What is laccase?

Laccase is one of the few enzymes that have been the subject of study since the end of the last century. The enzyme is a type of copper-containing polyphenol oxidase that was discovered in the exudates of the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883) and subsequently was demonstrated as a fungal enzyme as well (Bertrand, 1896; Laborde, 1896). At present, there is only one bacterium, *Azospirillum lipoferum*, in which a laccase-type phenol oxidase has been demonstrated (Givaudan et al., 1993). Laccase is one of a small group of enzymes called the large blue copper proteins or blue copper oxidases. The other members of this group are the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin. It has very recently been shown, in addition, that phenoxazinone synthase from *Streptomyces antibioticus* has several features in common with the blue oxidases (Freeman et al., 1993).

The blue oxidases have been intensively studied not least because they share with the terminal oxidases of aerobic respiration the ability to reduce molecular oxygen to water. It is therefore paradoxical that our knowledge of these proteins is so incomplete. An attempt to tease apart individual oxidation reactions in order to reduce molecular oxygen to water is coupled to four-electron reduction of oxygen the reaction mechanism cannot be entirely straightforward and has therefore been the subject of much investigation (Reinhammar & Malmstrom, 1981). With 1,2,4,5-tetramethoxybenzene as substrate the initial product is the carbon-centred cation radical formed by removing one electron from the aromatic nucleus (Kersten et al., 1990). The initial product is typically unstable and may undergo a second enzyme-catalysed oxidation (converting phenol to quinone with many substrates), may undergo non-enzymic reactions such as hydration or disproportionation and/or may partake in a polymerization reaction giving an amorphous insoluble melanin-like product. As one-electron substrate oxidation is coupled to four-electron reduction of oxygen the reaction cannot be entirely straightforward and has therefore been the subject of much investigation (Reinhammar & Malmstrom, 1981). In a sense, laccase must operate as a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxidized varies from one laccase to another (for instance cf. Wood, 1980a, and De Vries et al., 1986). Simple diphenols like hydroquinone (Fig. 1) and catechol are good substrates (for most laccases, but not all), but guaiacol and 2,6-dimethoxyphenol are often better (NB these are both methoxy-substituted monophenols!). *p*-Phenylenediamine (a diamine rather than a diphenol) is a widely used substrate and syringaldizine [N,N'-bis(3,5-dimethoxy-4-hydroxybenzyldene) hydrazine]; Fig. 1] is considered to be uniquely a laccase substrate (Harkin et al., 1974), as long as hydrogen peroxide is rigorously excluded since this compound is also oxidized by the manganese-dependent peroxidases produced by many lignolytic basidiomycetes. Thus laccase is an oxidase that oxidizes polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, but does not oxidize tyrosine (as the tyrosinases do). We do not presently know the full range of laccase substrates and still less the range of compounds that laccase activity can affect either directly or indirectly. Bourbonnais & Paice (1990) have shown that the artificial laccase substrate ABTS [2,2'-azino bis(3-ethyl benzthiazoline-6-sulphonate)] has the capacity to act as a mediator enabling the oxidation of non-phenolic lignin model compounds that are not laccase substrates on their own.

