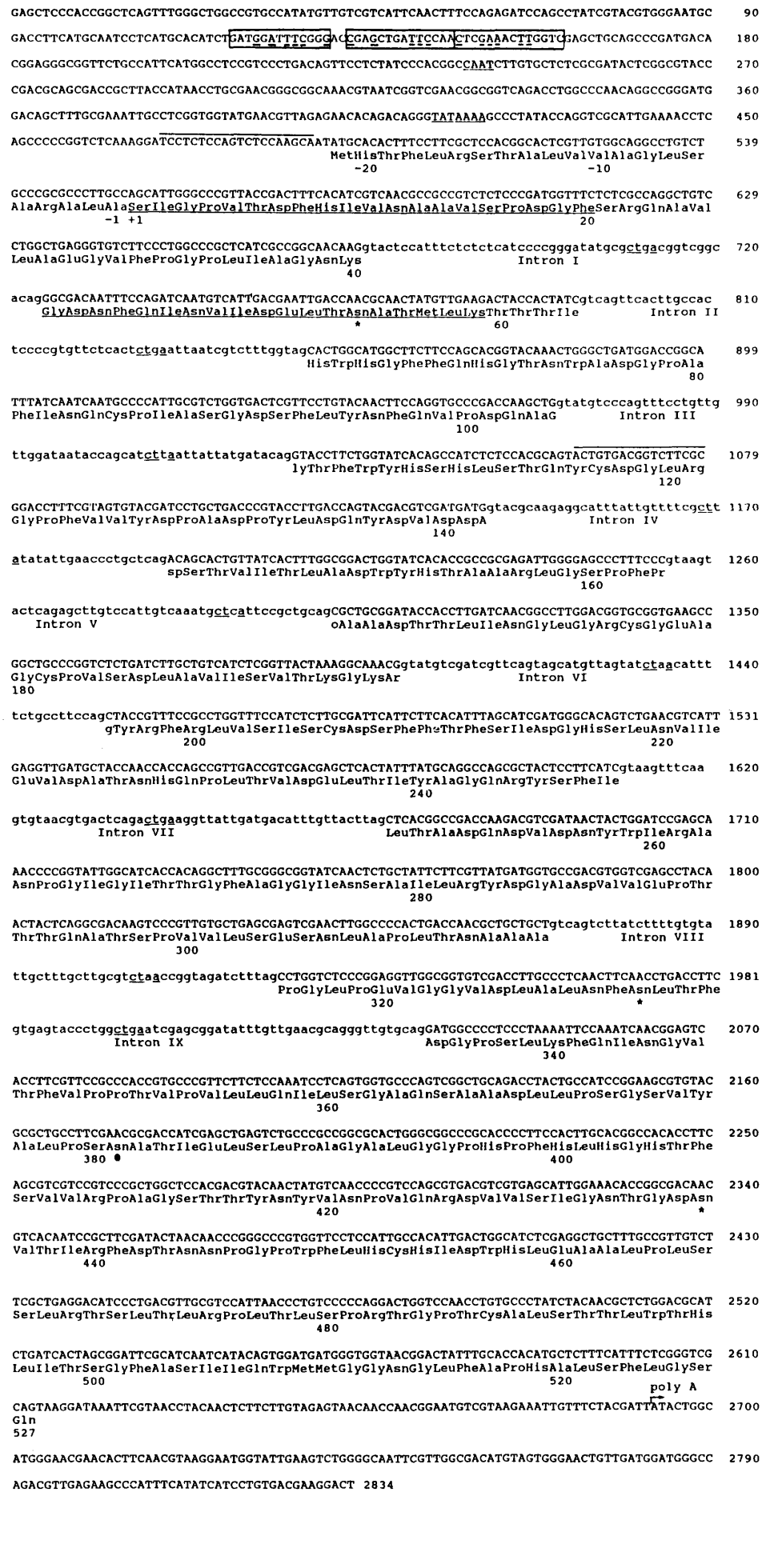
Abbreviations: AOase, ascorbate oxidase; HSE, heat shock element; ORF, open reading frame; PCR, polymerase chain reaction; PKDAO, porcine kidney diamine oxidase; PQQ, pyrroloquinoline quinone.

