AN INVESTIGATION OF

LACCASE-LIKE MULTICOPPER OXIDASES (LMCOs):

STRUCTURE AND FUNCTION STUDIES

By

James Todd Hoopes

(under the Direction of Jeffrey F. D. Dean)

ABSTRACT

This dissertation describes investigations into the structure and function of selected laccase-like multi-copper oxidases (LMCOs). A plant LMCO from *Acer pseudoplatanus* has been expressed in tobacco BY2 cell ands compared to the native LMCO isolated from *Acer pseudoplatanus* cell suspension cultures. It was found to be nearly identical to the native enzyme except with respect to the K_ms for certain substrates. A LMCO gene cloned from yellow-poplar (*Liriodendron tulipifera*) and expressed in transgenic tobacco cells showed ferroxidase activity. This marks the first report of ferroxidase activity associated with a plant LMCO, and suggests an alternative physiological function for these enzymes. Molecular modeling of the Fet3 LMCO gave insight into which amino acids could be involved in iron binding in LMCOs. Additionally, a new chromogenic substrate for laccases and peroxidases, 1,8-diaminonapthalene, was found to be more sensitive and less toxic than traditional substrates when used in SDS-PAGE zymograms.

INDEX WORDS: Dissertation, Laccase, Multi-Copper Oxidase, MCO, Copper,Iron Metabolism, Ferroxidase, Lignin, Lignification, Liriodendron tulipifera, Acer pseudoplatanus

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DEDICATION

For my Father who gave me my love of science. For my Mother who fought for my education.

PREFACE

Philosophy is nothing more than the persistent desire to think clearly. Would that man applied this practice to all his endeavors.

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CHAPTER 1

INTRODUCTION

Nomenclature:

The enzymatic reaction catalyzed by laccase (EC 1.10.3.2) is amongst the oldest studied biochemical activities and was first mentioned by Yoshida in 1883 (Yoshida, 1883). Around the same time, Bertrand did pioneering research on laccase isolated from Japanese lacquer tree sap (Bertrand, 1894, 1895), and in his descriptions coined the terms "laccase" and "oxidase" (Messerschmidt, 1997). As will be described in more detail below, similar enzymes have been found in a great many organisms and have been the subject of much study. However, for all the study, these enzymes remain quite mysterious with the exact physiological substrates and functions known only for the lacquer tree laccase and a handful of related enzymes (Omura, 1961; Askwith et al., 1994).

Part of the problem stems from the broad range of substrates that laccases and related enzymes are capable of utilizing *in vitro* (Fahraeus and Ljunggren, 1961). This promiscuity created considerable difficulties for the identification and classification of these enzymes. Consequently, on the basis of

their oxidative activities, copper content and blue color, laccase, ascorbate oxidase and ceruloplasmin defined the early blue-copper oxidase enzyme family.

As the name implies, blue-copper oxidase are characterized by their blue color caused by a strong absorbance band at approximately 600 nm related to the Type-1 copper center (Keilin and Mann, 1939). Traditionally, this group of enzymes was comprised of laccase, ascorbate oxidase, and ceruloplasmin, as well as cytochrome c oxidase and bilirubin oxidase, and more recently the yeast FET3 protein and hephaestin (Askwith et al., 1994; Conrad et al., 1999; Vulpe et al., 1999). Grouping these enzymes together, however, can be somewhat misleading. While ascorbate oxidase and laccase share a tremendous sequence and structural homology with each other, these enzymes share only small regions of similarity with ceruloplasmin and almost none at all with cytochrome c oxidase.

As additional enzymatic activities were used to help define enzyme families, laccase, ascorbate oxidase, tyrosinase and catechol oxidase were grouped together as similar enzymes (phenoloxidases) that could be distinguished on the basis of their substrate and inhibitor specificities (Mayer and Harel, 1979). Thus, structurally distinct enzymes – laccases and catechol oxidases – were linked by their ability to remove protons from many of the same phenolic

compounds, although laccases were thought to be distinguishable by their ability to oxidize para-substituted substrates. Adding to the confusion, the name catechol oxidase was also used interchangeably with names such as polyphenoloxidase, tyrosinase, catecholase or cresolase, for enzymes derived from a wide variety of plant and microbial sources. For a brief period, even the IUPAC Enzyme Commission grouped these enzymes together under the classification number EC 1.14.18.1 (Mayer and Harel, 1979; Mayer, 1987). However, with increasing availability of phenoloxidases from diverse sources it soon became apparent that substrate and inhibitor studies could not provide clear-cut distinctions between the different classes of enzymes (Kahn and Andrawis, 1986; Walker and Ferrar, 1998).

Much of this early ambiguity and imprecision in terminology is being removed by application of recently acquired structural information. Structurally, the enzymes placed in the original EC 1.14.18.1 category obviously comprised two distinct groups. Methane monooxygenase, tyrosinase and catechol oxidase are distinguished by a single binuclear copper center and no blue Type-1 coppers (Mayer et al., 1966; Sanchez-Ferrer et al., 1995). All of the copper ions in these enzymes are in Type-3 centers, which cycle from Cu^{2+} to Cu^{1+} and back during oxidation of the electron donor (Sanchez-Ferrer et al., 1995; McGuirl and Dooley, 1999). This is quite distinct from laccase,

ascorbate oxidase, and ceruloplasmin, which contain both Type-1 and Type-2 centers in addition to a Type-3 pair.

Today, with the increasing availability of genomic sequence information, enzymes are most reliably assigned to families on the basis of their sequence similarity and structural properties, and only secondarily on their specific enzymatic activities. These new methods of classification highlight misleading classifications in the older nomenclature where implied similarities between enzymes based on activity provide no insight into mechanisms of catalysis or physiological function. Consequently, the nomenclature evolved so that group of enzymes originally referred to as the blue copper oxidases, was absorbed into the more general category of multicopper oxidases. Thus, besides classical blue-copper oxidases (laccase, ascorbate oxidase and ceruloplasmin), the multicopper oxidase family contains the yeast FET3 protein, tyrosinase, catechol oxidase, methane monooxygenase, sulochrin oxidase, dihydrogeodin oxidase, phenoxazinone synthase, and bilirubin oxidase (Askwith et al., 1994; Messerschmidt, 1997; McGuirl and Dooley, 1999). The members of this enzyme group are obviously diverse in function and in structure, requiring only two criteria for inclusion – multiple copper ions in their catalytic centers and the ability to utilize diatomic oxygen as a co-substrate. The evolution of this category of enzymes and the characteristics that distinguish them have been

extensively review elsewhere (Mayer and Harel, 1979; Mayer, 1987; Messerschmidt, 1993; Solomon et al., 1996; Messerschmidt, 1997).

Recent acquisition of large amounts of genomic sequence data has enabled identification of numerous genes that appear to encode enzymes bearing the structural hallmarks of laccases (Wojtas-Wasilewska and Trojanowski, 1975; Kurtz and Champe, 1981; Banerjee and Vohra, 1991; de Silva et al., 1995; Claus and Filip, 1997; Klomp et al., 1997; Eck et al., 1999; Fernandez et al., 1999; Syed et al., 2002; Wartmann et al., 2002). Sequence comparisons of these putative enzymes show them all to contain the invariant copper-binding domains that were first identified in laccase. Thus, to distinguish this group of structurally related enzymes from unrelated members of the blue-copper and multicopper oxidase families, I have proposed the designation "laccase-like multicopper oxidase" or LMCO for this highly homologous group of enzymes. This terminology recognizes that these enzymes belonging to a conserved structural/mechanistic group, but does not bring with it any preconceived notions of substrate specificity or physiological function. It is presumed that more specific names will be developed for members of this class as precise physiological functions and metabolic substrates are identified. Therefore, unless referring to the specific enzyme (laccase) isolated from *Rhus vernicifera* tree sap, the term laccase-like

multicopper oxidase (LMCO) will be used throughout the remainder of this dissertation.

LMCOs and Lignin Biosynthesis:

As noted previously, laccase was first reported as the natural hardening agent in the lacquer (urushi) produced from the sap of the Japanese lacquer tree (*Rhus vernicifera*) (Yoshida, 1883; Bertrand, 1895). The sap from trees in the closely related genera *Rhus* and *Melanorrhoea* trees is used for a wide variety of purposes in Asia, including the manufacture of ornamental lacquerware in Japan, and consequently is production is of considerable economic importance (Dean and Eriksson, 1994). The names "laccol", to describe the alkylcatechol precursors of the polymeric lacquer, and "laccase", to describe the oxidative catalyst that initiated laccol polymerization, were first used by Bertrand (Bertrand, 1895). He also was the first to suggest that laccase might be a metallo-enzyme, although he mistakenly identified the critical metal as manganese. In 1939, Keilin and Mann demonstrated that the metal involved was not manganese, but copper (Keilin and Mann, 1939).

By the mid-1960's, laccase-like enzyme activities had been detected in extracts from a variety of plant species (Levine, 1966). Laborde and others found similar enzymes to be widely distributed in fungi, where they appeared

to be connected to the ability of fungi to breakdown the plant phenolic polymer, lignin (Laborde, 1897). Although distinctions between catechol oxidase and laccase activities in terms of substrate preferences were well known from the earliest characterizations of these enzymes, many early studies were confounded by difficulties encountered in dealing with less than purified enzyme samples (Mayer and Harel, 1979). Despite these problems, a great deal of speculation was made concerning the potential functions of laccase-like enzymes in various organisms. However, in the absence of clear metabolic substrates, these speculations did little to advance our understanding of these enzymes.

As research to uncover the physiological function of plant phenoloxidases progressed, the focus moved to *in vitro* investigations of potential metabolic substrates. Very early work had shown that fungal phenoloxidases could catalyze oxidative polymerization of various diphenols *in vitro* (Dean and Eriksson, 1994). Building on these observations, Erdtman proposed that the phenolic polymer, lignin, which is a major component of woody plant biomass, was synthesized via oxidative polymerization of hydroxyphenol precursors (termed dehydrogenation theory) to yield the phenolic polymer. Condensation reactions between phenoxyradical intermediates could then produce the vast array of polymer substructures

identified in lignin. Early studies suggested that plant laccases or peroxidases were the most likely candidates for Erdtman's oxidases.

It was Freudenberg and co-workers that identified the predominant lignin precursor (monolignol) in plants, coniferyl alcohol, and demonstrated its polymerization *in vitro* using a laccase-like enzyme isolated from a fungus (Freudenberg, 1950; Freudenberg and Dietrich, 1953; Freudenberg, 1959). Freudenberg and Richtzenhain achieved an important milestone using enzymes *in vitro* to form the first lignin-like dehydrogenation polymers (DHPs) from monolignols. Although the enzymes initially used for these experiments were crude preparations of solutions of LMCOs isolated from mushrooms, the experiments were repeated later with laccase-like activities isolated from plant extracts. Peroxidases, which required hydrogen peroxide as a co-substrate, were subsequently used by Higuchi and co-workers to prepare similar DHPs with a much faster rate of polymerization (Higuchi and Ito, 1958). In addition to their *in vitro* synthesis of DHPs, Freudenberg and co-workers used histochemical staining to localize LMCO and peroxidase activities in the lignifying xylem of spruce (*Picea abies*) (Freudenb.K, 1968). Thus, in the 1950's, LMCOs and peroxidases held equal footing as potential *in vivo* catalysts for lignin biosynthesis.

Over the next twenty years, very few LMCOs were identified in plants, and in 1967, Nakamura reported that a laccase preparation prepared from Japanese lacquer tree sap did not produce DHPs from coniferyl alcohol (Nakamura, 1960; Nakamura et al., 1965; Nakamura and Ogura, 1966). This leading Nakamura to claim that laccase was not the polymerizing enzyme responsible for lignification (Nakamura, 1967). Later histochemical work by Harkin and Obst using syringaldazine demonstrated peroxidase, but not LMCO activity, in the lignifying xylem from a number of tree species (Harkin and Obst, 1973). These two studies were sufficient to cause most researchers to abandon further work on LMCOs as the oxidative catalysts for lignification (O'Mally et al., 1993; Olson and Varner, 1993), and the only physiological function postulated for plant LMCOs was sap hardening as a wound healing response in lacquer trees (Dean and Eriksson, 1994).

In the early 1990's, researchers began detecting LMCO activity in a wider variety of plants, and in some cases these activities were associated with lignifying tissues (Bao et al., 1993; Cai et al., 1993). However, much of the credit for the resurgence of theories involving LMCOs in the lignification process can be assigned to work done with the enzyme isolated from cell suspension cultures of sycamore maple (*Acer pseudoplatanus*). Bligny and Douce (1983) purified a phenoloxidase abundant in supernatants from these

cultures, and demonstrated it to have all the salient characteristics of a LMCO (Bligny and Douce, 1983). Following up on their initial work, Bligny and Douce (1983) showed that additional copper had to be added to the culture medium to recover maximal amounts of enzyme activity from culture supernatants, although an inactive apoenzyme form was secreted even in the absence of sufficient copper (Bligny et al., 1986). However, the question remained as to the physiological function of the sycamore maple LMCO. Although studies of suspension-cultured cells haves many advantages, a major disadvantage is the extreme difficulty in assigning meaningful physiological roles to phenomena observed in culture.

In an effort to probe potential roles for this LMCO in nature, Sterjiades et al. showed that the purified LMCO from sycamore maple was capable of oxidizing monolignols (sinapyl alcohol, coniferyl alcohol, or *p*-coumaryl alcohol) to form water-insoluble DHPs (Sterjiades et al., 1992). Subsequent work showed that the DHPs produced by the sycamore maple LMCO were virtually identical to those produced *in vitro* using horseradish peroxidase. In addition, these DHPs contained substructure bonding similar to what is found in naturally occurring lignins. It was also found that the sycamore maple LMCO oxidized phenolic compounds containing multiple aromatic rings at a slower rate than did a peroxidase from the same organism. This observation

prompted researchers to suggest that the LMCO might be involved in the early stages of lignification, while peroxidases were involved in later stages or in response to environmental stresses where polymerization reactions would be shifted to oligomeric substrates (Sterjiades et al., 1993).

A LMCO isolated from loblolly pine (*Pinus taeda*) xylem was subsequently shown to satisfy all the criteria suggested by Yamamoto as necessary to causally link particular phenoloxidases with lignification: substrate specificity for monolignols, reaction products containing expected bond patterns, subcellular location, and temporal correlation. The pine LMCO was shown to produce DHPs from coniferyl alcohol, and the activity was localized by histochemical staining to lignifying xylem near the cambium (Bao et al., 1993). Peroxidase activity, on the other hand, was quite evident throughout pine stems and was not specifically localized to tissues undergoing active lignification.

In a study of lignified tissue development in *Zinnia elegans*, histochemical staining clearly demonstrated the tight correlation of LMCO activity with lignification of vascular tissues in stems (Liu et al., 1994). Activity staining of stem sections showed LMCO activity specifically in developing xylem elements undergoing active lignification.

These studies clearly showed that LMCO activity is spatially and temporally associated with lignification in plants. It is possible that, as suggested by Sterjiades et al (1993), LMCOs and peroxidases are separated temporally in their roles as catalysts for lignin deposition. And contrary to the earlier work of Nakamura (Nakamura, 1967), studies with a LMCO freshly extracted from young *Rhus typhina* stems demonstrated that this enzyme was able to oxidize coniferyl alcohol in vitro (Savidge and Udagama-Randeniya, 1992). One possible explanation for the inability of early workers to detect LMCOs in a wider variety of plants, or to demonstrate a capacity to oxidize monolignols, may have more to do with the nature of these enzyme, rather than their presence or absence. LMCOs often require special extraction procedures to protect them from inactivation be monophenolic compounds, and several LMCOs show unusual susceptibility to loss of activity through freezing when in crude preparations (Hoopes, unpublished). These characteristics could, in conjunction with the stability of the majority of peroxidases, account for the contradictory results seen in many of the earlier studies. Thus, by the mid-1990's, work from a number of laboratories had bolstered the proposed role for LMCOs in catalyzing lignin deposition (Liu et al., 1994; Dean et al., 1996).