Substrate oxidation by laccase is a one-electron reaction generating a free radical (Reinhammar & Malmstrom, 1981). With 1,2,4,5-tetramethoxybenzene as substrate the initial product is the carbon-centred cation radical formed by removing one electron from the aromatic nucleus (Kersten et al., 1990). The initial product is typically unstable and may undergo a second enzyme-catalysed oxidation (converting phenol to quinone with many substrates), may undergo non-enzymic reactions such as hydration or disproportionation and/or may partake in a polymerization reaction giving an amorphous insoluble melanin-like product. As one-electron substrate oxidation is coupled to four-electron reduction of oxygen the reaction mechanism cannot be entirely straightforward and has therefore been the subject of much investigation (Reinhammar & Malmstrom, 1981). In a sense, laccase must operate as a battery, storing electrons from individual oxidation reactions in order to reduce molecular substrates range with another type of (also copper-containing) oxidase – tyrosinase – notionally a monophenol mono-oxygenase, EC 1.14.18.1 (Mayer & Harel, 1979). Secondly, laccases are remarkably non-specific as to their reducing substrate and the range of substrates oxidized varies from one laccase to another (for instance cf. Wood, 1980a, and De Vries et al., 1986). Simple diphenols like hydroquinone (Fig. 1) and catechol are good substrates (for most laccases, but not all), but guaiacol and 2,6-dimethoxyphenol are often better (NB these are both methoxy-substituted monophenols!). *p*-Phenylenediamine (a diamine rather than a diphenol) is a widely used substrate and syringaldizine [N,N'-bis(3,5-dimethoxy-4-hydroxybenzyldene) hydrazine]; Fig. 1] is considered to be uniquely a laccase substrate (Harkin et al., 1974), as long as hydrogen peroxide is rigorously excluded since this compound is also oxidized by the manganese-dependent peroxidases produced by many lignolytic basidiomycetes. Thus laccase is an oxidase that oxidizes polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, but does not oxidize tyrosine (as the tyrosinases do). We do not presently know the full range of laccase substrates and still less the range of compounds that laccase activity can affect either directly or indirectly. Bourbonnais & Paice (1990) have shown that the artificial laccase substrate ABTS [2,2'-azino bis(3-ethyl benzthiazoline-6-sulphonate)] has the capacity to act as a mediator enabling the oxidation of non-phenolic lignin model compounds that are not laccase substrates on their own.

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The reactions catalysed by laccase

Laccase is a polyphenol oxidase (*p*-diphenol oxidase, EC 1.10.3.2). The reduction of oxygen to water is accompanied by the oxidation, typically, of a phenolic substrate. The difficulty in defining laccase by its reducing substrate is twofold. First, laccase has an overlapping substrate range with another type of (also copper-containing) oxidase – tyrosinase – notionally a monophenol mono-oxygenase, EC 1.14.18.1 (Mayer & Harel, 1979). Secondly, laccases are remarkably non-specific as to their reducing substrate and the range of substrates oxidized varies from one laccase to another (for instance cf. Wood, 1980a, and De Vries et al., 1986). Simple diphenols like hydroquinone (Fig. 1) and catechol are good substrates (for most laccases, but not all), but guaiacol and 2,6-dimethoxyphenol are often better (NB these are both methoxy-substituted monophenols!). *p*-Phenylenediamine (a diamine rather than a diphenol) is a widely used substrate and syringaldizine [N,N'-bis(3,5-dimethoxy-4-hydroxybenzyldene) hydrazine]; Fig. 1] is considered to be uniquely a laccase substrate (Harkin et al., 1974), as long as hydrogen peroxide is rigorously excluded since this compound is also oxidized by the manganese-dependent peroxidases produced by many lignolytic basidiomycetes. Thus laccase is an oxidase that oxidizes polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, but does not oxidize tyrosine (as the tyrosinases do). We do not presently know the full range of laccase substrates and still less the range of compounds that laccase activity can affect either directly or indirectly. Bourbonnais & Paice (1990) have shown that the artificial laccase substrate ABTS [2,2'-azino bis(3-ethyl benzthiazoline-6-sulphonate)] has the capacity to act as a mediator enabling the oxidation of non-phenolic lignin model compounds that are not laccase substrates on their own.
desirable to know what substrate(s) laccase oxidizes and what function in metabolism the enzyme performs.

**Laccase function**

*To delignify or not to delignify*

The plant laccases are thankfully outside the scope of this review, but it is worth noting that one proposed function for laccase in woody tissues is as a component of the lignin synthesizing system (Bao *et al.*, 1993). This is relevant because a function that has repeatedly been claimed for the laccases of basidiomycete fungi is that they are part of the enzymic machinery capable of mineralizing lignin that is apparently unique to this group of organisms.