The nucleotide sequence data reported in this paper have been submitted to EMBL Data Library/GenBank and have been assigned the accession number X52134.



were prepared against it as described by Niku-Paavola *et al.* (1988). A *Phlebia* cDNA gene library was screened with antibodies as described by Huynh *et al.* (1983). cDNA synthesis and DNA amplification were done according to Frohman *et al.* (1988). DNA sequencing was done with synthetic oligonucleotide primers and plasmid DNA templates using modified T7 DNA polymerase (USB). Protein sequencing was done as described by Saloheimo *et al.* (1989).

RNA hybridization. P. radiata RNA was isolated as described by Saloheimo *et al.* (1989). The Hybond N nylon membrane (Amersham) was used in blotting and hybridization according to the manufacturer's instructions.

Results

Isolation and sequence of the P. radiata laccase gene

A *P. radiata* cDNA library constructed in λ gt11 (Saloheimo *et al.*, 1989) was screened with polyclonal antibodies against the laccase of the fungus. A positively reacting clone with an insert of about 800 bp was isolated. The cDNA library was further screened with the insert of this clone as a probe. The longest cDNA isolated in the second round of screening was about 1·5 kb, which could not contain the whole coding region of the gene. A genomic library was therefore constructed from *P. radiata* DNA in the vector λ EMBL3. This library was screened by plaque hybridization with the isolated cDNA as a probe. The chromosomal laccase gene was isolated and transferred from a λ clone into Bluescript M13+ as a 1·6 kb *Sac*I and a 2·0 kb *Asp*718 fragment. The 5′ end of the gene not present in the isolated cDNA clones was sequenced from the chromosomal plasmid constructions.

The 5′ end of the laccase cDNA was then cloned using the polymerase chain reaction (PCR). Two oligonucleotides based on chromosomal gene sequence (Fig. 1) were used in a PCR reaction. The template in this reaction was cDNA synthesized from *Phlebia* mRNA isolated from mycelium induced for production of ligninolytic enzymes. A DNA fragment comprising the 5′ end of the cDNA was obtained, cloned into Bluescript M13+ plasmid and sequenced. Comparing this sequence with the corresponding region in the chromosomal laccase gene revealed no differences except for the three introns that are in this area.

The region sequenced from the chromosomal and cDNA copies of the gene spans over 2834 bp of DNA (Fig. 1), which includes about 500 bp of the promoter region. A putative TATA box TATAAAA is found 77 bp and a putative CAAT element 253 bp upstream from the translation start site.

A gene encoding a manganese-dependent peroxidase of *Phanerochaete chrysosporium* has been found to contain heat-shock regulatory elements (HSE) in its promoter and shown to be regulated by heat shock (Godfrey *et al.*, 1990). Three sequences homologous to the eukaryotic consensus HSEs (C--GAA--TTC--G; Pelham, 1982) can be found in the promoter of the *Phlebia* laccase gene (boxed in Fig. 1). These sequences show a homology of 6/8, 5/8 and 6/8 to the consensus, although in the sequence farthest upstream there is only one base between the GAA- and TTC-like elements. Furthermore, the HSE-like element farthest downstream in the laccase promoter shows an identity of 10/14 base pairs to the most proximal HSE-like element of the Mn-peroxidase promoter of *Phanerochaete* (Godfrey *et al.*, 1990). Whether the *Phlebia* laccase gene is regulated by heat shock remains to be determined.