LMCOs and Lignin Degradation:

Although the laccase isolated from *R. vernicifera* sap was the first identified LMCO, researchers studying LMCOs in fungi quickly outpaced plant researchers with respect to the completeness of their data and the sheer number of LMCOs discovered. This is no doubt a result of the highly stable activity of fungal LMCOs, which is likely related to the fact that these enzymes are generally secreted into the environment to carry out their physiological functions. That many of these enzymes were identified in basidiomycetes growing on lignocellulosic substrates suggested that fungal LMCOs might have some role in the metabolism of lignin and lignin-associated biopolymers by these organisms.

Bavendamm was the first to show that lignin-degrading white-rot fungi could be distinguished from brown-rot fungi by the formation of a colored band around colonies growing on tannin-impregnated agar plates (Bavendamm, 1928). This test, termed Bavendamm's reaction, produced a colored ring of oxidized tannins around white-rot fungi capable of degrading lignin, and it was found that in almost all cases, a positive Bavendamm test, indicating a white-rot fungus, was correlated with the ability of the fungus to degrade lignin (Ishihara, 1980). Since hydrogen peroxide is not added the Bavendamm test, the phenoloxidases it detected were considered likely to be LMCOs. However,

this assumption cannot be considered conclusive as no efforts were made to scavenge any hydrogen peroxide that might have been released by the fungi themselves (Joslyn, 1949).

More recent results described a LMCO from C*oriolus versicolor* that was able to decrease the amount of insoluble milled wood lignin (MWL) with a concomitant increase in more soluble, low molecular weight components (Konishi and Inoue, 1971). However, at almost the same time, a LMCO from *Polyporus versicolor* (now thought to be the same species) was shown to have an oxidation potential too low to remove electrons from many of the non-phenolic substituents found in naturally occurring lignins (Reinhammer, 1972; Kersten et al., 1990). Thus, it was unclear from these conflicting results whether LMCOs played any direct role in lignin degradation.

However, LMCOs retained interest as a major contributor to lignin degradation of lignin when Ander demonstrated that a mutant of the white-rot fungus, *Sporotrichum pulverulentum,* lacking secreted phenoloxidase activity could not degrade lignin unless a purified LMCO was added to the cultures (Ander and Eriksson, 1976). The seemingly conclusive findings were marred, however, by the fact that wild-type *S. pulverulentum*, does not produce detectable LMCO under ligninolytic conditions. This anomaly, coupled with the discoveries of lignin peroxidase (LiP) and manganese peroxidase (MnP),

contributed greatly to the dismissal of LMCO as a major participant in lignin degradation (Glenn et al., 1983; Kuwahara et al., 1984).

However, as with lignin deposition, the story was more complicated than initially thought, and a number of recent investigations have once again provided strong support for LMCO involvement in lignin degradation by white-rot fungi. Contrary to Reinhammer's work (Reinhammer, 1972), fungal LMCOs have been shown capable of catalyzing reactions necessary for the breakdown of lignin *in vitro*, such as the cleavage of alkyl-phenyl and C_a-C_b cleavages of phenolic dimers used as lignin models (Eriksson et al., 1990; Higuchi, 1990). In work reviewed by Thurston (1994), a LMCO was implicated in Mn(III)-catalyzed lignin degradation in an in vitro study using purified LMCO and manganese peroxidase from *Rigidoporus lignosus*. In fact, in a survey of white-rot fungi by De Long (1992), LMCO plus either manganese peroxidase (MnP) or lignin peroxidase (LiP) has been suggested as the predominant system for the degradation of lignin by fungi (Thurston, 1994).

Still, the apparent inability of LMCOs to directly oxidize non-phenolic compounds was a major obstacle to consideration of these enzymes as major players in lignin degradation. In naturally occurring lignins, phenolic subunits do not comprise the majority of carbon-carbon linkages. In fact, only about

one in three C-9 subunits in spruce mill wood lignin has a free phenolic hydroxyl group (Bourbonnais and Paice, 1990). It would seem unlikely then that an enzyme capable of only working on about one-third of the available units of lignin would be the major catalyst for lignin degradation. However, LMCOs have recently been shown to catalyze oxidation of non-phenolic substrates via mediating compounds. For example, after they were oxidized by a LMCO from C*oriolus versicolor* both Remazol Blue and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) were able to oxidize phenolic and non-phenolic components of lignin (Bourbonnais and Paice, 1990). While not physiologically relevant, Remazol Blue and ABTS demonstrated provided a possible mechanism via which LMCOs might break down lignin *in vivo*. This theory was given further relevance when a fungal LMCO, in combination with a mediator (ABTS), was shown capable of demethylating and deliginifying kraft pulp (Bourbonnais and Paice, 1992). It was shown that the LMCO from *T. versicolor* alone was incapable of substantial delignification of kraft pulp. However, whereas the LMCO was able to catalyze lignin degradation only with addition of an exogenous mediator, supernatants from lignolytic cultures of the same fungus also required addition of a mediator in order to catalyze de-lignification of kraft pulp. Presuming physiological relevance for the LMCO-

mediator system, these suggest that any endogenous mediators in this system have either limited solubility or are unstable.

It should be noted that a system employing mediating compounds would help to explain how degradation could be initiated within the intact lignin polymer, rather than just at its surface. Enzymes, such as LMCOs, are too large to penetrate the lignin polymer, but low molecular weight organic radicals generated by the LMCO could defuse into the lignin to begin its degradation. Good evidence for this type system has been demonstrated in the fungus, *Pycnoporus cinnabarinus*, where a low molecular weight metabolite capable of acting as a natural mediator for lignin degradation has been isolated from culture supernatants (Eggert et al., 1995; Eggert et al., 1996; Eggert et al., 1997; Goodell et al., 1997).

Despite these recent data supporting LMCOs as major components of the ligninolytic systems of white-rot fungi, there still data against LMCO involvement in lignin degradation. For example, the predominant LMCO secreted by the white-rot fungus, *Fomes annosus,* seems to have little role in the delignification caused by this fungus (Thurston, 1994). Addition of thioglycollic acid, a copper chelater and potent inhibitor of the *F. annosus* LMCO, had no apparent affect on fungal growth or its ability of the fungus to degrade high molecular mass lignosulphonate. Similarly, in a study with C. *versicolor*, a

LMCO-specific antibody was used to inhibit LMCO activity in culture. Despite inhibition of the LMCO, the fungus continued to grow normally, and showed no detectable reduction in its ability to degrade lignin.

Although these studies seem contradictory at first glance, if the minimal requirements for lignin degradation are the presence of at least two of the three known phenoloxidases produced by fungi, or LMCO alone in combination with a mediator of some type, there may not be any real contradictions. Fungi, such as *C. versicolor* and *F. annosus,* might be able to switch to a lignin degradation system based on LiP and MnP if LMCO activity is removed or rendered dysfunctional. Such a mechanism might also explain the early work Ander regarding the complementation phenoloxidase mutants in *S. pulverulentum* with a LMCO despite the fact that this fungus does not produce detectable LMCO activity under lignolytic conditions. The possibility that fungi have more than one mechanism for lignin degradation would seem advantageous given the different environments and substrates with which these organisms must cope.

It should be noted that three known species of white-rot fungi – *Perenniporia medulla-panis, T. cingulata*, and *P. sordida* – do not fit this simple theory. These fungi appear to produce only MnP under lignolytic conditions. However, MnP has been proposed to use chelated Mn(III) as a low molecular

weight mediator, and thus these fungi may find it sufficient to employ a mechanism akin to that proposed for fungi reliant on LMCOs.

Microbial LMCOs:

LMCOs have been identified and isolated from a number of bacterial species (Alexandre and Zhulin, 2000). The first credible hint that LMCOs might exist in these organisms came in a report of laccase-like phenoloxidase activity in a melanizing isolate of the bacterium, *Azospirillum lipoferum* (Givaudan et al., 1993). *A. lipoferum* is a nitrogen-fixing bacterium associated with the roots of many plant species, particularly rice and other grasses. The purified phenoloxidase was later confirmed as a LMCO (Alexandre and Bally, 1999; Diamantidis et al., 2000).

The function of this LMCO is as yet unknown; however its presence does not appear to be critical for the survival of this bacterium. Because of the melanizing activity of this enzyme, a phenomenon arising from the oxidation of phenolic compounds near the bacterium, it has been postulated that the LMCO may play some protective role for the organism (Givaudan et al., 1993), while not being absolutely necessary for survival in general. Melanization related to a bacterial LMCO has also been observed in the marine bacterium,

Marinomonas mediterranea (Solano et al., 2000); however, no physiological function has been for advanced for melanization in this species either.

Several recent studies have implicated bacterial LMCOs in metal metabolism. For example, a LMCO identified in *E. coli* is involved in the tolerance of this organism to high concentrations of copper (Grass and Rensing, 2001). Whether this protection is mediated through direct action on copper ions or through modification metabolic pathways for other critical metals, such as iron, is still a subject of debate as the *E. coli* enzyme was demonstrated to have ferroxidase activity (Kim et al., 2001). That bacterial LMCOs can oxidize other metals was shown in various species of *Pseudomonads,* which use these enzymes to oxidize manganese (Brouwers, 1999). Even the A. *lipoferum* LMCO may be involved with metal metabolism if its function is to oxidize phenolic siderophores that the bacterium secretes to assist in the uptake of vital metals (Saxena et al., 1989; Shah et al., 1992).

As previously mentioned, the FET3 protein from *S. cerevisiae* is a highly conserved LMCO whose ferroxidase activity is a critical component of a high-affinity iron-uptake system (Askwith et al., 1994; Askwith et al., 1996). Recent studies have identified multicopper oxidases with ferroxidase activity as critical components of iron-uptake systems in *Chlamydomonas reinhardtii* (La Fontaine et al., 2002); Herbek, 2002 #1295}, although structurally these enzymes bear

greater resemblance to ceruloplasmin and other eukaryotic MCOs that do not strictly fall into the LMCO family.

LMCO Substrates:

As mentioned earlier, one of the unusual characteristics of LMCOs is their enzymatic activity against broad range of substrates. Thus, LMCOs isolated from different organisms have been shown to oxidize compounds as diverse as hydroquinone, *p*-anisidine, guaiacol, syringaldazine, 4-methylcatechol, catechol, α-napthol, ABTS, *p*-phenylenediamine, 2,7-diaminofluorene, toluquinone, 4-hydroxyindole, 2,6-dimethoxyphenol, pyrogallol, N,N-dimethyl-1,4-phenylendiamine, 1,8-diaminonapthalene, DOPA, *p*-cresol, iron, manganese, and copper (Fahraeus and Ljunggren, 1961; Mayer and Harel, 1979; Dean and Eriksson, 1994; Hoopes and Dean, 2001). All known LMCOs oxidize simple o- and *p*-substituted diphenols. In fact, by Enzyme Commission definition LMCOs (*p*-diphenol:O2 oxidoreductase) are distinguished from catechol oxidases by their ability to oxidize para-substituted diphenols. However, this enzymatic definition cannot be used to define LMCOs as many, if not all, LMCOs oxidize *m*-substituted diphenols, as well as many arylamines. Additional caution against taking the definitions strictly come from work by Gregory and Takeo (1973) that showed some catechol

oxidases could oxidize certain para-substituted diphenols (Gregory and Bendall, 1966; Mayer and Harel, 1979). Savidge and co-workers identified a catechol oxidase-like enzyme in extracts of spruce xylem that could oxidize para-substituted phenolic compounds (Savidge and Udagama-Randeniya, 1992). This observation alone casts doubts on the stringency of substrate-based tests used to distinguish LMCOs from catechol oxidases in much of the old literature.

It should also be noted that, contrary to what was written in one review of plant and fungal LMCOs it was (Benfield et al., 1964), LMCOs are quite capable of oxidizing monophenols, such as p-cresol. This conclusion was undoubtedly based on reports that of a partially purified LMCO that was incapable of oxidizing p-cresol (Keilin and Mann, 1939), an observation that conflicted with work from Yakushiji (1937) and Higuchi (1958), who both reported LMCOs that could utilize p-cresol as a substrate (Higuchi and Ito, 1958; Fahraeus and Ljunggren, 1961). More recently, a LMCO purified from *P. versicolor* was also shown to be capable of oxidizing p-cresol (Bollag et al., 1988).

Perhaps a factor contributing to the confusion is the fact that LMCOs tends to be rapidly inactivated by the products of monophenol oxidation. However, this inactivation phenomenon can be minimized through the addition of certain

protecting agents, such as glycerol. Although the exact nature of the protection afforded by these agents has not been defined, it is likely to be related to a scavenging effect on the monophenoxy radicals produced in the reaction. This scavenging effect would protect amino acids in the LMCO active site from coupling with the reaction products.

As with almost all metalloenzymes, LMCOs are inhibited by azide and cyanide anions; however, specific LMCO inhibitors have thus far eluded researchers. This remains a major obstacle to the facile discrimination of LMCO and catechol oxidase. However, the cationic detergent, CTAB, and ferulic acid are much more effective inhibitors of catechol oxidases than they are of LMCOs. Additionally, carbon monoxide has been reported to completely inhibit catechol oxidases at concentrations that show little affect on LMCO activity (Mayer, 1987; Allan and Walker, 1988).

LMCO Structure and Spectroscopic Characteristics:

All LMCO exhibit a basic ß- sheet based structural design first identified in the copper-containing electron-transfer protein, plastocyanin (Messerschmidt et al., 1992; Ducros et al., 1998). These proteins contain three structural domains connected by short intervening loops. (Figure 1) Domain 1 comprises two four-stranded ß-sheets that form a ß-sandwich structure. Domain 2 is

formed from one six-stranded and one five- stranded ß-sheet, while Domain 3 contains two five-stranded ß-Sheets that form a partial ß-barrel.

The four cuprous (Cu^{2+}) ions in LMCOs are arranged in two coordination groups. A single Type-2 copper and the Type-3 copper pair are arranged in a trinuclear cluster with all three ions within 4 Å of one another. The remaining Type-1 copper is separated from the trinuclear cluster by some 12 Å. Perturbations in Type-2 or Type-3 coppers have little or no effect on the Type-1 copper, but have great influence on other members of the trinuclear cluster. Anion binding studies confirm that the Type-2 and Type-3 coppers interact with each other directly (Andreasson et al., 1976; Spira-Solomon et al., 1986).

As previously mentioned, the Type-1 copper gives the enzyme its characteristic blue color by virtue of its strong absorbance around 600 nm. This copper center also has an unusually low hyperfine splitting constant, A_z 40 to 95 x 10^{-4}, that easy to detect using EPR spectroscopy. The Type-2 copper site has no optical absorbance, but exhibits a hyperfine splitting constant greater than 140 x 10^{-4}. The Type-3 copper pair is coupled antiferromagnetically, which renders them essentially EPR silent. However, the Type-3 pair does have an optical absorbance around ca. 330 nm. These four copper ions are coordinated to eleven invariant amino acids, ten histidines and a cysteine. One additional amino acid ligand interacts can interact with the

Type-1 copper in certain LMCOs, and so far only limited variation – Met, Leu, or Phe – has been seen in this position.

There is some confusing literature describing significant structural variations in certain LMCOs. For example, a LMCO from *Phlebia radiata* was reported to be missing the Type-3 copper pair, with a PQQ prosthetic group replacing the copper as the electron accumulation site (Karhunen et al., 1990). No other LMCOs have shown evidence of containing such PQQ group. Curiously, the gene for the *P. radiata* LMCO appears to retain the conserved copper-binding site for the Type-3 copper pair (Saloheimo et al., 1991). In another report of an unusual LMCO, an enzyme from C. *versicolor* was reported to contain only two copper atoms (Morohoshi, 1991). Of course, such reports must be regarded with caution given the difficulty of accurately determining the stoichiometry of metalloenzymes. It is quite possible that copper ions were lost from these enzymes during purification as a certain amount of lability has previously been demonstrated for copper centers in LMCOs. To date there have been no corroborating studies of either of these two unusual enzymes.