To give the context of this discussion, it is necessary to sketch our present knowledge of lignin breakdown. Complete mineralization of lignin has yet to be achieved *in vitro*. The medium of lignolytic cultures of the best studied lignolytic fungus, *Phanerochaete chrysosporium*, contains two sorts of haemoprotein peroxidases (lignin peroxidase and manganese-dependent peroxidase) that are able to cleave key bonds in a range of compounds that resemble or mimic different parts of the enormous diversity of structure found within lignin (Farrell *et al.*, 1989; Gold *et al.*, 1991; Datta *et al.*, 1991). Some of the most studied strains of this fungus are good lignin degraders but do not synthesize laccase. Therefore, lignin can be degraded without using laccase.

Unfortunately, this conclusion cannot be extended to eliminate a role for laccase in ligninolysis in those lignolytic fungi that do secrete this enzyme. One of the best papers on this subject is still that of Ander & Eriksson (1976) that clearly demonstrates diminished ability to degrade lignin in laccase-minus mutants of *Sporotrichum pulverulentum*.†, coupled with recovery of lignolytic ability in laccase-plus revertants. Further support for a role for laccase in lignin degradation comes from a series of studies showing that laccase can take part in many of the reactions required for ligninolysis (e.g. Lundquist & Kristersson, 1985; Kersten *et al.*, 1990; Bourbonnais & Paice, 1990, 1992). Two recent reports also implicate laccase in Mn(III)-catalysed lignin degradation. In an *in vitro* system using pure enzymes from *Rigidoporus lignosus*, laccase and manganese peroxidase act synergistically to degrade radiolabelled lignin (Galliano *et al.*, 1991). Archibald & Roy (1992) have shown that pure laccase from *Coriolus versicolor* can produce Mn(III)

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† The fungi are organisms for serious study by microbiologists, but they are also at the mercy of taxonomists whose capacity to create havoc in the literature is untold. The less familiar reader should therefore note the following. *Sporotrichum pulverulentum* is *Phanerochaete chrysosporium* strains where nobody has yet observed the sexual stage. A number of fungi commonly used in this field suffer from a difference in political correctness between North America and the UK. Although Europe espoused SI units in a collective moment of insanity, it is our Canadian and US colleagues who switch to a new name at the drop of a hat. Thus *Coriolus* spp. in most European labs become *Trametes* strains (and in the older literature see *Pobporus*) and *Agaricus bisporus* occasionally becomes *Agericus brunescens*. Even more bizarre, *Aspergillus nidulans* (not the most obscure of fungi) has its laccase sequence described in a paper with this name used in the title (*Aramayso & Timmerhalse*, 1990), but an entry in the sequence database (GenBank) under *Emeriaella nidulans*.

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**Fig. 1.** (a) The typical laccase reaction, where a diphenol (hydroquinone shown here) undergoes a one-electron oxidation to form an oxygen-centred free radical. This species can be converted to the quinone in a second enzyme-catalysed step or by spontaneous disproportionation. Quinone and free radical products undergo polymerization. (b) The laccase reaction with syringaldizine as substrate, where the initial product is a free radical. The quinone formed by a second one-electron oxidation (again by a second enzymic step and/or by disproportionation) is deep purple in colour and not apparently prone to polymerization.

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Oxygen. In fact, it appears that bound oxygen intermediates are also involved (Messerschmidt *et al.*, 1992, and references cited therein), but detailed discussion of the reaction mechanism is beyond the scope of this review. At the physiological level it would be highly
chelates from Mn(II) in the presence of a phenolic ‘accessory’ substrate.

On the other hand, some data indicate little association between laccase activity and ligninolysis. The laccase of *Fomes annosus* can be efficiently inhibited by thioglycollic acid (a copper chelator) without apparent detriment to either the growth of the fungus or its ability to cleave high molecular mass lignosulphonate (a lignin derivative produced in some processes of wood-pulp bleaching for paper manufacture; Haars & Huttermann, 1980). In an elegant study with *Coriolus versicolor*, Evans (1985) showed that lignin degradation continued unaffected when laccase activity was inhibited by a specific antibody.