Sequences of two peptides digested from the laccase of *Phlebia radiata* with lysyl endopeptidase can be identified in the putative amino acid sequence derived from the gene studied (Fig. 1). Thus the gene isolated very probably codes for the *Phlebia* laccase described earlier (Niku-Paavola *et al.*, 1988). One of the peptide sequences is preceded in the ORF of the gene by 21 amino acids, reminiscent of a typical signal sequence with a positively charged N-terminus followed by a hydrophobic region. For this reason it is likely that this amino acid sequence is derived from the N-terminal peptide of the protein.

The molecular mass of the mature laccase protein derived from the ORF of the gene is 56440 Da, and that of the isolated laccase is 64 kDa (Niku-Paavola *et al.*, 1988). The difference (11·8%) is probably due to glycosylation. The putative laccase has four potential *N*-glycosylation sites (Asn-Xxx-Ser/Thr), at positions 54, 330, 381 and 436. The region containing the first *N*-glycosylation site was included in one of the two peptide sequences determined from the protein and, interestingly, Asn54 did not give an identifiable signal. This suggests that this glycosylation site is used *in vivo*.

RNA hybridization

P. radiata was grown in a medium with a low nitrogen content, to induce the production of ligninolytic enzymes (Saloheimo *et al.*, 1989) and mycelial samples were collected after 1, 2 and 3 d of growth. RNA was isolated

Fig. 1. Nucleotide sequence of the *P. radiata* laccase gene with the putative laccase amino acid sequence. Putative TATA and CAAT boxes are underlined in the promoter region of the gene. HSE-like sequences are boxed, with underlining of the bases homologous to the consensus (C--GAA--TTC--G). Introns are shown in lower case. Putative splicing signals are underlined in the introns. Sequences of oligonucleotides used in the PCR amplification of the 5′ end of the laccase cDNA are overlined. Peptide sequences obtained from the *Phlebia* laccase protein are underlined in the putative amino acid sequence. Potential *N*-glycosylation sites are shown by asterisks. The polyadenylation site observed is shown in the 3′ flanking area.

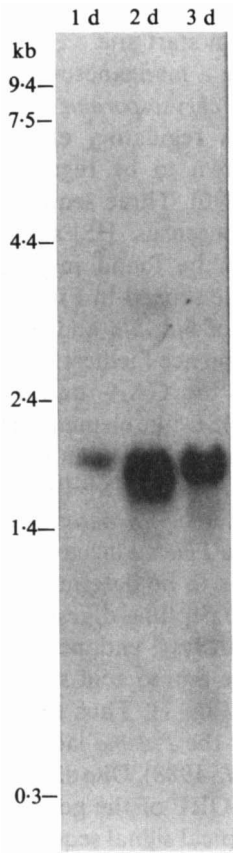


Fig. 2. Northern hybridization of *P. radiata* RNA isolated from mycelium induced for production of ligninolytic enzymes. Mycelium was grown for 1, 2 or 3 d as indicated at the top. RNA samples of 2 μg were used in each case. RNA Ladder (BRL) fragments were used as size standards.

and Northern hybridization with the laccase cDNA as a probe was performed from these samples (Fig. 2). Equal loading of RNA to the hybridized gel lanes was controlled by staining a similarly loaded RNA gel with acridine orange (data not shown). A single transcript of about 1·9 kb is seen at each timepoint. This is in good agreement with the size of the isolated laccase gene.

Production of laccase activity by *P. radiata* in bioreactor cultivations similar to that used for the RNA isolations has been described (Kantelinen *et al.*, 1989). Typically, the laccase activity starts to appear after day 1, reaching its peak value on day 3, after which it starts to decrease. The laccase mRNA levels (Fig. 2.) are consistent with the enzyme activity profile. Some laccase mRNA is transcribed on day 1. After 2 d of growth, as the enzyme activity is still increasing, the mRNA level is highest. On the third day the mRNA level has started to decrease. The above suggests that the regulation of the laccase gene of *Phlebia* occurs to at least some extent at the level of transcription. This has also been shown for two other types of ligninolytic enzymes, the lignin peroxidase (Tien & Tu, 1987) and Mn-peroxidase (Pribnow *et al.*, 1989) of the white-rot fungus *Phanerochaete chrysosporium*, and is generally the case for regulated fungal genes.