LMCO Mechanism:

Although the details of the electron-transfer reactions in LMCOs remain somewhat obscure, much evidence supports a two-cycle mechanism for electron transfer to oxygen, but a four-cycle accumulation of electrons from the donor substrates. In the initial step, the Type-1 center is reduced by a single electron abstracted from the donor substrate (Andreasson and Reinhammar, 1976). The role of the Type-2 copper is less clear, but this center has been implicated in the abstraction of electrons from donor substrates, as well as for temporary storage of an electron before net transfer to the Type-3 center. However, work by Messerschmidt (Messerschmidt, 1992) suggests that the Type-2 copper may only be involved in the breaking of the O-O bond of the peroxide intermediate. The Type-3 copper center is the binding site for the O_2, and it is from this center that electrons are transferred in pairs to the acceptor. It is the paired nature of the copper ions in the Type-3 site that is thought to allow the accumulation of electron pairs prior to transfer. However, the order and timing of electron transfers to the Type-3 copper ions is unclear. Once the first electron pair is transferred to O2, a tightly bound and relatively stable peroxide intermediate is formed. Transfer of a subsequent pair of electrons further reduces the peroxide intermediate to yield water (Andreasson et al., 1973; Pecht et al., 1977; Allendorf et al., 1985; Spira-Solomon et al., 1986).

Although there is some evidence for a three-electron reduced species of O2 as a bound intermediate, this observation still needs corroboration (Andreasson et al., 1976). It may be that the LMCO catalytic center operates with four completely independent electron transfer cycles or some combination of single and multiple electron cycles. However, there is no debate over the number of electrons abstracted from each molecule of donor substrate (Allendorf et al., 1985).

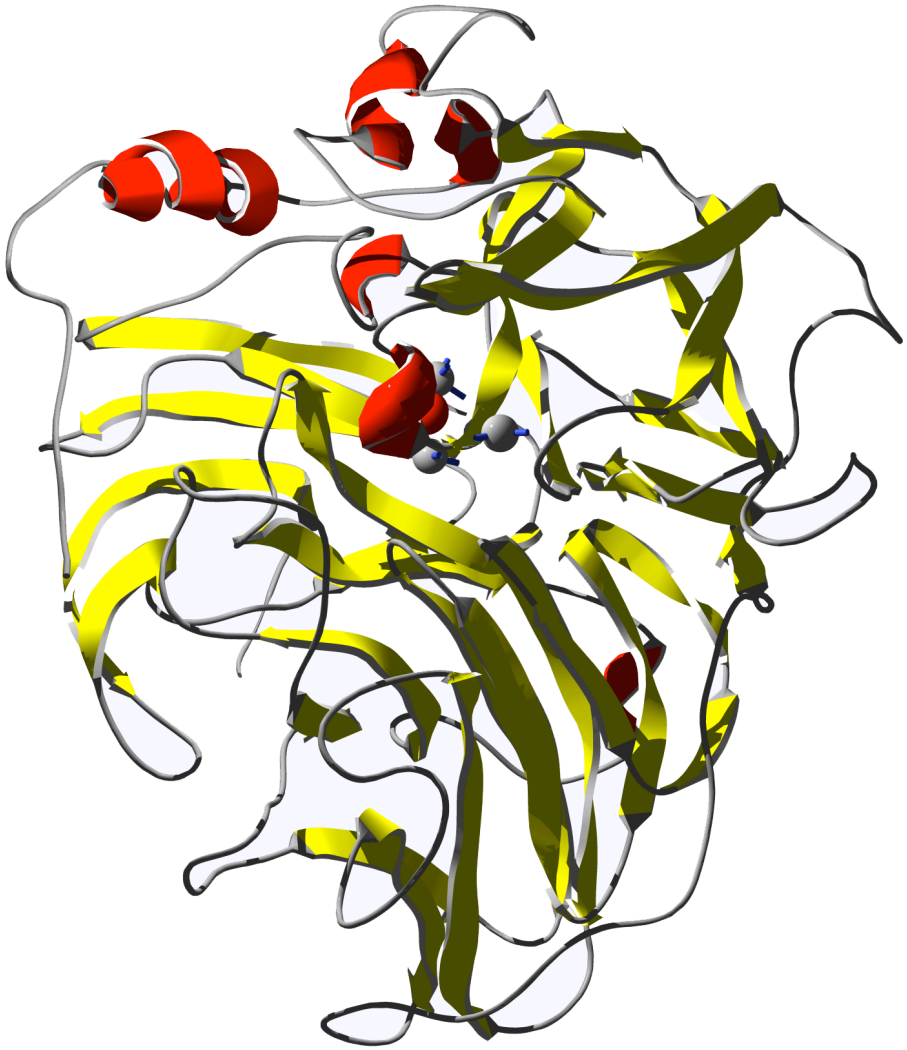

Figure 1 Laccase from C. *Coprinus* in standard orientation. Type II Cu 2+ depleted (red sphere) Domain 1 far left. Domain 2 top right. Domain 3 bottom right. β-sheet shown in yellow, α-helix shown in red. Image generated from the crystal structure of Ducros et al. 1998.

CHAPTER 2

Staining Electrophoretic Gels for Laccase and Peroxidase Activity Using 1,8-Diaminonaphthalene[1]

[1]1Hoopes etal. Analytical Biochemistry 293, 96–101 (2001)
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ABSTRACT

A new chromogenic substrate for laccases and peroxidases, 1,8-diaminonapthalene, was used to detect phenoloxidase activity in gels after SDS-PAGE. This substrate has several advantages over other widely used phenoloxidase stains in that it is inexpensive, and the oxidized product has both high molar absorptivity and very low solubility. Furthermore, neither the substrate nor the product is known to have toxicity problems of the type associated with many other phenoloxidase stains. The sensitivity of detection using 1,8-diaminonapthalene was comparable to that obtained using the most sensitive stains commonly used for phenoloxidases, e.g. 3,3-diaminobenzidine, and was close to that attainable for protein detection using silver staining. Zymograms developed with 1,8-diaminonapthalene can be used with video densitometry to monitor the specific enzymatic activity of phenoloxidases during enzyme purification.

It is widely appreciated that although phenoloxidases and peroxidases are relatively specific with respect to their electron-acceptors, they are often non-specific with respect to their electron donors (Maehly, 1955; Mayer and Harel, 1979; Yaropolov et al., 1994; Xu et al., 1996). While sometimes making it difficult to identify the physiological functions of these enzymes, this biochemical promiscuity makes them easily detectable using a wide variety of chromogenic substrates, and extensive lists of potential substrates have long been available for peroxidases (Kastle and Porch, 1908; Joslyn, 1949). Because phenoloxidases and peroxidases can often make use of the same chromogens, differentiating these enzymes from one another – for example, distinguishing laccases from those peroxidases able to slowly oxidize substrates in the absence of H_2O_2 – can be next to impossible if done solely on the basis of enzymatic analyses using a catalog of substrates and inhibitors (Mayer and Harel, 1979). Thus, assays based on the activity staining of electrophoretic gels (zymograms) have become an important method for assessing the complexity of phenoloxidase and peroxidase constituents in protein mixtures, as well as for following these enzymes throughout the course of a purification protocol (Rescigno et al., 1997). Although a great many chromogenic substrates have been used as detection agents in zymograms, most have characteristics that make them less than desirable for this purpose. In

general, zymogram stains should be stable in the absence of enzyme, but react quickly when the enzyme is present to produce insoluble reaction products having high molar absorptivity (Macchenko, 1994). In addition, it is desirable that the stain be inexpensive and have minimal toxicity. A number of highly sensitive zymogram stains, such as 1-napthol, 3-amino-9-ethyl-carbazole (AEC) and 3,3-diaminobenzidine (DAB), are available for peroxidases; however, most are acutely toxic and must be handled with care (Macchenko, 1994). Fewer reagents are available for detecting laccase activity with comparable sensitivity, due in part to the lower redox potential of laccases, particularly plant laccases, which limits their ability to oxidize compounds such as AEC and DAB. Syringaldazine (Harkin and Obst, 1973), while an effective substrate for fungal laccases, is expensive and only poorly oxidized by most plant laccases (Sterjiades et al., 1993). Tetramethylbenzidine and a two-component stain consisting of p-phenylenediamine and pyrocatechol work well for the histochemical localization of peroxidase activity (Imberty et al., 1985), but are not as consistently effective for the detection of plant laccases in a gel format (Mayer and Harel, 1979). Modified Nadi reagent (Nachlas et al., 1958) is an effective reagent for detecting nearly any laccase (Sterjiades et al., 1993), but it requires two substrates, one of which, N,N,N'N'-tetramethyl-*p*-phenylenediamine, is easily auto-oxidized and is considered acutely toxic.

There are similar concerns associated with the use of catechol and p-phenylenediamine (Nachlas et al., 1958). Other laccase substrates, such as ABTS (2,2'-azinobis(3-ethylbenzathiazoline-6 acid) (Srinivasan et al., 1995) or 4-amino-N,N-diethylaniline (Rescigno et al., 1997)suffer from having relatively soluble products that diffuse away from gels with prolonged incubation. Laccase-catalyzed polymerization of monolignols "in-gel", followed by staining with reagents specific for lignin, is physiologically relevant for plant laccases and does not suffer from diffusion of the reaction product, but is costly with respect to the substrates (Sterjiades et al., 1996).

Insoluble polymers, such as the pigments found in the walls of many fungal spores, are common products of laccase activity in a wide variety of organisms (Dean and Eriksson, 1994; Andersen et al., 1996; Butler and Day, 1998). A spore color mutant of *Cochliobolus heterostrophus* was shown to be deficient in laccase activity and to accumulate 1,8-dihydroxynaphthalene, the putative precursor of the spore wall melanin (Tanaka and Tajima, 1992). This observation suggested that certain fungal metabolites, such as 1,8-dihydroxynaphthalene, might be useful in zymogram staining for laccase activity. Although 1,8-dihydroxynaphthalene is not commercially available, 1,8-diaminonaphthalene (DAN) is an inexpensive chemical that may be obtained from a variety of sources. As described in this paper, we took

advantage of the general ability of laccases to oxidize arylamines and show that DAN has properties that make it an excellent zymogram stain for laccases.

MATERIALS & METHODS

Enzymes and Chemicals:

Horseradish peroxidase (HRP) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Laccase was purified from *Acer pseudoplatanus* cell suspension cultures as described previously(Sterjiades et al., 1992), and purity was verified by SDS-PAGE and mass spectrometry. All other chemicals and reagents used in this study were of ACS grade or better, and were used without further purification.

Protein and Enzyme Assays:

Protein was quantified using the ELS protein assay kit (Boehringer Mannheim, Indianapolis, IN) against BSA standards. Laccase activity was determined by measuring the rate of oxidation for 5 mM 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) in 100 mM sodium acetate (pH 5.0) as increased absorbance at 420 nm and considering 36000 $M^{-1} \cdot cm^{-1}$ as the molar absorptivity of the product at his wavelength (Sterjiades et al., 1993). Peroxidase activity was determined in an identical manner, but with the

addition of 5 mM H_2O_2 to the reaction mixture.

Gel Electrophoresis:

Isoelectric focusing (IEF) was performed using pre-cast agarose gels, pH 3-10 (BMA, Rockland ME). Acetic acid (0.5 M) was used as the anode buffer, while a premixed amino acid buffer from Serva (Crescent Chemical Co., Hauppauge, NY; catalogue #42986) was used as the cathode buffer (Allen et al., 1984). Gels were run at 20 W constant power for 45 min at a constant temperature of 4°C. Trypsinogen (Sigma) was used as a marker for post-stain alignment of gels run under identical conditions, but stained using different methods. Serva Test mix 9 (Crescent Chemical Co.) was used to provide pI markers. Prior to electrophoresis, the salt concentration in each sample was reduced by concentrating the proteins against a 10 kDa MWCO centrifugal filter and then diluting the sample with 0.5 ml water. This process was repeated three times, after which the concentrated proteins were subjected to electrophoresis. There was no apparent loss of enzyme activity to the filtrates from this procedure.

SDS-PAGE was performed using a modified Laemmli protocol (Laemmli, 1970). Laccase and HRP samples from 1.6 mg/ml and 4 mg/ml stocks, respectively, were mixed 1:1 with 2X Laemmli sample buffer containing no reducing agents and loaded directly onto the gel without heat denaturation.

For the purposes of densitometric protein quantification, a series of BSA standards from which to generate a standard curve were included. These standards were prepared using sample buffer containing reducing agent and were heat denatured prior to electrophoresis, as recommended in the Laemmli protocol. For estimating apparent molecular masses, ProSieve Mark 12 standards (BMA, Rockland, ME) were used to generate a linear standard curve of the Rf values for each standard protein versus their molecular masses. Rf values were calculated as the distance moved by each protein from the top of the resolving gel divided by the distance between dye front and the top of the resolving gel. This process was carried out using the "Molecular Weight" module of the AlphaEase™ software provided with the ChemImager 4000 video imaging system (Alpha Innotech Corp., San Leandro, CA).

Gel Staining

Following electrophoresis, gels were incubated in 50 mM sodium acetate (pH 5.0) containing 1% dimethylsulfoxide and 2 mM DAN. DAN, from a 1M stock in 100% dimethylsulfoxide, was added to the buffer immediately prior to immersion of the gel. Gels containing laccase were routinely incubated at 40°C for a period of time sufficient to develop activity bands to the desired intensity - usually ca. 30 min. In the case of gels containing HRP, hydrogen peroxide was added to a final concentration of 5mM and the

incubation temperature was maintained at 20°C in order to more easily control the intensity of staining. The incubation time was usually about 5 min for peroxidases. To halt activity staining, the gels were immersed for 10 min in 50% (v/v) methanol containing 10% (v/v) acetic acid at 50°C. Gels were usually immersed in 20% trichloroacetic acid (TCA) to darken the DAN oxidation product. Where desirable, activity bands were further enhanced by counter-staining the gels with Coomassie brilliant blue dye using protocols developed to detect proteins. Briefly, gels were immersed in 10% (v/v) acetic acid containing Coomassie G-250 (0.6 g/L) for 2 h, and subsequently destained in 50% (v/v) methanol, 10% (v/v) acetic acid until the background was clear.

Densitometric analyses were preformed using the AlphaEase™ software and ChemImager 4000 imaging system. Briefly, in "View" mode, the initial image was magnified until it filled the entire screen. With the "Saturation View" on, the "Pattern Adjustment" control was used to adjust the area outside of the gel to highest white level possible, while the darkest band in the gel was not allowed to reach camera saturation. In this way, the widest possible response range was achieved for each individual gel. The "Spot Densitometry" mode was then used to acquire raw data in the case of activity stained bands, or to create a standard curve for protein quantification.

RESULT AND DISCUSSION

Figure 1 depicts an IEF gel stained first for phenoloxidase activity using DAN and subsequently enhanced by Coomassie counter-staining. The smeary banding pattern in the laccase lane is typical for IEF with this particular enzyme preparation, as numerous isoform bands focus around pH 5 (Sterjiades et al., 1993). As proteins cannot be fixed in gels prior to activity staining, it is important to note that there is some diffusion of enzyme from the gel during the activity staining procedure. This is a general problem for zymograms, and is more of a problem with IEF gels, particularly those using an agarose matrix, due to their larger pore size and the relative thickness of the gels. The problem can be further exacerbated by longer incubation times and elevated temperatures, such as those routinely used to stain for laccase versus peroxidase activity. This diffusion of protein can be seen in the staining intensity differences for the trypsinogen bands in the left and right panels of Figure 1.

The sensitivity of activity staining with DAN was compared with that of Coomassie protein staining using HRP in an SDS-PAGE format (Figure 2). Identical gels containing a concentration series of HRP were stained for protein only (panel A), for activity only (panel B), or for activity followed by enhancement with Coomassie stain (panel C). While protein bands were not

visible with Coomassie staining at a load below 3.0 µg total protein, DAN-oxidizing activity was still faintly visible at a 50 ng load, and easily detectable at 50 ng with Coomassie-enhanced DAN staining. Based on the total protein load, Coomassie-enhanced DAN staining was at least 60 times more sensitive than Coomassie staining alone. However, it is clear from the gel stained for protein (panel A) that the HRP sample was actually a mixture of proteins with HRP constituting only a small fraction of the total material. Densitometric analysis suggested that only about 15% of the total protein migrated with the same R*f* as the predominant peroxidase activity band. Thus, based on densitometric estimations of HRP content, the sensitivity of the Coomassie-enhanced DAN staining appeared to be more than 400 times that of Coomassie staining alone.