Thus, although laccase can (directly or indirectly) cleave a significant proportion of the structures found in lignin, the role of laccase in ligninolysis remains unresolved, but the widespread occurrence of this enzyme in wood rotting fungi is unlikely to be coincidental. The fact that laccase tends to polymerize phenolic substrates in *vitro* does not eliminate the possibility that it contributes to lignin breakdown as the lignin and manganese peroxidases also tend to polymerize substrates in *vitro*. A recent survey of white rot fungi suggests that manganese peroxidase in combination with either laccase or lignin peroxidase may be the necessary (minimum) complement of oxidative enzymes for lignin degradation (De Jong *et al.*, 1992). As a number of authors have pointed out (e.g. Bollag *et al.*, 1988; Wood, 1985), the enzyme could well have a function during lignolytic growth other than direct involvement in lignin cleavage. All the enzymes putatively involved in lignin cleavage produce highly reactive (and hence highly toxic) species from which the fungal mycelium must be protected. It may be that (one of) the function(s) of laccase is to scavenge these compounds by promoting polymerization, before they can enter the hypha. In laccase non-producer strains of fungi such as *Phanerochaete chrysosporium*, this protective function would require an alternative mechanism and could conceivably be achieved by secretion of glucan ‘slime’ (Bes *et al.*, 1987).

**Laccase function in morphogenesis**

In some fungi, laccase has a well understood function that is unrelated to ligninolysis. The best example is perhaps *Aspergillus nidulans* in which two laccases with different functions have been characterized. The product of the *aA* gene is a laccase uniquely involved in formation of the green colour of the conidium (Clutterbuck, 1972). A second laccase is localized in the hülle cells and cleistothecial primordia and again is involved in pigment synthesis (Herrmann *et al.*, 1983). In a number of fungi such as *Daldinia concentrica* and *Leptinotus edodes*, laccase activity is associated with pigment formation in structures that are more rigid than a simple mycelial aggregate. Fruiting body formation may involve ‘phenol oxidase-catalysed formation of extracellular pigments...coupled to oxidative polymerization of cell wall components strengthening cell-to-cell adhesion’ (Bu’Lock, 1967; Leatham & Stahlmann, 1981).

The formation of rhizomorphs (mycelial strands formed from large numbers of tightly adpressed hyphae) is a different developmental change that is associated with laccase synthesis in *Armillaria mellea* (Worrell *et al.*, 1986). It is possible that here too, laccase is responsible for making a polyphenolic glue that sticks the hyphae together. This idea must be treated with some caution, however, as in laboratory liquid cultures of *Armillaria mellea*, although laccase activity accumulates in the medium co-ordinately with the onset of rhizomorph formation, manipulation of medium pH can substantially alter the amount of laccase activity that accumulates without having any significant effect on the mass of rhizomorph tissue formed (Rehman & Thurston, 1992). If laccase is required for rhizomorph synthesis therefore, the amount of laccase activity produced in the medium at the optimal pH for growth (pH 5) is in very considerable excess. There are two well-studied fungi in which laccase synthesis is regulated in relation to fruit body development, but in a fashion that gives no clear indication of the function of the enzyme. In *Schizophyllum commune*, the dikaryotic strains that are able to develop fruit bodies can secrete high levels of laccase but the co-isogenic monokaryotic strains cannot. Curiously, however, the condition for maximum laccase synthesis is culture in the dark at 30 °C, whereas fruit body formation is greatest at 25 °C in the light. The laccase activity in the medium accumulates during the period of glucose consumption after which it is inactivated—both synthesis and activity of the enzyme are controlled (De Vries *et al.*, 1986). In the cultivated button mushroom *Agaricus bisporus*, laccase activity accumulates during vegetative growth in precise parallel with mycelial mass, but undergoes rapid inactivation shortly after the onset of fruit body formation (Wood, 1980a, b). This accumulation/inactivation cycle is repeated with successive flushes of fruit body development when the fruit bodies are removed as in commercial production. If deep boxes are used for mushroom growth it can be seen that the extent of the oscillations in laccase activity are amplified in the mycelium furthest from the fruiting surface (Smith *et al.*, 1989). Whilst no experimental test has yet been devised to characterize the function of laccase in these organisms, it is very plausible that in *Agaricus bisporus*, laccase is somehow involved in lignolytic growth as the cycles of fructification appear to involve alternating periods of lignin breakdown and cellulolysis—endo-cellulase activity oscillates in phase with fruit body enlargement and out of phase with laccase (Smith *et al.*, 1989). An interaction between laccase and tyrosinase may also play a part in substrate colonization by some basidiomycetes (Ainsworth & Rayner, 1991).