Discussion

Homology of the P. radiata laccase to other multicopper oxidases

The primary structures of six multicopper oxidases, human ceruloplasmin (Ortel *et al.*, 1984), laccases of *Neurospora crassa* (Germann *et al.*, 1988), *Aspergillus nidulans* (Aramayo & Timberlake, 1990) and *Coriolus hirsutus* (Kojima *et al.*, 1990) and the ascorbate oxidases (AOase) of cucumber (Ohkawa *et al.*, 1989) and pumpkin (Esaka *et al.*, 1990) have been reported previously. Most importantly, the crystal structure of the zucchini AOase has been published (Messerschmidt *et al.*, 1989). It is a dimeric protein with a molecular mass of 140 kDa. Each subunit of the dimer is divided into three domains with a similar polypeptide folding of a β-barrel type. The domains are thus clearly related to the small copper-containing blue proteins such as plastocyanins of plants (Colman *et al.*, 1978) and azurins of bacteria (Adman *et al.*, 1978). Therefore it would seem probable that the AOase has evolved through a triplication of an ancestral gene coding for a small copper protein (Messerschmidt *et al.*, 1989).

The primary structure of the zucchini AOase has not been published. However, the known amino acid sequence of the AOase from cucumber is about 90% homologous to the zucchini enzyme (Ohkawa *et al.*,1989; Messerschmidt *et al.*, 1989). Assuming that the structure of cucumber AOase is very similar to that of the zucchini enzyme we have compared the fungal laccases with the cucumber sequence. The *Neurospora*, *Phlebia* and *Coriolus* laccases are about 30% homologous to this AOase. The laccases of the ligninolytic fungi, *P. radiata* and *C. hirsutus*, show an identity of 63%. Interestingly, the homology of these two laccases to the *Neurospora* laccase is no greater than it is to the AOase. In the *A. nidulans* laccase, homology to the other oxidases can be detected only in the active-site regions.

The alignment of *Phlebia* and *Coriolus* laccase and the cucumber AOase sequences is shown in Fig. 3. The AOase has previously been aligned with *Neurospora crassa* laccase by another group (Messerschmidt & Huber, 1990). Our alignment is different from the previous one in the middle domain of AOase and shows greater homology between AOase and *Phlebia* laccase. Alignment of the proteins by hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) gives support to the