Laccase activity may be detected with similar sensitivity using Coomassie-enhanced DAN staining.

Figure 3 shows identical SDS-PAGE gels containing a concentration series of *A. pseudoplatanus* laccase stained for protein using Coomassie dye (panel A), or for activity using Coomassie-enhanced DAN staining (panel B). Again, the diffuse banding pattern is typical for this enzyme preparation when analyzed by SDS-PAGE (Sterjiades et al., 1992).

Zymograms can provide a rapid, high-resolution technique for screening

multiple biological samples for the enzyme activity of interest. Coupled with video densitometry to provide for semi-quantitative analysis, the technique is particularly useful for optimizing enzyme purification protocols because it enables researchers to calculate for the enzyme of interest a "specific enzymatic activity" (enzymatic activity per mg of a specific polypeptide) in samples that have only been partially purified (Smith et al., 1997). In contrast, the specific activity data presented for partially purified enzyme in most protein purification papers is technically the "specific catalytic activity" (enzymatic activity per mg of total protein). It is not uncommon to find with metalloenzymes, such as laccases, that a particular chromatography resin will yield the polypeptide of interest in very high purity, but with low residual activity due to stripping of the catalytic metal or some structural perturbation. A different resin may return the enzyme activity in higher yield, but also removes fewer contaminating proteins, so as to yield a pool with comparable specific catalytic activity. Densitometric analysis of zymograms allows the researcher to differentiate between these two scenarios and select the resin best suited to meet the needs of the project.

To demonstrate the utility of this technique, two sets of identical gels were stained for protein content, laccase activity, or laccase activity with Coomassie counter-staining (Figure 4). One set of gels (panels A1, B1, C1)

was loaded with a sample of purified *A. pseudoplatanus* laccase, or increasing amounts of the same laccase to which a fixed amount of crude *E. coli*, strain K-12 protein extract had been added. In the second set of gels (panels A2, B2, C2), in addition to the same purified laccase sample, lanes were loaded with a fixed amount of laccase that had been partially inactivated by heating at 70°C for 5, 10 and 20 minutes. To these heat-inactivated samples, a fixed amount of crude *E. coli* extract was added prior to sample preparation. All of the gels contained a set of BSA standards with which to calibrate the densitometer for protein quantitation, while the purified laccase sample in each gel allowed for calibrated quantification of enzyme activity for comparison with aqueous assays of laccase activity. In Figure 5, the true specific enzymatic activity for purified laccase added to each sample (black bars) is compared to the specific enzymatic activity as calculated from the densitometry data (gray bars). The specific catalytic activity measurements for each mixture of laccase and *E. coli* crude extract are also depicted (white bars). Clearly, the specific enzymatic activity values calculated from the densitometric measurements provide a more accurate indication of the enzyme quality in protein mixtures than do the specific catalytic activity values.

It was not a surprise that DAN proved an excellent laccase substrate given its resemblance to 1,8-dihydroxynapthalene, a known physiological

substrate for a fungal laccase (Tanaka and Tajima, 1992), and the normal tendency of aromatic amines to be easily oxidized. The production of an insoluble, colored polymer via the enzyme-catalyzed oxidation of DAN is analogous to what occurs during the oxidative polymerization of aniline to form polyaniline and, in fact, treatment of polyaniline with a strong acid is the method used to produce the dye, Aniline Black. This suggests that a similar reaction is responsible for the darkening and change in color, i.e. brown to black (data not shown), of the initial DAN polymer (polyDAN) when the gels are immersed in 20% TCA. Although the exact mechanism by which Coomassie dye binds to proteins is as yet unknown, it has been shown that proteins rich in arginine, histidine, and lysine bind the dye best (Tal, 1985), while tyrosine, tryptophan, and phenylalanine residues also make significant contributions to the binding (Compton and Jones, 1985). This suggests that the positively charged, polyaromatic nature of the DAN reaction product makes it an ideal target for the Coomassie dye binding that makes this detection technique so sensitive.

Diaminobenzidine (DAB) is one of the most widely used zymogram substrates for detecting peroxidase activity because its oxidation also produces a darkly colored insoluble product. However, DAB is not a particularly good substrate for plant laccases and, in addition, its use carries with it health

concerns as the chemical is currently classified as acutely toxic. In addition to being extremely sensitive for laccase activity, the DAN staining procedure described here detects peroxidase activity with a sensitivity equivalent to that obtained with DAB, but the MSDS (Material Safety Data Sheet) information for DAN suggests a much reduced health risk associated with its use. These properties all suggest that DAN should prove a useful substrate for the study of laccases, peroxidases and other phenoloxidases.

Acknowledgements – This research was funded by U.S. Department of Energy grant DE-FG01-99ER20336.

Figure 1

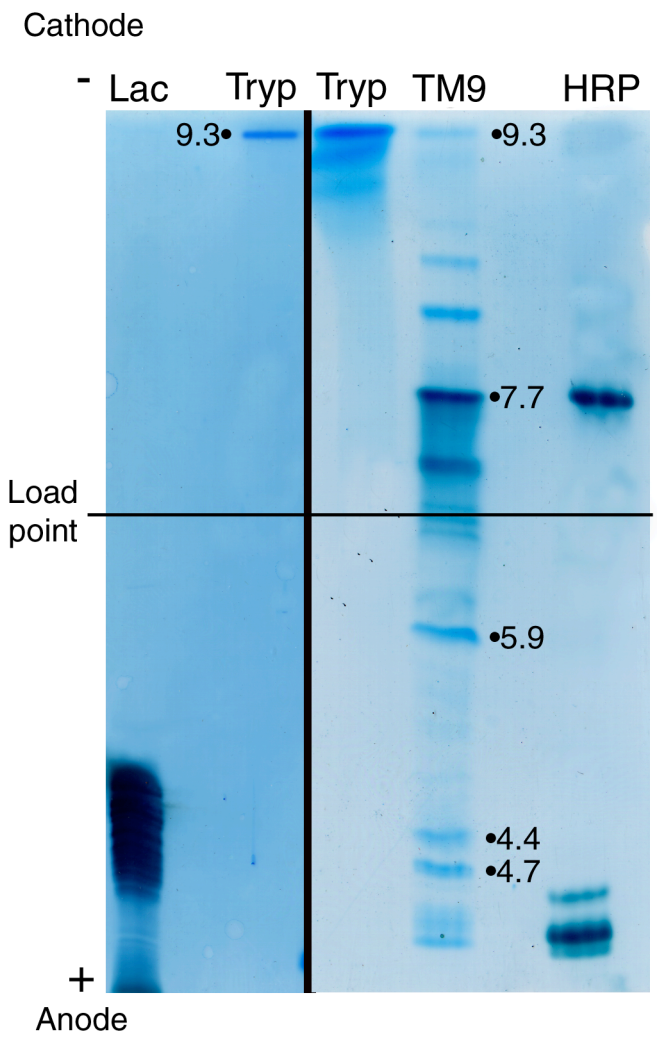

Figure 1. Activity staining of laccase and peroxidase with DAN after isoelectric focusing. From left to right, the lanes were loaded with 20 µg of A. *pseudoplatanus* laccase (Lac), 10 µg trypsinogen (Tryp, 2 lanes), 15 µl of Serva test mix 9 (TM9), and 8 µg of horseradish peroxidase (HRP). After electrophoresis, the gel was split, and the left most two lanes were incubated for 30 min. in staining buffer without H_2O_2, while the remaining three lanes were stained 5 min. in the presence of H_2O_2. The gel was counter-stained with Coomassie G-250 to visualize the marker proteins and enhance the activity bands.

Figure 2

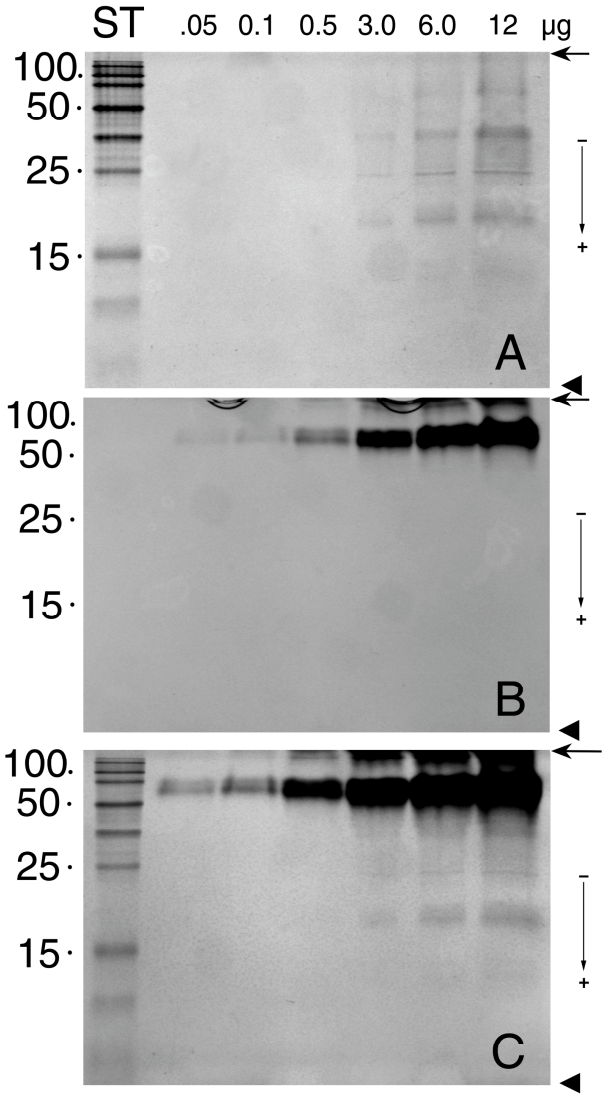

Figure 2. Activity staining of peroxidase with DAN after SDS-PAGE. Identical 12.5% acrylamide gels were loaded with 0.05, 0.1, 0.5, 3.0, 6.0, and 12.0 µg of HRP and run as described. Protein alone was visualized by staining with Coomassie G-250 (panel A). Peroxidase activity was detected by TCA-enhanced DAN staining either without (panel B) or with (panel C) Coomassie G-250 counter-staining. The standards lane (ST) in each case contained 10 µl of BMA ProSieve protein standards. Note that the HRP samples were not fully denatured, and thus may not be compared directly to the marker proteins for a determination of molecular mass. The top of the resolving gel is indicated by the horizontal arrow to the left of each panel, while the ending position of the dye front is indicated by the triangle.

Figure 3

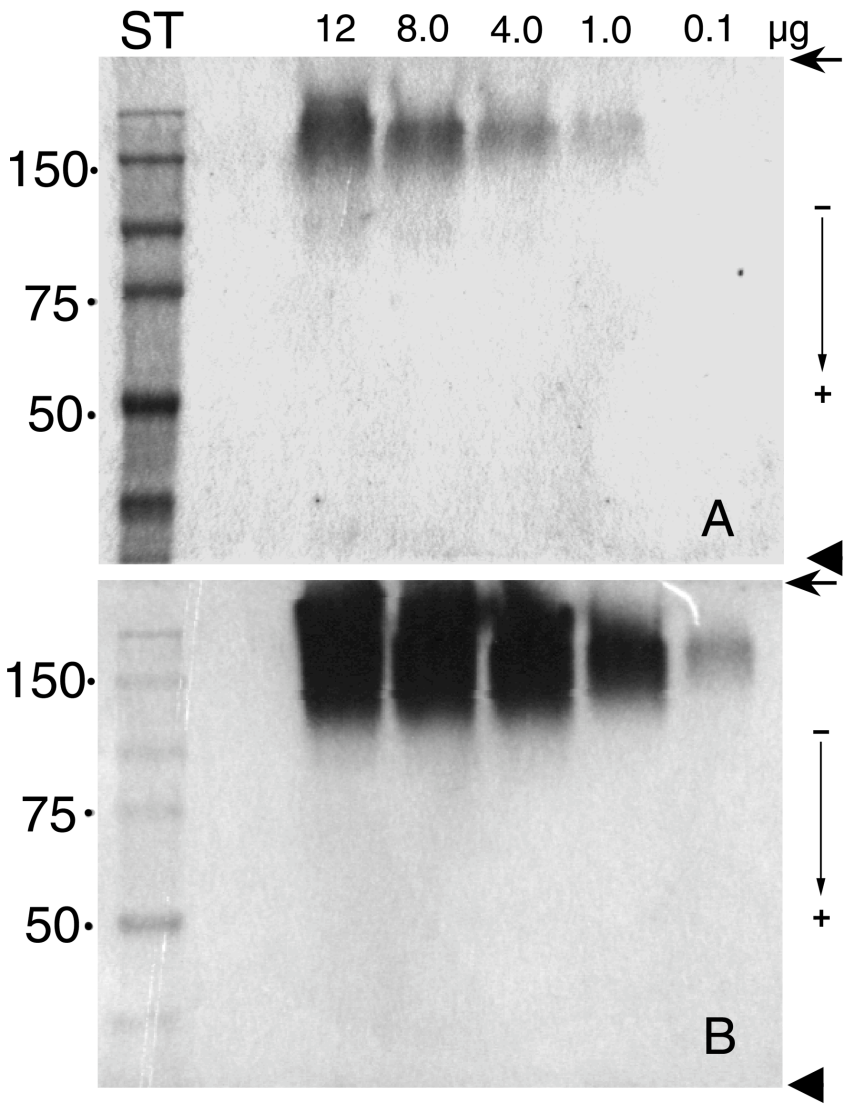

Figure 3. Activity staining of laccase with DAN after SDS-PAGE. Identical 7.5% acrylamide gels were loaded with 12.0, 8.0, 4.0, 1.0, and 0.1 µg of *A. pseudoplatanus* laccase and run as described. Protein was visualized by staining with Coomassie G-250 (panel A), while laccase activity was detected using Coomassie-enhanced DAN staining (panel B). The standards lane (ST) in each case contained 10 µl of BMA ProSieve protein standards. The top of the resolving gel is indicated by the horizontal arrow to the left of each panel, while the ending position of the dye front is indicated by the triangle.