**Laccase in plant pathogens**

*Botrytis cinerea* causes soft rot infections in many horticultural crop plants (carrot and cucumber, for instance) as well as the ‘noble rot’ and ‘grey rot’ of grapes. This fungus produces extracellular laccases that are involved in the pathogenic process because cucurbitacins, tetracyclic triterpinoids produced by the cucumber, protect the plant from infection (Bar-Nun & Mayer, 1989, 1990) and this protection is mediated by specific repression of laccase
synthesis by the fungus (Viterbo et al., 1993). There remains, nevertheless, a lot that is not understood about the part played by laccase in pathogenesis of B. cinerea. The induction system is complex involving concerted action of phenolic and pectic substances (Marbach et al., 1985), there appear to be multiple forms of laccase that are induced to different extents in different media and these different forms have quantitatively different substrate specificities (Marbach et al., 1984).

Evidence of a role for laccase in pathogenesis has also been obtained in the chestnut blight fungus Cryphonectria parasitica (Rigling & Van Alfen, 1991). There are hypovirulent strains of this fungus in which the diminution of virulence is associated with the presence of a double-stranded RNA of viral origin. These strains are repressed for laccase synthesis by prevention of accumulation of laccase mRNA. It remains to be determined how specific this effect is, although overall growth is similar in virulent and hypovirulent strains.

The structure of laccase enzymes

Studies of purified enzymes

Table 1 shows a list of fungal laccases that have been purified over the last 30 years and the relevant references. They are all extracellular glycoproteins. It is important to note that many of these enzymes show very considerable heterogeneity after purification (Fig. 2). This is an inevitable consequence of the extracellular location of these proteins, as fungal media often contain substantial proteolytic and glycosidic activities (Wood, 1985) such that a significant proportion of enzyme molecules become modified by trimming of carbohydrate or nicking of the polypeptide chain (Perry et al., 1993a). The typical laccase is a 60–80 kDa molecule of which 15–20% is carbohydrate (although the sugar composition of the glycan moiety has only been analysed in a few examples, such as Podospora anserina and Botrytis cinerea). In sedimentation analysis, the Agaricus bisporus laccase appears to be substantially larger (100 kDa), but this is not in accord with data from electrophoretic analysis under denaturing conditions (that gives a value of about 65 kDa) or gene sequence analysis (see below). It may be that this laccase is a dimer of identical subunits (where the size computed from sedimentation analysis is an underestimate because of imperfect correction for the part protein/part carbohydrate composition of the molecule). Similarly, the molecular mass value for the Aspergillus nidulans conidial laccase obtained from gel filtration (110 kDa) could indicate that this enzyme is a dimer – this method does not reliably measure molecular mass as Stokes' radius (which determines migration rate in a gel matrix) is not simply related to mass for non-spherical molecules.

The other obvious exception is laccase 1 of Podospora anserina which is a tetramer of identical subunits (from which the free 80 kDa laccase III is derived). It should be noted, in this context, that care must be taken to fully denature some laccases prior to estimation of molecular mass. The Schizophyllum commune laccase migrates during SDS-PAGE with an apparent molecular mass of 36 kDa, but after boiling migrates as a 64 kDa species. Laccase I of Armillaria mellea behaves in the same way, but the heat required for complete denaturation is less (Rehman, 1991). These are presumably very compact and/or non-spherical molecules prior to denaturation.