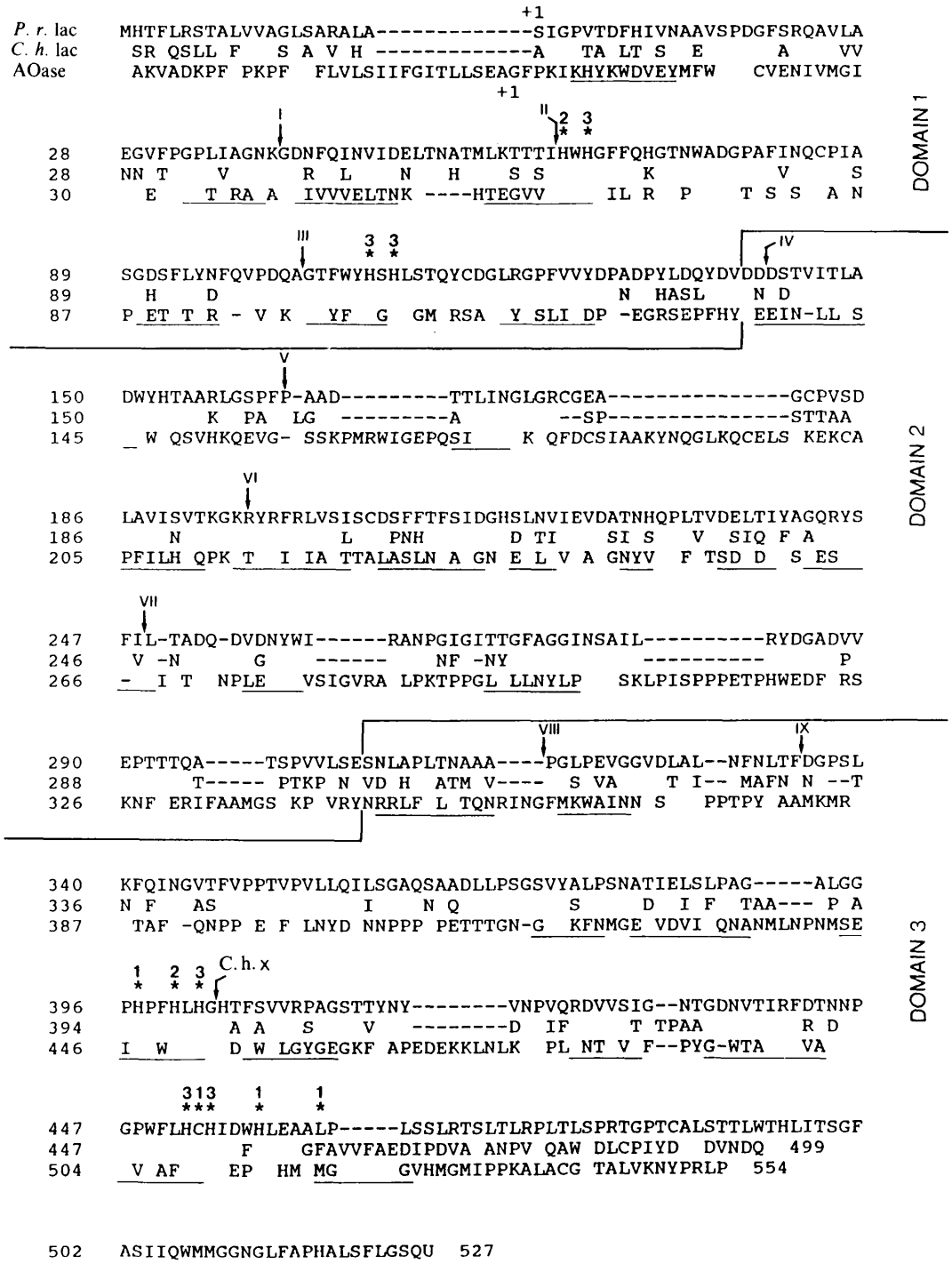


Fig. 3. Alignment of the amino acid sequences of *Phlebia radiata* laccase (*P. r.* lac), *Coriolus hirsutus* laccase (*C. h.* lac) and the ascorbate oxidase of cucumber (AOase). Conserved amino acids have not been marked, and gaps are indicated by -. The domain structure of the cucumber AOase is indicated. Amino acids working as copper ligands in the active site of AOase are shown by asterisks and numbers indicating the copper configuration which these ligands are parts of. β-Strands forming the basic fold of AOase are shown by underlining of the AOase sequence. Locations of introns in the laccase genes are shown by arrows with the intron numbering by Roman numerals (*C. h.* X refers to the tenth intron of the *Coriolus* laccase genes).

alignment presented here in regions of low homology (B. Henrissat & M. Saloheimo, unpublished). As the laccase sequences can be aligned with the AOase throughout their whole lengths, it is likely that the basic architecture of their polypeptide folding is similar to that of the AOase.

The three repetitive structural domains and the locations of the β-strands forming-the basic fold of the cucumber AOase are shown in the alignment in Fig. 3. The homology of the three proteins compared is highest in the N-terminal domain 1 and lowest in the middle domain 2. Moreover, the proteins are best conserved in the central parts of each domain. This is not unexpected, since the active-site regions of the AOase are located in the central parts of domains 1 and 3. Domain 2 contains many deletions in the laccases when compared to the AOase of cucumber. An extension of about 30 amino acids in the C-terminus of the *Phlebia* laccase is revealed by the alignment.