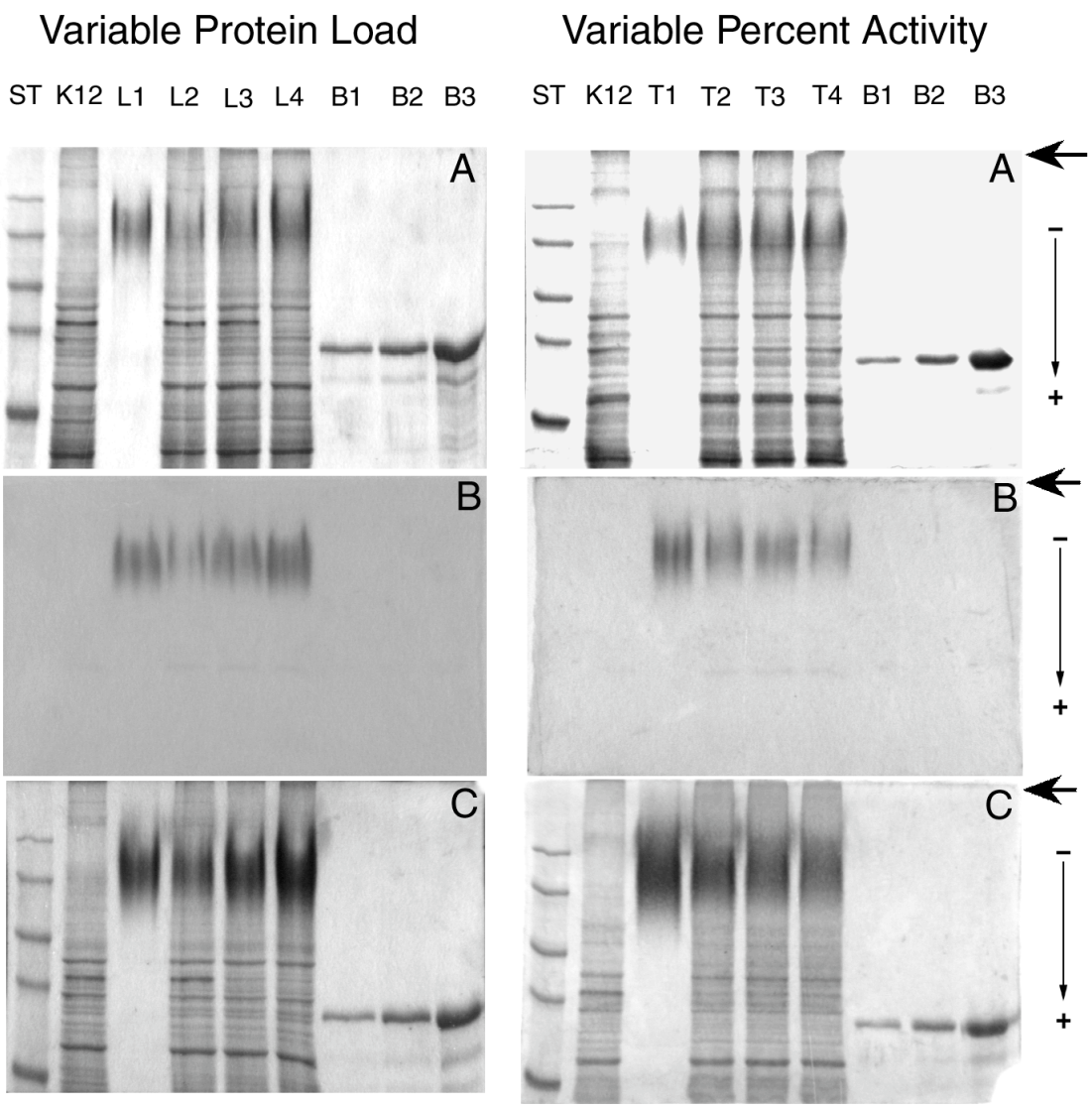

Figure 4 SDS-PAGE analysis of laccase in gels stained for protein (panels A) or stained for activity using 1,8-diaminonaphthalene without (panels B) or with (panels C) Coomassie counter-staining. Three identical gels (A1, B1, C1) were loaded with either pure laccase (L1 = 1.6 µg) or different amounts of laccase (L2, L3, L4 = 0.8, 1.6, 3.2 µg, respectively) each containing 30 µg of *E. coli* crude protein extract. Similarly, three identical gels (A2, B2, C2) were loaded with either pure laccase (T1 = 1.6 µg) or laccase (1.6 µg) that had been incubated at 70°C for 5, 10 or 20 min (T2, T3, T4, respectively) before being spiked into 30 µg of *E. coli* crude protein extract. All gels contained BSA calibration standards (B1, B2, B3 = 0.5, 1.0, 3.0 µg, respectively). The top of the resolving gel is indicated by the horizontal arrow to the left of each panel.

Figure 5

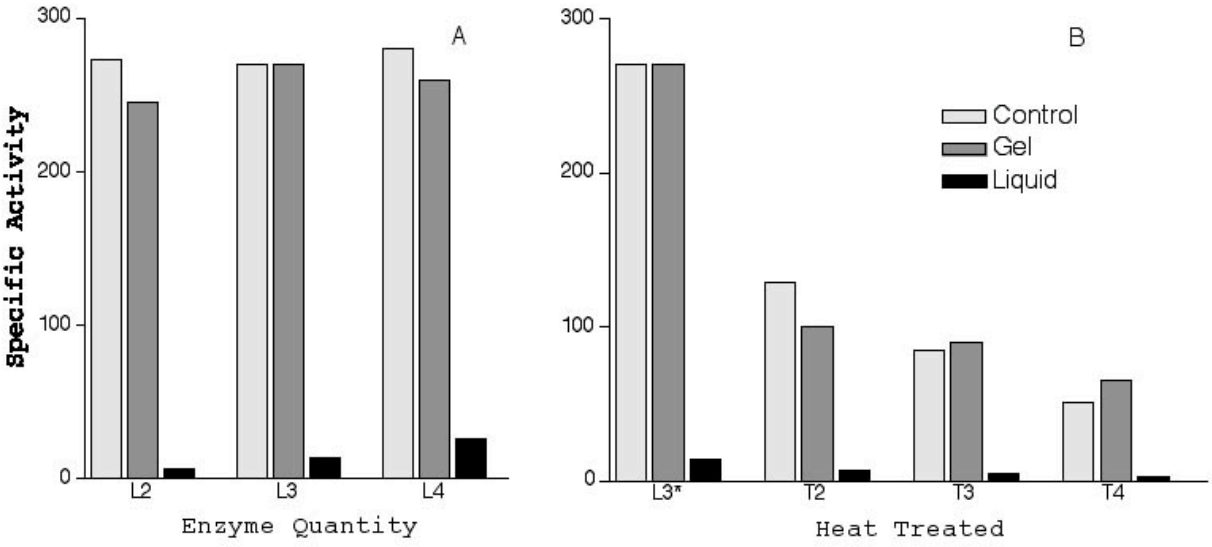

Figure 5. Comparison of densitometric determination of specific enzymatic activity from zymograms versus specific catalytic activity from liquid assays. Specific enzymatic activities calculated from the laccase activity measured by liquid assay in samples to which 30 µg of *E.coli* crude extract had been added and the known amount of laccase protein added (light grey bars) were compared to the specific enzymatic activities calculated from densitometry measurements of the gels depicted in Figure 4, panels A and B (dark gray bars). Specific catalytic activity values (black bars) calculated from the laccase activity measurements and the total protein content of the samples (including 30µg of *E. coli* crude extract) are shown for comparison.

CHAPTER 3

Altered glycosylation of a LMCO from *Acer pseudoplatanus* expressed in suspension-cultured tobacco cells[1]

[1] [1]Hoopes and Dean To be Sumitted to *FEBS Letters*

Abstract

A LMCO gene cloned from *Acer pseudoplatanus* has been expressed in Tobacco BY2 cell and compared to the native LMCO isolated from Acer pseudoplatanus cell suspension cultures. The recombinant Acer LMCO showed a Pi 5.3-5.6, nearly identical to that of the native Acer isozyme pool. The banding pattern apparent in the native was also present in the recombinant enzyme. The Km and Vmax of both the native isozyme pool, and the recombinant single isoform LMCOs were reasonably close. The molecular mass for both the native and recombinant LMCO was determined by MALDI-MS to be 90.1 and 82.9 kDa respectively. The percent carbohydrate by mass of the recombinant and native enzymes was 27.6% and 33.5% respectively. Enzyme samples of the native LMCO determined to be pure by mass spectroscopy and SDS PAGE gel were shown to give multiple peaks when bound and eluted from a Pharmacia Mono-S cation exchange column. IEF and MALDI-MS analysis of the separate peaks showed little detectable differences between the peaks. However, the peak pattern was reproducible and elution volume was consistent if a singe peak was rerun under identical conditions.

Abbreviations – ABTS, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); DEAE, diethylaminoethyl ; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; LMCO, laccase-like multicopper oxidase; MALDI-MS,

matrix-assisted laser desorption ionization mass spectrometry; MS medium, Murashige and Skoog medium; MWCO, molecular weight cutoff; PBS, phosphate-buffer saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride

Introduction

Laccase (EC 1.10.3.2), the stereotype for the multicopper oxidase (MCO) enzyme family, was described in 1883 as a "diastase," or enzyme activity, in sap from the Japanese lacquer tree (*Rhus vernicifera*) (Yoshida, 1883). The sap from this tree is used to create the lacquerware that is so highly prized throughout Asia, and laccase was found to be the catalyst for the curing process in this material. However, it remained for a French researcher, Bertrand, who was interested in replicating this lacquer in Europe, to coin the names "laccase" and "laccol" for the enzyme and substrate in this biopolymer system (Bertrand, 1895).

Through the years, researchers have identified a variety of related enzymes that utilize multiple copper ions in the electron-transfer reactions they catalyze. They are generally referred to as multicopper oxidase (MCOs), or sometimes as blue copper oxidases for those that contain Type-1 copper centers which impart a blue color to the purified enzyme (Malmstron, 1997). Specific physiological functions and substrates are not yet known for many

members of the MCO family, yet the term "laccase" has been used widely to describe monomeric enzymes that contain four copper atoms arranged in the three known electromagnetic configurations for bioinorganic copper (Mayer and Staples, 2002). Since the unsaturated alkylcatechols (laccols) originally described by Bertrand (Bertrand, 1895) are unlikely to be natural substrates for the enzymes produced by organisms other than a handful of plant species, we have proposed the term "laccase-like multicopper oxidase (LMCO) as a general identifier for this group of enzymes.

Physiological substrates have only been proven for a handful of the known LMCOs, primarily because most members of this enzyme family are capable of oxidizing a broad range of substrates, including phenols, diphenols, and phenyldiamines, as well as metal ions including iron, manganese and copper (Askwith et al., 1994; Brouwers, 1999; Stoj and Kosman, 2003). This breadth of potential substrates, while making possible numerous physiological functions and industrial applications, presents a considerable problem with regards to identification and characterization of LMCOs. Many of these compounds are also substrates for other classes of oxidative enzymes, such as phenoloxidases and peroxidases, which can make identification of specific enzymes difficult in crude solutions. Consequently, there has been much work devoted to finding specific inhibitors and substrates

with which to distinguish different classes of these various enzymes (Mayer and Harel, 1979).

Complicating efforts to identify physiological functions for LMCOs is the fact that these enzymes exist as multigene families in most eukaryotic genomes, and in many cases multiple LMCO gene products are produced simultaneously in the same tissues (Wahleithner et al., 1996; Yaver and Golightly, 1996; Munoz et al., 1997; Mansur et al., 1998; LaFayette et al., 1999). This task is also made more difficult because the possible physiological functions proposed for LMCOs have expanded considerably in recent years (Mayer and Staples, 2002). In order to clearly define enzymatic activity with respect to specific substrates and kinetic parameters, researchers must have access to large amounts of purified, homogeneous enzyme, and it appears that for many of the LMCOs identified to date this will require heterologous expression of single genes in host cells that do not produce large amounts of endogenous LMCOs under culture conditions.

Efforts to express in bacteria or yeast the enzymes encoded by LMCO genes from plants have met with little success, but active enzyme can be recovered from transgenic plant cells (Dean et al., 1998; LaFayette et al., 1999). In the case of the LMCO gene cloned from *Acer pseudoplatanus* (LaFayette et al., 1995), heterologous expression leads to secretion of large

amounts of the enzyme into culture medium. In the study described here, purified samples of this heterologously expressed enzyme were characterized with respect to physical and enzymatic parameters and compared to the pool of LMCO isozymes that were purified from suspension-cultured *A. pseudoplatanus* cells.

Materials and methods

Plant materials:

Unless otherwise specified all chemicals used in this study were of ACS grade or better. Coniferyl alcohol was synthesized as previously described (Weymouth et al., 1993). Suspension cultures of *A. pseudoplatanus* cells were grown in 2800 ml Fernbach flasks each containing 500 ml of the medium described by Bligny et al. (Bligny and Douce, 1983). Cells were grown in dark at 24°C on an orbital shaker (110 rpm), and transfers (1:10, v:v) were made every seven days. Cultures used for enzyme purification were allowed to grow for two weeks prior to collection of the spent medium.

Suspension-cultured cells of *Nicotiana tabacum* L. cv. Bright Yellow 2 (BY2) were grown in 2800 ml Fernbach flasks containing 500 ml of Murashige-Skoog (MS) medium (Life Technologies, Bethesda, MD). The medium composition was myo-inositol (100mg/L), thiamine-HCL (1mg/L), potassium

phosphate (monobasic) (370 mg/L), 2,4-D (0.2 mg/L), and sucrose (30 g/L). The medium was adjusted to pH 5.7, and cells were grown in dark at 24°C on an orbital shaker (110 rpm). Cells were transferred on to fresh medium on a weekly basis at a 1:10 (v:v) ratio.

Enzyme purification:

The following protocol was used to purify the Acer LMCO secreted by transgenic tobacco cells grown in liquid culture. In the case of the mixed LMCO isozymes in the spent medium of A. *pseudoplatanus* cell suspension cultures, a final cation exchange column (Mono-S) was used as described for a final clean-up step. Medium from cell suspension cultures was filtered through Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA), and the supernatant was centrifuged (1500 x g) for 15 min at 4°C. The crude supernatant was again filtered through Miracloth and phenylmethylsulfonyl fluoride (PMSF) was added from a 100 mM ethanol stock to a final concentration of 1mM. This "crude preparation" was treated with solid ammonium sulfate to reach 55% saturation, and stirred 24 h at 4°C. The solution was then centrifuged (24,000 x g) for 30 min at 4°C, after which the solution was filtered through Miracloth to yield the "soluble ammonium sulfate fraction." All subsequent chromatography steps were performed using fast-

protein liquid chromatography (FPLC) (Amersham Biosciences, Piscataway, NJ).

This supernatant was applied to a butyl-Spherilose (Isco, Inc., Lincoln, NE) column (5 x 20 cm) equilibrated with 50 mM sodium acetate buffer, pH 6.5, containing ammonium sulfate to 55% saturation. The column was washed with loading buffer until the A_{280} of the eluate showed no further change, and the enzyme was eluted in a single step using 50 mM sodium acetate buffer, pH 6.5, containing ammonium sulfate to 22.5% saturation. Column loading, washing and elution were all performed at a flow rate of 5 ml/min.

Fractions from the butyl-Spherilose column containing phenoloxidase activity were pooled. The sample was re-buffered into 10 mM Tris-HCl, pH 7.0, by dialysis, and then concentrated to a suitable volume by ultrafiltration against a 10-kDa molecular weight cutoff (MWCO) membrane (PM-10, Millipore Corp., Billerica, MA). Enzyme was loaded onto a DEAE-Spherilose column (1.5x 20) equilibrated with 10 mM Tris-HCl, pH 7.0, buffer. The column was washed with 10 mM Tris-HCl, pH 7.0, and enzyme was eluted with a 200 ml linear gradient from 0-250 mM NaCl in 10 mM Tris-HCl, pH 7.0. All steps were performed at a flow rate of 2 ml/min.

Active fractions from the DEAE-Spherilose column were pooled and re-buffered against 10 mM sodium acetate, pH 5.0. The LMCO purified from

transgenic tobacco cells was stored after this step at -20°C. The mixture of isozymes from A. *pseudoplatanus* cells was loaded onto Mono-S column (Amersham Biosciences), and washed with 10 mM sodium acetate, pH 5.0. Enzyme was eluted with a 60 ml linear gradient from 0-150 mM NaCl in 10 mM sodium acetate, pH 5.0. Steps were performed at a flow rate of 5 ml/min.

Protein and enzyme activity assays:

Protein was quantified against bovine serum albumin standards using the ELS protein assay kit. (Boehringer Mannheim Corp., Indianapolis, IN). LMCO phenoloxidase activity was routinely monitored by following the oxidation (A_{420}) of 10 mM 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) in 50 mM sodium acetate buffer, pH 5.0, buffer at 30°C. For determination of the optimal pH for phenoloxidase activity the oxidation of 10 mM pyrogallol at 30°C was monitored at a wavelength of 450 nm. Low pH range reactions were carried out in 100 mM sodium citrate/phosphate buffer, while high pH reactions were carried out in 100 mM monobasic/dibasic phosphate buffer. The two curves overlapped in the range from pH 5.0 to 7.0. Halide salt inactivation studies were carried out using 10 mM pyrogallol (A_{450}) in 100 mM citrate/phosphate buffer, pH 6.5 to which halide salts were added to final concentrations of 1, 2, 10, 20, 100, 500, and 750 mM. NaCl was also tested

at a concentration of 1750 mM. Temperature stability studies were carried out using 10 mM pyrogallol (A_{450}) in 100 mM citrate/phosphate buffer, pH 6.5. Samples were incubated for 10 min at each test temperature, then transferred to an ice bucket to cool. Reaction rates were calculated from the change in absorbance detected in the first minute of the reaction using a diode-array UV-Vis spectrophotometer equipped with HP Biochemical Analysis Chemstation Software (Mdl. 8452A, Agilent Technologies, Palo Alto, CA).