Copper content

The measured copper content of the purified laccases varies between four and about two atoms per enzyme molecule (subunit). There are two reasons why the lower values must be regarded with caution. As discussed above, purified preparations of some fungal laccases include a fraction of molecules that are demonstrably damaged and therefore unlikely to retain their full complement of copper. Secondly, as discussed by Reinhammar & Malmstrom (1981), although the copper ions in these proteins are regarded as firmly bound, selective depletion of one copper centre (type 2) can be achieved experimentally and may occur during purification. It has also been found that type 1 copper centres that lack a ligand, are as is true for all the fungal laccases (see below), are relatively unstable (Karlsson et al., 1989).

For the laccase of Phlebia radiata a novel combination of prosthetic groups has been proposed (Karhunen et al., 1990; Saloheimo et al., 1991). This laccase is one in which about two atoms of copper per enzyme molecule are found and the claim is that these work in concert with a pyrroloquinoline quinone (PQQ) cofactor. This combination of copper and PQQ in fungal enzymes has since been questioned (Klinman et al., 1991; Maccarone et al., 1991). In addition, as discussed below, all the laccases for which complete amino acid sequence has been deduced, including the P. radiata sequence, show conservation of the eleven residues involved in binding four copper atoms. The problem is accentuated by the presence of numerous compounds in some fungal culture fluids with similar spectroscopic properties to PQQ. At present, it seems that there is insufficient evidence to sustain any proposal other than that active laccase enzymes contain four copper atoms per molecule/subunit.

Studies of laccase cDNA and gene sequences

During recent years laccase gene and/or cDNA sequences have been described from five sources of which the first to be reported was the ascomycete fungus Neurospora crassa (Germann & Lerch, 1986; Hermann et al., 1988). Subsequently, the sequences from another ascomycete, Aspergillus nidulans (Aramayo & Timberlake, 1990), and three basidiomycetes, Coriolus hirutus (Kojima et al., 1990), Phlebia radiata (Saloheimo et al., 1991) and Agaricus bisporus (Perry et al., 1993b) have been published. These sequences show a common pattern in that they all encode polypeptides of approx. 520–530 amino acid residues including an N-terminal secretion peptide. In addition, the one cysteine and ten histidine residues involved in binding the four copper atoms found in each laccase molecule are conserved, together with a small amount of sequence around the four regions in which the copper ligands are clustered. It is in the copper binding amino acid sequence that the diversity is found. For example, one region contains a polypeptide with two Cys residues and four His residues, the other contains a polypeptide with three Cys residues and six His residues.
### The structure and function of fungal laccases

#### Table 1. Properties of some purified fungal laccases

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of enzymes</th>
<th>Mol. mass (kDa)</th>
<th>Carbohydrate content (%)</th>
<th>Copper content (atoms per molecule)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Polyergus (Coriolus) versicolor</em></td>
<td>2</td>
<td>60 ~ 65</td>
<td>14</td>
<td>4</td>
<td>Mosbach (1963)</td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em> (conidial)</td>
<td>1</td>
<td>110 80</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>1 2</td>
<td>72 72</td>
<td>80</td>
<td></td>
<td>A. M. Mayer, pers. comm.</td>
</tr>
<tr>
<td><em>Sclerotium commune</em></td>
<td>1</td>
<td>62-64</td>
<td></td>
<td></td>
<td>De Vries <em>et al.</em> (1986)</td>
</tr>
<tr>
<td><em>Pycnoporus coccineus</em></td>
<td>1</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Armillaria mellea</em></td>
<td>2 2</td>
<td>59 59</td>
<td>9.3 9.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Monocillium indicum</em></td>
<td>1</td>
<td>100 72</td>
<td></td>
<td></td>
<td>Thakker <em>et al.</em> (1992)</td>
</tr>
</tbody>
</table>

* *Agaricus bisporus* is shown as having 1(2) laccases because the two laccase genes lc1 and lc2 are both expressed, but encode virtually identical proteins (Perry *et al.*, 1993b). As argued in the text, copper content < 4 is most probably an experimental artifact. The 390 kDa laccase of *Podospora anserina* is a tetramer of identical subunits. The 100 kDa form of the *Agaricus bisporus* enzyme may be a dimer of 65 kDa subunits, which might also apply to the 110 kDa form of the *Aspergillus nidulans* laccase.