The active site of AOase contains four copper atoms, one type 1 or blue, one type 2 or normal, and two type 3 or binuclear (Messerschmidt *et al.*, 1989). The type 1 copper configuration is clearly homologous to the only copper atom in the small copper proteins (Colman *et al.*, 1978; Adman *et al.*, 1978). The ligands for this copper (two His, Met and Cys) that lie in the C-terminal domain 3 are conserved in the fungal laccases with one exception: Met 519 of the AOase is replaced by a leucine or a phenylalanine. The type 2 and type 3 copper atoms are in close proximity, forming a trinuclear complex. The type 2 copper has two histidines and the two type 3 coppers have six histidines serving as their ligands (Messerschmidt *et al.*, 1989). All of these amino acids are conserved in all of the fungal laccases.

The *P. radiata* laccase has been suggested to contain two copper atoms per protein molecule, in type 1 and type 2 configurations as indicated by electron paramagnetic resonance (Karhunen *et al.*, 1990*b*). As stated above, all the six ligands for type 3 copper atoms are conserved in the sequence of this laccase and also their surroundings are rather well conserved. Thus it would seem probable that the geometry of the active site of the *P. radiata* laccase is different from AOase in such a way that it is unable to bind the type 3 copper atoms.

Possible binding site of the prosthetic group PQQ

Recently, it has been suggested that the laccase of *P. radiata* is a quinoprotein, in that it contains PQQ as its prosthetic group (Karhunen *et al.*, 1990*b*). PQQ is a two-electron-transferring group found in many oxidoreductases of microbial, plant and animal origin. It is bound covalently to its apoenzyme in eukaryotic quinoproteins (Duine, 1988). van der Meer *et al.* (1988, 1989) have

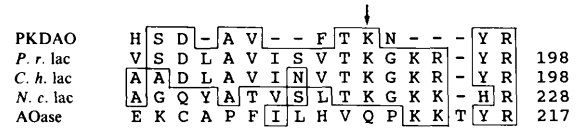


Fig 4. Alignment of the sequence of the peptide carrying PQQ in porcine kidney diamine oxidase (PKDAO) with homologous sequences in *Phlebia radiata*, *Coriolus hirsutus* and *Neurospora crassa* laccases and cucumber AOase. Homologous amino acids are boxed. The lysine suggested to be bonded to PQQ in PKDAO is indicated by an arrow. Amino acid numbering is shown.

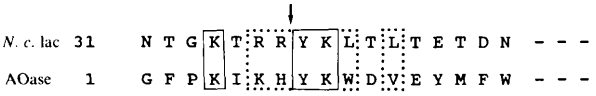


Fig. 5. Alignment of the *Neurospora crassa* laccase and cucumber AOase protein sequences (Messerschmidt & Huber, 1990) in the region containing the only intron of the laccase gene (Germann *et al.*, 1988). Identical amino acids are boxed with a solid line and similar ones with a broken line. The laccase gene intron position is shown by an arrow. The first β-strand of AOase is represented by a dashed line. Amino acid numbering is indicated.

suggested that PQQ forms an amide bond with a lysyl residue in the apoprotein of porcine kidney diamine oxidase (PKDAO). They have also determined a short segment of amino acid sequence around the lysine proposed to carry PQQ.