Enzyme kinetics:

All spectroscopic measurements were performed with a diode-array spectrophotometer as described above. Scan rate was set for 30 times per minute, and all kinetic determinations were generated from initial rate calculations over the time period best suited to each set of substrates and conditions. All measurements were made at 30°C unless otherwise specified.

Determination of kinetic parameters for the plant LMCOs required higher substrate concentrations than are typically used in assays of fungal LMCO activity, which necessitated special considerations some of the substrates. Stock solutions of ABTS, 500 mM in 10 mM citrate/phosphate buffer, pH 6.5, were prepared and allowed to sit at 4°C for two to four days prior to use. This minimized the level of auto-oxidation contributing to the

baseline of reactions with this substrate. Coniferyl alcohol oxidation was monitored as a reduction in absorbance at 310 nm. Reactions were performed in 0.5 or 0.1 cm path length cuvettes as required (Starna Cells, Atascadero, CA). For all substrates, solutions were made up in volumetric flasks, and frozen as aliquotted stocks. A fresh aliquot of substrate stock was thawed and used each day.

To determine K_m and V_{max} values for pyrogallol, six measurements were taken for each substrate concentration, and this was repeated at three different enzyme concentrations to generate three curves. The K_m value was taken as the average values generated by the least-squares fit of the Michaelis-Menten equation to each data set. Calculations were performed using Prism (Graph Pad Software, San Diego, CA). The K_m and V_{max} values for other phenolic substrates were calculated from triplicate measurements at different substrate concentrations using a single enzyme concentration.

Kinetic parameters for oxygen were determined by following LMCO-catalyzed pyrogallol oxidation under different oxygen concentrations in sealed glass cuvettes. Prior to taking measurements, the buffer and substrate were equilibrated with respect to temperature and oxygen concentration for 8 hours. The sealed cuvettes were purged with the same gas mixture for 1 min prior to addition, by syringe, of substrate and enzyme.

Gel electrophoresis:

Denaturing SDS-PAGE was routinely performed as described by Laemmli (Laemmli, 1970), and molecular mass determinations were made using ProSieve protein markers (BMA Bioproducts, Rockland, ME). Proteins were visualized by staining with Serva Blue W (Crescent Chemical Co., Inc., Islandia, NY) according to the manufacturer's recommendations. To detect LMCO activity after SDS-PAGE, sample preparation was modified by omitting heat denaturation of the proteins. In general, samples destined for such zymogram analyses were concentrated by acetone precipitation (3x vol cold acetone), after which the pellets were solubilized in SDS-PAGE sample buffer containing no reducing agents. Samples were subjected to centrifugation at 20,000 x g for 2 min prior to loading onto gels.

Isoelectric focusing was performed using precast pH 3-10 agarose gels (FMC Bioproducts, Rockland, ME). Electrode buffers were 0.5 M acetic acid (anode) and SERVA pre-mixed cathode buffer (Crescent Chemical Co., Inc.). Gels were run at 20 W constant power for 45 min on a flat-bed apparatus with temperature maintained at 5°C. Serva test mix 9 (Crescent Chemical Co., Inc.), as well as B-lactoglobulin, amyloglucosidase, and trypsinogen (Sigma-Aldrich Chemical Co., St. Louis, MO) were used as IEF mobility standards

according to the manufacturer's recommendations. Prior to electrophoresis, samples were concentrated against a 10-kDa MWCO membrane (Gelman Sciences, Ann Arbor, MI), subjected to two water washes, and loaded directly onto the gel. Activity staining of IEF gels was performed using 1,8-diaminonaphthalene (DAN) as previously described (Hoopes and Dean, 2001).

Antibodies and western blotting:

Monoclonal antibodies were generated against LMCO that had been purified from A. *pseudoplatanus* cell-suspension cultures as described by Sterjiades et al. (Sterjiades et al., 1992) and subsequently deglycosylated by treatment with hydrofluoric acid (Mort and Lamport, 1977). Western blot analyses were performed as previously described (Dean et al., 1994) after transfer of proteins separated by SDS-PAGE gel to PVDF-Plus membrane (Micron Separations, Inc., Westborough, MA). After blocking, membranes were incubated for 4 h in the primary antibody (1:500 dilution in PBS cotaining 0.1% Tween-20). Binding of the primary antibody was detected using goat anti-mouse:alkaline phosphatase conjugate (Sigma-Aldrich) at a dilution of 1:20,000 in PBS-Tween. The secondary antibody was allowed to incubate for 1 h, and the blot was subsequently washed with water three times (1 min. each).

Bands tagged with alkaline phosphatase were visualized as per the protocol of Gallagher et al (Gallagher et al., 1992).

Mass spectrometry:

Purified protein was analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) using a Hewlett-Packard G2025A TOF mass spectrometer (Agilent Technologies, Palo Alto, CA). Samples were ionized using an ultraviolet nitrogen laser (@337nm) with a pulse width of 3 ns. The acceleration voltage was 28 kV, the extractor voltage was 7 kV, and pressure was maintained at ca. 1 x 10^{-6} Torr. External calibration of the instrument was routinely performed using a mixture of standard proteins. The solvation matrix for all samples was 100 mM sinapinic acid dissolved in a solution of 80% (v/v) acetonitrile, 0.08% (v/v) trifluoroacetic acid.

Protein sequencing:

Purified LMCO that had been electrophoretically transferred to a PVDF membrane was subjected to amino acid sequencing at the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia.

Results

The mixture of LMCO isozymes from *A. pseudoplatanus* cell-suspension cultures, as well as the single isozyme secreted from transgenic tobacco cells, were amenable to roughly the same purification scheme. The only difference was the addition a final cation-exchange (Mono-S) chromatography step to purify the enzyme from *Acer* cells. Yields and specific activities for LMCO preparations from these protocols are shown in Table 1, and agreed with previous work on these enzymes (Bligny and Douce, 1983; Sterjiades et al., 1992). The enzyme preparations were judged to be pure by virtue of the single band detected by protein staining after SDS-PAGE in both the heat-denatured (Figure 1) and non-denatured (Figure 2) formats.

Molecular masses for the LMCOs from *Acer* and transgenic tobacco cells were determined by SDS-PAGE as 93 kDa and 85 kDa, respectively (Figure 1). MALDI-MS analysis of the two purified LMCO preparations indicated that each sample contained a single protein, with masses of 90.1 kDa and 82.9 kDa for the *Acer* and transgenic tobacco enzymes, respectively. Amino-terminal sequence analysis was the same for proteins from both sources. This indicated that the tobacco cells processed the *Acer* signal sequence correctly, and that an N-terminal truncation was not responsible for the size difference seen in the proteins from the two sources. Monoclonal antibodies

prepared against the deglycosylated LMCO mixture from *Acer* cells were capable of detecting the LMCO from transgenic tobacco cells (Figure 1). The size difference seen for the LMCOs from the two sources was also seen in SDS-PAGE where proteins were not denatured, and under these conditions the enzymes ran with apparent molecular masses of 128 kDa and 111 kDa for the Acer and tobacco enzymes, respectively (Figure 2). Isoelectric points for the LMCOs from *Acer* and transgenic tobacco cells were determined as 4.8 and 5.3, respectively (Figure 3).

When the mixture of purified LMCO isozymes from *Acer* cells was eluted from the Mono-S column using a 10x shallower NaCl gradient the preparation could be resolved into a series of overlapping activity peaks (Figure 4). Peaks pooled separately, and re-run under identical conditions eluted predominantly as a single peak having approximately the same retention volume as in the original run. Isoelectric focusing showed that the mean pI for the enzyme in these pools ranged from about 4.7 to 5.1 for pools eluting at higher salt concentrations (Figure 4). MALDI-MS analysis of fractions 5 and 6 found masses of 89.4 kDa and 88.5 kDa, respectively, for the proteins.

In agreement with previous work, the optimum pH for the LMCO preparation from *Acer* cells was found to be 7.0, and the same was true for the enzyme isolated from tobacco cells (*data not shown*). No detectable difference

was noted in the activity of either LMCO in 100 mM citrate/phosphate versus 100 mM phosphate buffer at overlapping pH values. Temperature stability experiments showed both LMCO preparations as relatively stable up to ca. 65°C (Figure 5).

LMCO activity was highly sensitive to sodium fluoride no matter the enzyme source (Figure 6). However, sodium salts of chlorine and bromine had little, if any, impact on activity. Both LMCO preparations retained less than 0.1% of their initial activity in the presence of 3.2 mM azide, and the I_{50} for sodium azide was 37μM for either enzyme. For samples treated with sodium chloride, fluoride, or bromide, but not azide, enzyme activity was completely recovered by extensive dialysis at 4°C.

As determined from plots of activity versus substrate concentration (Figure 7), the Acer and transgenic tobacco LMCOs had essentially indistinguishable K_m values for pyrogallol (12.5 mM). K_m values were also determined for ABTS, hydroquinone, catechol and 4-methylcatechol (Table 2). Pyrogallol was the co-substrate for determining the K_m for oxygen, which proved to be approximately 0.02 mM for the LMCOs from both sources. This value was in agreement with a value previously published by Bligny et al. (Bligny and Douce, 1983).

During the acquisition of kinetic data it was noticed that reactions with certain phenolic substrates exhibited a slight increase in rate (hysteresis) during the first few seconds of the reaction. Further investigation found that both LMCO preparations exhibited this phenomenon to some extent; however, the rate with which the catechol reaction reached steady-state was unusually slow. For low substrate concentrations (1-10mM), it was necessary to discard the first 150 seconds of the reaction, and the rate of reaction was determined from the zero-order fit to the remaining time points in the linear portion of the trace. At higher substrate concentrations, the rate began to decay shortly after the onset of reaction due to rapid depletion of oxygen. Consequently, for high substrate concentrations the rate was calculated from the tangential curve at the point of maximum activity.

Discussion

Heterologous expression systems will be critical for the production of sufficient quantities of plant LMCOs that their biochemical and enzymatic characteristics can be determined. Plant LMCOs have not yet been expressed in an active form in any system other than transgenic plant cells. However, even in these in systems, care must be taken to make certain that studies performed with the recombinant enzyme reflect the situation with the enzyme

as it is produced in the native organism. It was immediately apparent from SDS-PAGE analyses there was a substantial mass difference between the LMCOs produced by Acer and transgenic tobacco cells (Figure 2), which raised the possibility that the recombinant enzyme might exhibit substantially different characteristics than that recovered from *Acer* cells. Mass spectrometry verified the mass difference as being approximately 7.2 kDa. Amino-terminal sequencing confirmed that the signal sequence was correctly cleaved from the recombinant enzyme and no aberrant truncation had occurred at this end of the polypeptide. If the apparent 7.2 kDa mass shift resulted from a truncation at the carboxyl-terminal end of the polypeptide, it would require the removal of approximately sixty-one amino acids. A highly conserved, copper-binding domain is located thirty-five amino acids from the C-terminus (Figure 8), and loss of this binding domain through truncation would render the enzyme completely inacive. Because the recombinant protein retained its enzymatic activity it was hypothesized that the mass difference must have resulted from a difference in post-translational modification, mostly likely glycosylation.

The LMCO produced by suspension-cultured *A. pseudoplatanus* cells is known to be highly glycosylated, and an in-depth study of the structure of the glycosyl moieties identified a number of unique structures (Takahashi and Hotta, 1985). Further study showed that addition heterogeneity is added to

these structures through trimming and incomplete capping (Tezuka et al., 1993). In our study, comparison of the known mass of the polypeptide with the mass of the enzyme purified from *Acer* cells, as determined by MALDI-MS, indicates that 33.5% of the protein mass is carbohydrate. For the recombinant enzyme this figure drops to 27.7% carbohydrate. This suggests that 11 or 12 of the 16 potential sites for N-linked glycosylation are likely occupied on the protein produced by *Acer* cells (LaFayette et al., 1995).

In *Acer* cell suspension cultures there is evidence for LMCOs that differ in predicted mass by less than 100 daltons. Such a mass difference is far smaller than can be resolved by common analytical techniques used to asses the purity of a protein This situation has been further complicated by the tendency of highly glycosylated enzymes to produce somewhat smeary bands in SDS page gels and broad humps in MALDI-MS.

MALDI-MS analysis of the LMCO purified from *Acer* cells indicated the presence of multiple polypeptides differing in mass by less than 100 Da (*data not shown*). While some of these forms might be attributable to glycosyl modifications, such as those previously described (Tezuka et al., 1993), it seems likely that polypeptides corresponding to different LMCO isoenzymes also made a contribution. In fact, since glycosyl chains do not typically add

charges to modified proteins, the Mono-S elution profile depicted in Fig. 4 suggests that at least 4 or 5 major LMCO isozymes are secreted by *Acer* cells.

Until such time as additional *Acer* LMCO genes are cloned and expressed in transgenic tobacco cells we cannot say whether the isoenzymes differ significantly in their kinetic characteristics. Catechol, resorcinol and hydroquinone represent the three possible isomers (ortho-, meta-, and para-substituted) forms of dihydroxybenzene. While the ortho- and para-substituted forms are generally excellent substrates for LMCOs, the meta-substitutes isomer is generally not. The two enzyme preparations had roughly the same affinity for the ortho-substituted isomer, catechol, as well as several other substrates, which suggests that the previously mentioned glycosyl modification has not did not greatly impact the active site of the recombinant enzyme with respect to substrate binding. However, the 3-fold difference displayed by the two LMCO preparations with respect to their K_m values for hydroquinone (1,4-dihydroxybenzene) suggests that significant differences may exist between isoenzymes for particular substrates. Whether such substrate discrimination, if it exists in intact A. *pseudoplatanus* trees, carries a physiological significance remains to be seen.