† The laccases of *Botrytis cinerea* were previously described as very much smaller proteins (38 and 36 kDa; Marbach *et al.*, 1984). These values have now been revised by A. M. Mayer and his colleagues.

Acid residues and their general distribution in the polypeptide chain (two pairs of histidines in the N-terminal domain and the other liganding residues all near the C-terminus) that the laccases are all similar, and in which property they closely resemble the ascorbate oxidase subunit. There is also recognisable homology with domains of the significantly larger mammalian plasma protein ceruloplasmin (Germann & Lerch, 1986; Messerschmidt & Huber, 1990; Saloheimo *et al.*, 1991).

The similarity with ascorbate oxidase is most significant. This protein has been crystallized and analysed by X-ray diffraction to very high resolution (Messerschmidt *et al.*, 1989, 1992) allowing *Neurospora crassa* laccase three-dimensional structure to be predicted by molecular modelling based on a closely similar protein (Messerschmidt & Huber, 1990). Secondly, the arrangement of the copper centres can begin to be understood. The very large corpus of physicochemical study of the blue oxidases had shown that laccase and ascorbate oxidase contain three types of copper that can be distinguished by their spectroscopic and paramagnetic properties – type 1 (which confers the greenish-blue colour on these proteins), type 2 and type 3, the latter being a pair of copper atoms (Reinhammar & Malmström, 1981). This approach was already predicting close proximity of the type 2 and two type 3 copper atoms as a trinuclear cluster (Allendorf *et al.*, 1985). The crystal structure shows the type 2 and type 3 copper atoms disposed almost equidistant, the binuclear pair being 3-4 Å apart and 3-9 and 4-0 Å from the type 2, making a trinuclear cluster (Messerschmidt *et al.*, 1989; Messerschmidt & Huber, 1990). The kinetic, spectroscopic and EPR data (Reinhammar & Malmström, 1981), showing types 1 and 2 copper involvement in electron capture/transfer and the type 2 and type 3 copper involvement in oxygen binding, can now be related to a well-described three-dimensional structure (Messerschmidt *et al.*, 1989, 1992; Messerschmidt & Huber, 1990). It should also be noted that there is one striking difference between ascorbate oxidase and the laccases. Although both proteins bind type 1 copper with one cysteine and two histidines, ascorbate oxidases have a fourth amino acid ligand, a methionine residue, that is absent from all the laccases sequences so far known. This
Analysis of the amino acid sequence of the *Neurospora crassa* laccase (Messerschmidt & Huber, 1990) and the *Phlebia radiata* laccase (Saloheimo et al., 1991) both indicate that the laccase sequences can fold into the three domain $\beta$-barrel structure described for the ascorbate oxidase subunit. There is nevertheless quite limited similarity among the ascorbate oxidase and laccase primary sequences (Perry et al., 1993b). The aligned sequences of the five laccases and two ascorbate oxidases show only 7% identical residues. Surprisingly, the *Aspergillus nidulans* and *Neurospora crassa* sequences are no more similar to the basidiomycete laccases than are the cucumber and squash ascorbate oxidases (about 50% similar amino acid residues in paired alignments); indeed the *Aspergillus nidulans* sequence is the outlier of the whole group. This raises the intriguing possibility that future studies of these sequences will lead to understanding of the different substrate specificities and functions of laccases in different organisms, but it may also be that if rigid substrate specificity is not required, a major constraint is removed from the architecture of an enzyme. This argument cannot be sustained as a major factor, however, as if it were overriding, the most distantly related in evolutionary terms would be the most different and this is not what is observed.

**Outlook**

Recombinant DNA studies have combined with study of a larger number of purified laccase proteins to give a good idea of the structure of a typical laccase and its similarity to ascorbate oxidase and caeruloplasmin. Manipulation of recombinants will hopefully also lead to a better understanding of the factors controlling laccase synthesis (which area has not been considered here) and from this might come a few better insights into the biological function(s) of laccase in the fungi. It is perhaps of some small comfort that the biology of caeruloplasmin in mammals is proving equally difficult to understand (Schilsky et al., 1992).

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**References**