Amino acid stretches homologous to the PQQ-carrying peptide of PKDAO can be found in the laccases of *Phlebia radiata*, *Coriolus hirsutus* and *Neurospora crassa* (Fig. 4). It is interesting to note that the highest homology is found between PKDAO and the laccases of *Phlebia* and *Coriolus* (8 identical residues out of 11). The above suggests that the amino acid carrying PQQ in *P. radiata* laccase could be Lys193. The prosthetic group would thus be situated in the middle domain 2 of the enzyme (Fig. 3). The PQQ-carrying lysine of PKDAO is conserved in *N. crassa* laccase, but not in cucumber AOase. In addition, this region of AOase shows rather low homology to the other proteins. To our knowledge PQQ analysis has not been performed for *Coriolus* or *Neurospora* laccases. However, their different copper content (four atoms per enzyme molecule) from the *Phlebia* laccase suggests a different active site structure from the *Phlebia* protein. PQQ was not found when the structure of the zucchini AOase was solved (Messerschmidt *et al.*, 1989).

Janes *et al.* (1990) recently showed that bovine serum amine oxidiase, which has been suggested to be a quinoprotein, has 6-hydroxydopa instead of PQQ as its cofactor. They suggested that this could also be true for other mammalian copper oxidases that have been characterized as quinoproteins. Whether this concerns PKDAO or *Phlebia* laccase remains to be demonstrated.

The intron/exon structure of the P. radiata laccase gene

The protein-coding region of the *P. radiata* laccase gene is interrupted by nine introns. This number is relatively large for a fungal gene (Ballance, 1986). However, the lignin peroxidase genes sequenced from *Phanerochaete chrysosporium* (e.g. Brown *et al.*, 1988) have eight introns and in the lignin peroxidase gene of *Phlebia radiata* the number is at least eight (M. Saloheimo, unpublished). The *Coriolus hirsutus* laccase gene contains ten introns (Kojima *et al.*, 1990). The positions of nine of these are conserved in the *Phlebia* laccase gene without a single base change when the alignment presented in Fig. 3 is used as a guideline. The most proximal intron of the *Coriolus* gene has no counterpart in the *Phlebia* sequence. As is typical for the genes of filamentous fungi (Ballance, 1986), introns in the *Phlebia* laccase gene are short, 47–59 bp in length. Sequences at the intron/exon junctions and splicing signals (Fig. 1) follow the fungal consensus (Ballance, 1986).

In genes coding for proteins with the $\alpha\beta$-barrel type of folding, introns are non-randomly positioned in regions between the repeated α-helix-β-strand structures of the protein (e.g. Lonberg & Gilbert, 1985; Buisson *et al.*, 1987). Therefore it has been suggested that these proteins could have evolved by duplications of exons that code for structural domains of the protein (Lonberg & Gilbert, 1985). We have indicated the locations of introns in the *Phlebia* and *Coriolus* laccase genes and the locations of β-strands forming the basic fold of AOase in Fig. 3. Provided that the β-strands of the laccase are in the positions suggested by the alignment in this figure, then it appears that the introns are non-randomly placed in the laccase gene. Five out of the nine introns conserved in the *Phlebia* and *Coriolus* laccase genes (I, III, IV, VI and VIII) are at or only a few nucleotides away from positions corresponding to N-termini of β-strands in the AOase structure. The tenth intron of the *Coriolus* genes is also in a similar position. In addition, alignment of the region containing the only intron of the laccase gene of *Neurospora crassa* with AOase reveals that this intron is adjacent to an N-terminus of a β-strand in the AOase structure (Fig. 5). The above would suggest that the laccases of ligninolytic fungi could have evolved by a mechanism similar to that suggested for the $\alpha\beta$-barrel proteins. Moreover, intron IV in the *Phlebia* laccase gene and the intron of the *Neurospora* laccase gene are near locations corresponding to N-termini of domains 1 and 2 of the AOase. Thus, these introns might have been involved when the β-barrel was triplicated to form the present AOase.

In this paper we have presented hypotheses concerning the active site structure of the *P. radiata* laccase. Verifying any of these hypotheses will require experimental data gained by site-directed mutagenesis of the laccase gene and heterologous production of mutant laccase-proteins. Therefore, we are presently studying the expression of the *Phlebia* laccase gene in another fungus, *Trichoderma reesei*.

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