Table 1

Native LMCO

	Act. ODS/min. ml	Vol. Mls	Toal Act. Ods/Min.	Protein mg/µl	Total protein mg	Specific Act.*	Fold Purified
Crude	2.69	1500	4035	16..4	24.6	4.6	1
Ammonium Sulfate	2.36	1800	4248	9.2	16.6	7.1	1.5
Butyl Column	10.48	405	4200	40.0	16.2	7.3	1.6
DEAE Column	51.41	60	3085	166.0	10	8.5	1.9

Recombinant LMCO

Crude	0.59	3980	2356	21.9	87.16	0.8	1.0
Ammonium Sulfate	0.57	4100	2348	16.8	68.8	0.9	1.3
Butyl Column	6.51	270	1758	184.5	49.8	1.0	1.3
DEAE Column	62.47	25.5	1592	98	2.49	17.7	23.6
MONO-S Cloumn	200	6.5	1270	222	1.44	25.0	35.0

* $\mu m \cdot Min^{-1} \cdot mg^{-1}$

Table 2

	Native LMCO		Recombinant LMCO	
Substrate	Km	SEM	Km	SEM
Catechol*	20.2	4.1	21.8	3.97
Resorcinol	**		**	
Hydroquinone*	30.2	1.7	104.1	3.1
4-Methylcatechol	5.5	0.63	4.92	0.9
ABTS	17	1.6	14.3	0.95

*value determined from maximal rates under test conditions
** no activity

Figure 1

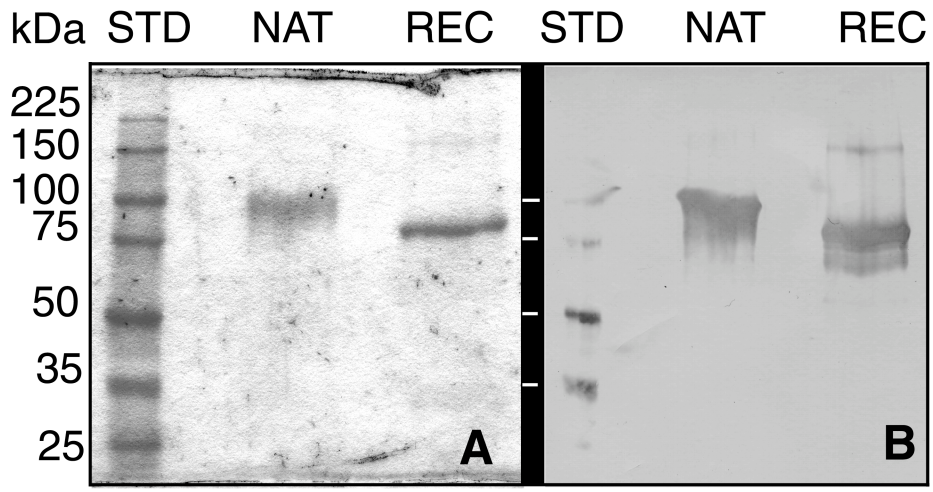

Figure 1SDS-PAGE analysis of purified native and recombinant Acer LMCOs. Panel A shows a gel stained for protein. Panel B shows a western blot of an identical gel. Primary monoclonal antibodies raised against deglycosylated purified native *Acer* LMCO

Figure 2

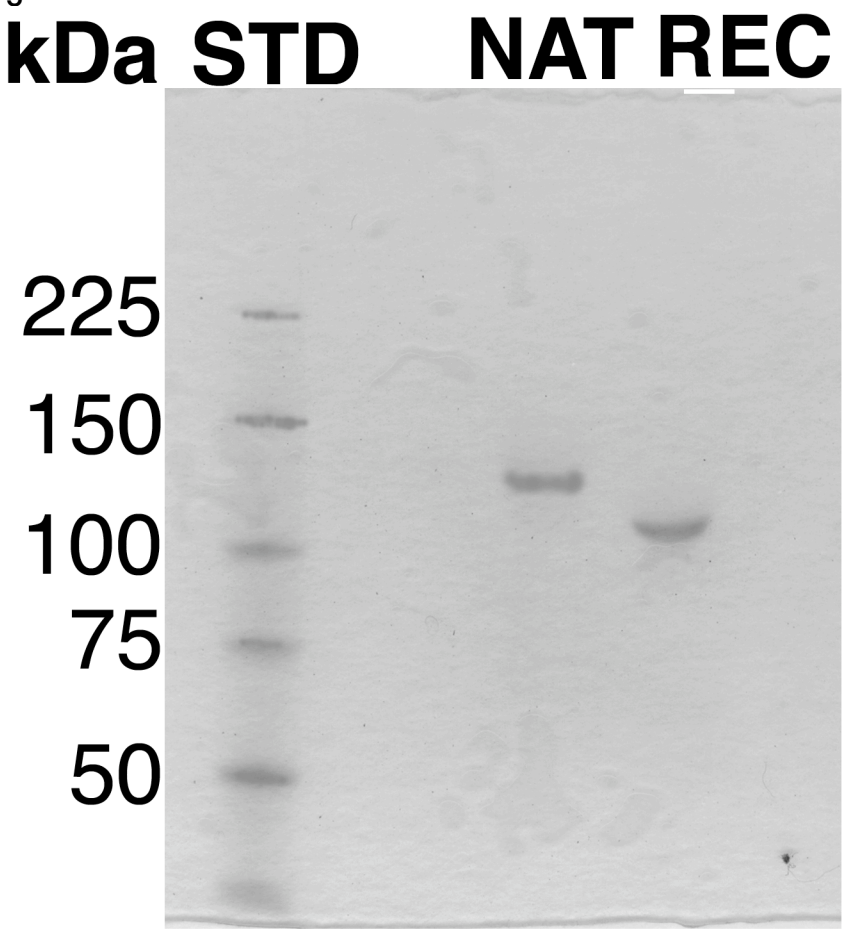

Figure 2 Semi-Native SDS-PAGE gel loaded with 1.5 µg each of native and recombinant LMCO. Gel stained with coomassie.

Figure 3

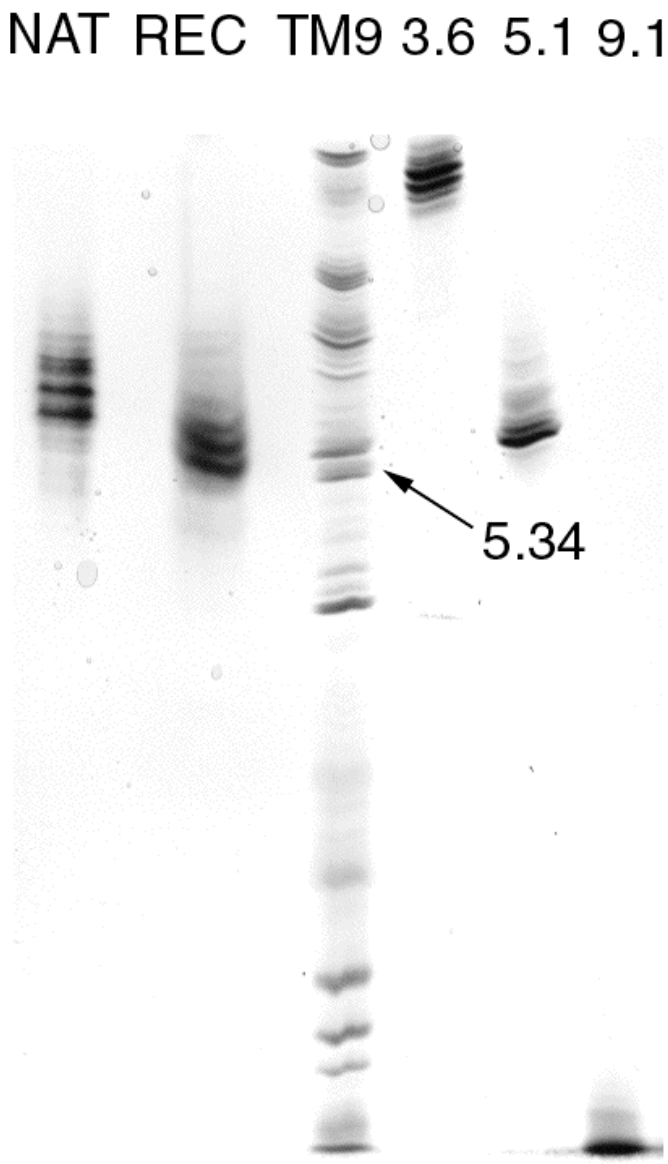

Figure 3 IEF gel stained with coomassie lane 1 and 2 contain 10µg of purified native and recombinant LMCO respectively. Lane 3 contain 15 µl of Serva Test Mix 9 IEF standards. Lanes 4-6 contain 20µg of Trypsinogen, B-Lactoglobuli, and Amyloglucosidase respectively.

Figure 4

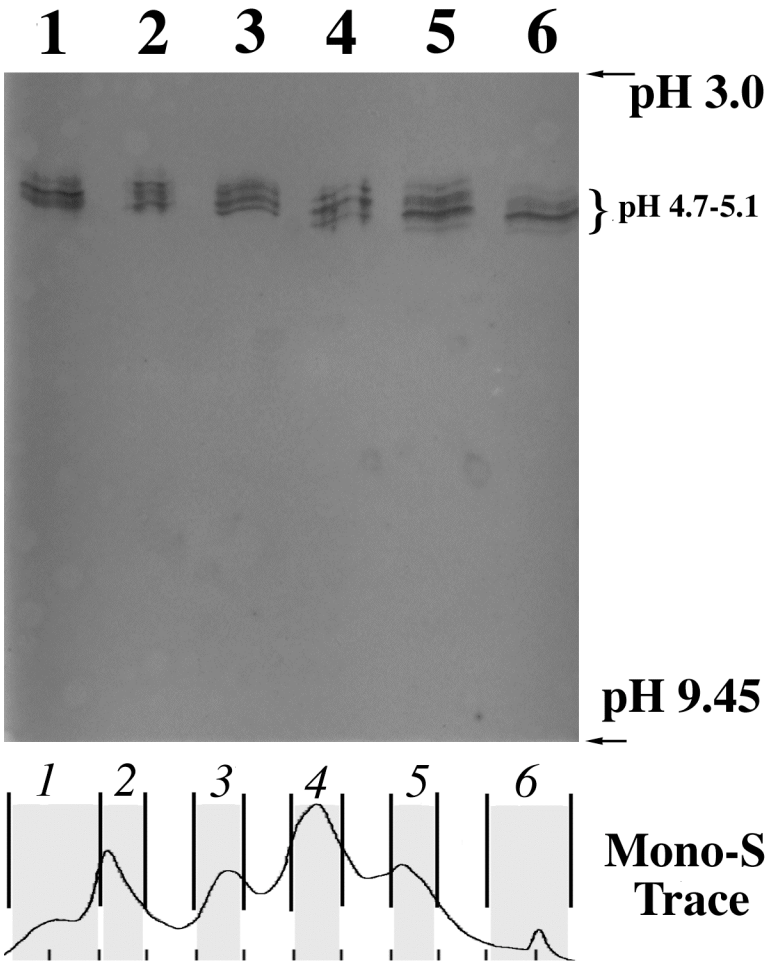

Figure4 IEF gel of native *Acer* LMCO stained with coomassie fractionated by Mono-S column. Lanes 1-6 contain pooled fractions from the Pharmacia mono-S coloumn run loaded with equivalent activity .

Figure 5

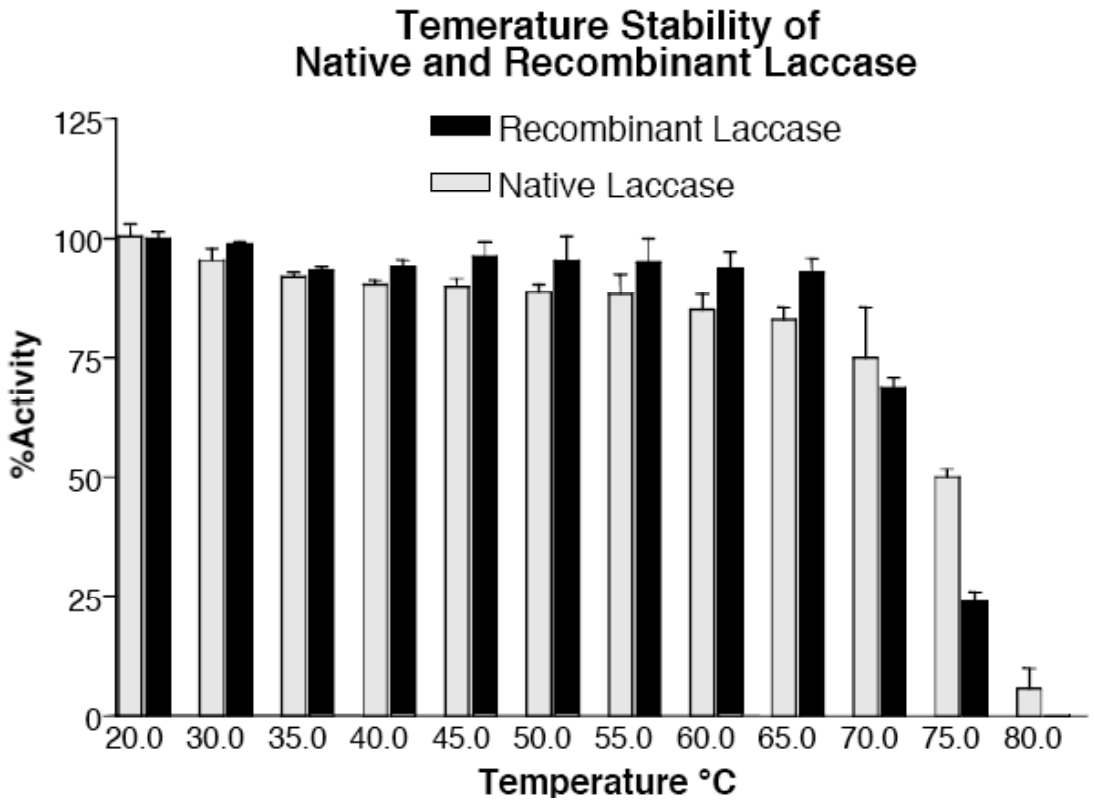

Figure 5 Graph representing percent activity of native (grey) and recombinant (black) LMCO after a 10 min. exposure to the temperature on the X axis.

Figure 6

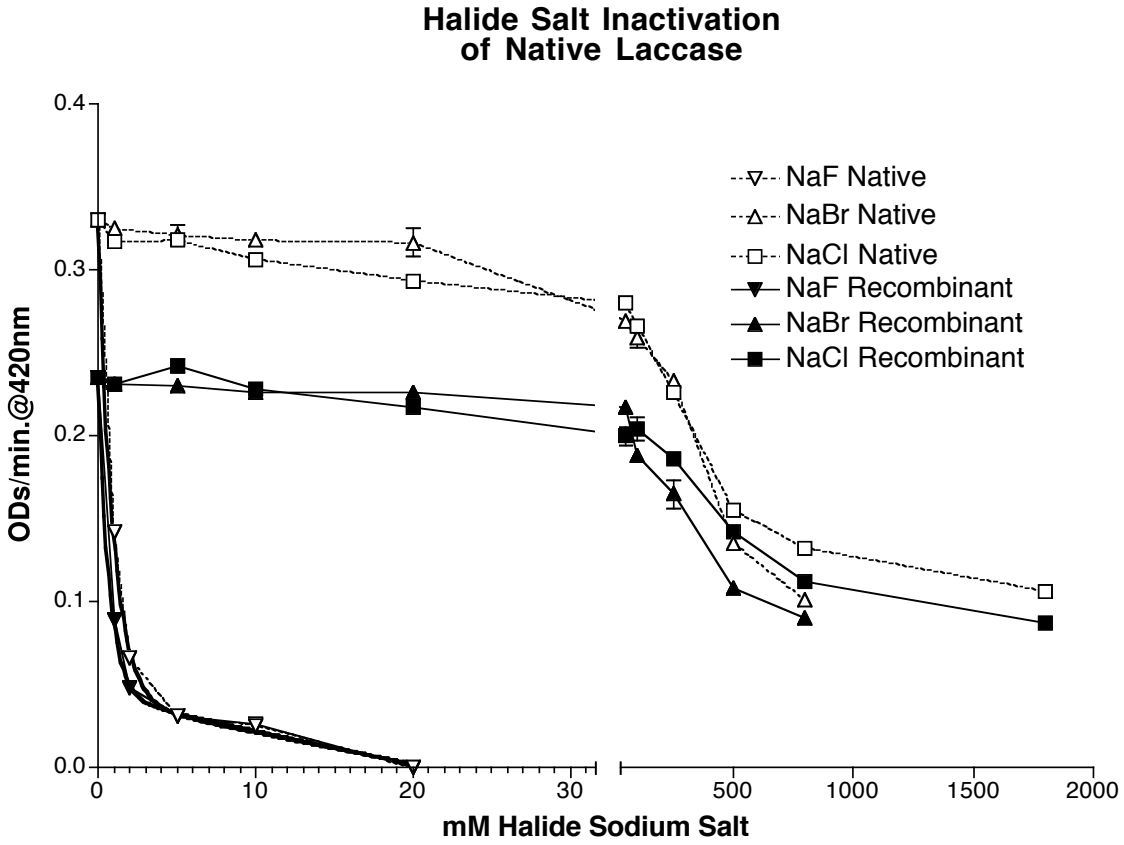

Figure6 Rate of LMCO in the presence of various halide salts. Solid lines represent the recombinant LMCO while the doted line represents the native LMCO. All reactions were carried out at 30°C and monitored by the increase in absorbance of 420 nm cause by oxidation of ABTS by the LMCO

Figure 7

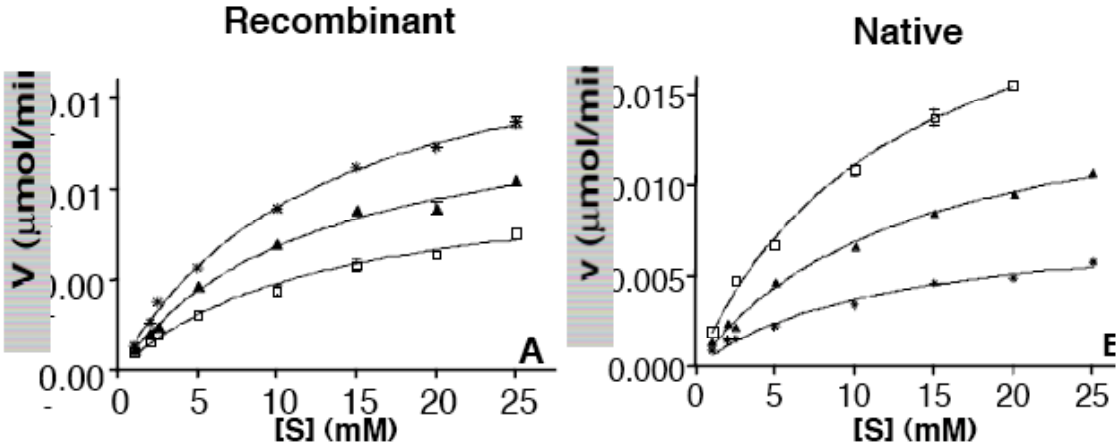

Figure 7 Lines represent non-linear fits of the Mchaelis-Menten equation using a lest squares y weighted fit. The reported kM is an average of the three fits shown. All data collected as increase in absorbance of pyrogallol at 450 nm. Reactions carried out at 30°C in triplicate.

Figure 8

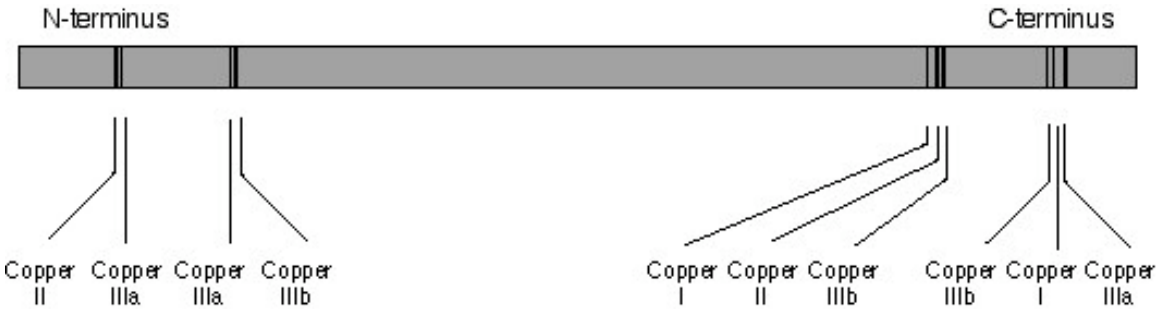

Figure 8 This is a scale schematic of a LMCO primary sequence. Black line represent conserved histidines involved in copper binding. The number associated with each histidine denote which copper it interacts with. It should be noted that these numbers represent the coordination geometries. The residue immediately preceding the first copper site is thirty two residues from the N-terminus. The residue immediately following the last copper site is nineteen residues from the C-terminus.

CHAPTER 4

Ferroxidase Activity in a Laccase-like Multicopper Oxidase from Yellow-poplar (*Liriodendron tulipifera* L.)[1]

[1] Hoopes etal. Plant Physiology and Biochemistry 42 (1): 27-33 (2004)
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Abstract

Ferroxidase activity was detected in a laccase-like multicopper oxidase (LMCO) produced in transgenic tobacco cells expressing a LMCO cDNA (Lt*lacc*2.2) cloned from yellow-poplar (*Liriodendron tulipifera*). This marks the first report of ferroxidase activity associated with a plant laccase, and suggests that some members of this plant enzyme family may have physiological functions based on activities other than their more widely recognized phenoloxidase activity. Recent work with laccase-like multicopper oxidases from bacteria, yeast and mammals has shown that metal oxidase activities in these enzymes can be important for their primary physiological functions. With respect to ferroxidase activity in certain plant LMCOs, it is proposed that the high levels of LMCO expression in plant vascular tissues may reflect the need for high-efficiency iron uptake pumps in tissues that undergo lignification during normal development. Such iron uptake pumps would function to minimize levels of free iron so that reactive oxygen species do not reach toxic levels when H_2O_2 is generated for peroxidase-mediated monolignol coupling during lignin deposition.

Introduction

Laccase (EC 1.10.3.2), a copper-dependent phenoloxidase isolated from the sap of the Japanese lacquer tree, *Rhus vernicifera* (Yoshida, 1883; Bertrand, 1894) was the first characterized member of the enzyme superfamily commonly referred to as the multicopper oxidases (MCOs) (Messerschmidt, 1997). Multiple catalytic copper centers and oxidative activity are the two hallmarks for members of the MCO superfamily, a group of enzymes that has grown rapidly in recent years to contain, in addition to laccase, such other enzymes as ceruloplasmin, tyrosinase, catechol oxidase, methane monooxygenase, sulochrin oxidase, dihydrogeodin oxidase, phenoxazinone synthase, and bilirubin oxidase (Mayer and Harel, 1979; Messerschmidt, 1997). Enzymes in the MCO family are diverse in both function and structure, and representatives have been identified in eubacteria, fungi, plants and animals (Messerschmidt, 1997). Although some of these enzymes oxidize specific, identified substrates, and have been named accordingly, most of the better known MCOs are capable of oxidizing multiple potential substrates, and this has created significant confusion in enzyme nomenclature (Mayer and Harel, 1979; Mayer, 1987). Strictly speaking, the name laccase should be reserved for those members of the MCO family that are found in plant saps containing the unsaturated alkylcatechols ("laccol," also known as "urushiol")

that are their natural substrates (Du et al., 1984; Du et al., 1984). However, the name has frequently been used to identify any MCO that contains four copper atoms in a specific arrangement of Type-1, Type-2 and Type-3 electromagnetic centers (Messerschmidt, 1997). In recognition of the multiplicity of functions likely performed by the numerous MCOs appearing in plant genomes, we have elected to abandon our previous use of "laccase" to cover all monomeric, four-copper MCOs in favor of "laccase-like multicopper oxidases" or "LMCOs." Following the traditions of enzyme nomenclature, more descriptive names may be assigned once specific catalytic reactions and metabolic functions are defined for the various LMCOs.

LMCOs have been identified in bacteria, fungi and animals(Messerschmidt, 1997), where they function in metal oxidation (de Silva et al., 1995; Brouwers, 1999; Kim et al., 2001) and lignin degradation (Eggert et al., 1996), as well as detoxification of antimicrobial agents (Mayer et al., 2001). Additional LMCOs have been cloned and characterized from several plant species (LaFayette et al., 1995; Kiefer-Meyer et al., 1996; LaFayette et al., 1999; Sato et al., 2001), yet their physiological functions in these organisms remain unclear (Boudet, 2000). It has been argued that these enzymes may be involved in lignin deposition by virtue of their ability to polymerize monolignols (Sterjiades et al., 1992), their association with plant

vascular tissues (Bao et al., 1993; Liu et al., 1994), and the impact that antisense laccase gene expression has on xylem tissue formation (Ranocha et al., 2002). Yet, definitive evidence demonstrating a role for these enzymes as primary catalysts of lignin polymerization remains elusive.

Saccharomyces cerevisiae uses a pair of LMCOs, encoded by the *fet*3 and *fet5* genes, as components of high-affinity iron uptake systems that regulate flux of this critical metal through the plasmalemma and vacuolar membrane, respectively (Askwith et al., 1996; Urbanowski and Piper, 1999). Like many organisms, *S. cerevisiae* acquires most of its iron in the ferrous state via a relatively non-specific divalent metal transporter (*fet4*) (Dix et al., 1994). Yet, when external conditions, such as elevated levels of copper or zinc, lead to unbalanced uptake of divalent metals, expression of the MCO-dependent Fet3 system is induced. The LMCO in this iron "pump" oxidizes the divalent ferrous ion to the trivalent ferric form, which is immediately passed through the membrane by an associated permease (Stearman et al., 1996). The specificity of this iron uptake system appears to derive at least in part from the paucity of trivalent metals ions circulating in the environment, and it enables yeast to balance iron intake against that of other divalent metal ions. Due to its higher affinity for iron than the Fet4 system, the Fet3 system is also critical for survival

of yeast when extracellular iron concentrations drop to very low levels (de Silva et al., 1995).

Discovery of the LMCO-dependent iron uptake systems in yeast has had a profound impact on our understanding of eukaryotic iron metabolism in general (Aisen et al., 2001). For example, the ferroxidase activity associated with ceruloplasmin, an abundant MCO found in mammalian blood serum, has been proposed to work in conjunction with circulating transferrin to minimize levels of circulating "free" iron, thereby limiting the generation of reactive oxygen species (ROS) (Harris et al., 1995). Subsequent work has identified hephaestin, the MCO component of an iron pump that appears to be critical for moving iron from intestinal enterocytes into the circulatory system (Vulpe et al., 1999).

This study marks the first demonstration of a ferroxidase activity associated with a plant LMCO. The enzyme in this case is normally expressed in the lignifying xylem tissue of a deciduous hardwood tree, yellow-poplar (*Liriodendron tulipifera*). On the basis of this enzyme's ferroxidase activity, we discusses how its potential involvement in an iron uptake system could represent an alternative to monolignol polymerization as the physiological function for these enzymes in higher plants.

Results

Heterologous expression of laccase

Previous studies from this lab and others have shown that multiple LMCO genes are expressed simultaneously in lignifying plant tissues (Kiefer-Meyer et al., 1996; LaFayette et al., 1999; Sato et al., 2001). In one of these, four LMCO cDNAs (Lt*lacc2.1, 2.2, 2.3, 2.4*) from the lignifying xylem tissues of yellow-poplar (*L. tulipifera*) were cloned and expressed in suspension-cultured transgenic tobacco cells (LaFayette et al., 1999) to make possible the independent characterization of the individual gene products. Unfortunately, in contrast to the stable transgene expression found in tobacco cells transformed with a constitutively expressed LMCO gene from sycamore maple (*Acer pseudoplatanus*) (Dean et al., 1998), the levels of enzyme activity in tobacco lines transformed with constructs of the yellow-poplar genes under control of a dual CaMV 35S promoter declined rapidly during extended culture. In cell lines receiving the yellow-poplar constructs, activity dropped to nearly undetectable levels within six months of the initial transformation event. Continued presence of the LMCO transgenes in cell lines that lost expression was confirmed by PCR analysis of genomic DNA, but RT-PCR showed that gene transcription was lost over time in culture (*data not shown*). These results were consistent over three independent transformation experiments and more than

sixty cell lines that initially expressed one or the other of the four yellow-poplar LMCOs. Adding to the difficulty this presented for obtaining sufficient protein for enzyme characterization, the growth rate (g fresh weight·wk^{-1}) of transformed cell lines appeared to be inversely proportional to the level of yellow-poplar LMCO activity initially expressed. Those cell lines that expressed the yellow-poplar LMCOs at the highest levels generally grew at no more than half the rate of cells lines transformed with empty vector controls. This was in contrast to tobacco cell lines transformed with the sycamore maple LMCO gene, which continued to grow at the same rate as untransformed cells. These results suggested that the activities of the constitutively expressed yellow-poplar LMCOs might be impeding normal cellular functions in the transformed cell lines.

For the current study, tobacco cells were again transformed with the dual 35S:Lt*lacc*2.2 construct for expression of the yellow-poplar enzyme, and kanamycin-resistant cells lines were selected within 6-8 weeks of the bombardment. Cell lines expressing the LMCO transgene were identified by their ability to oxidize a phenoloxidase substrate (0.1% aqueous ABTS) applied directly to the surface of calli. The cells did not release diffusible LMCO activity into the surrounding medium, as was the case for cells expressing the sycamore maple (Ap*lacc1.1*) gene (Dean et al., 1998). The cell

line expressing the highest level of phenoloxidase activity was expanded to production-scale (300 g cells·week^{-1}) within 12 weeks of the original transformation event.

Detection of ferroxidase activity:

Lysates from harvested and pooled cells were concentrated and partially purified by a combination of ammonium sulfate precipitation and ultrafiltration. Overall yield of LMCO phenoloxidase activity from 900 g of transformed cells was 0.12 units. Ferroxidase activity was detected in the concentrated extract using the ferrozine assay described in the Methods section. The specific ferroxidase activity in the concentrated enzyme pool was 18.3 nkatal·mg^{-1} protein and the apparent K_m of the enzyme for Fe^{2+} was 40 μM. The apparent K_m of the yeast FET-3 protein for Fe^{2+} is about 5 μM (Hassett et al., 1998), while ceruloplasmin displays nontypical, diphasic kinetics with respect to Fe^{2+} such that two K_m values, 0.6 μM and 50 μM, were determined for high (210 nM) and low (21 nM) concentrations of the enzyme, respectively (Osaki, 1966). The K_m values for ceruloplasmin ferroxidase activity seem particularly relevant for comparison with the yellow-poplar LMCO given that these two enzymes function within the relatively iron-rich milieu of living organisms, while the yeast enzyme must deal with naturally low levels of iron that exist in the

environment. In addition, limited amounts of the partially purified yellow-poplar enzyme prevented testing of kinetic parameters at high enzyme concentrations.

Zymogram analysis showed that the ferroxidase and phenoloxidase activities of the yellow-poplar LMCO co-localized in SDS-PAGE gels (Fig. 1). In this gel system, the Lt*lacc2.2* LMCO migrated significantly faster than the Ap*lacc1.1* LMCO, but not quite as quickly as the *E. coli yacK* gene product. Gels stained for phenoloxidase activity in the presence of 5 mM H_2O_2 showed no detectable increase in band intensity or the appearance of additional bands in any of the lanes, verifying that peroxidases were not responsible for any of the bands detected (*data not shown*). The multiple bands of ferroxidase activity for the *E. coli* enzyme appeared to be artifacts of the gel system since samples subjected to heat denaturation ran as a single band detectable by protein staining.

Structural modeling of laccase:

ClustalW alignment of amino acids encoded by Ap*lacc1.1*, Lt*lacc2.2* and part of the yeast Fet3 protein (truncated to remove the membrane spanning domain that is absent from the other LMCOs) illustrated the high degree of homology between these proteins (Fig. 2). Comparison of the amino acid residues between the two plant MCOs showed 41% identity and

60% similarity, while comparisons of either plant sequence with the yeast sequence found about 20% identity and 35% similarity. A similar alignment of the Ltlacc2.2 protein with a laccase from C*oprinus cinereus*, for which a crystal structure is available (Ducros et al., 1998), was used to generate the structural model shown in Fig. 3.

Discussion

This is the first report of ferroxidase activity associated with an LMCO from higher plants. Two independent studies recently reported that a ceruloplasmin-like MCO is involved with high-affinity iron uptake in the green alga, *Chlamydomonas reinhardtii* (Herbik et al., 2002; La Fontaine et al., 2002). Thus, it may well be that MCO-dependent iron uptake systems are as broadly distributed across the plant kingdom as they are across other eukaryotes.

From the time that the first plant LMCO gene was cloned (LaFayette et al., 1995), sequence comparisons, such as that depicted in Fig. 2, have suggested it would be difficult to distinguish LMCOs whose primary physiological function was to oxidize phenolic substrates from those that oxidize iron or other metals. Such distinctions would be impossible to draw using plant extracts that contain multiple LMCO isozymes. Although previous attempts in this lab to demonstrate ferroxidase activity in the plant LMCOs from

A. pseudoplatanus and *Rhus vernicifera* were unsuccessful, Fig. 1 clearly shows that both phenoloxidase and ferroxidase activities are associated with the Lt*lacc*2.2 gene product. Thus, plant LMCOs are capable of catalyzing the same range of enzyme activities noted for MCOs in other organisms.

Unlike the sycamore maple (Aplacc1.1) enzyme, which was secreted by transgenic tobacco cells (Dean et al., 1998), yellow-poplar and *Zinnia elegans* LMCOs expressed in transgenic tobacco cells were not released by protoplasting, but only by complete cell rupture (J.F.D. Dean, unpublished data). Since the yellow-poplar LMCO was fully soluble in cell lysates, the enzyme must have been retained in an intracellular compartment of the transgenic tobacco cells. If this intracellular localization results in a Ltlacc2.2 enzyme having little or no glycosylation, it could help explain the observation that in SDS-PAGE gels the Ltlacc2.2 enzyme had a migration rate closer to the *Escherichia coli yac*K MCO than the Aplacc1.1 enzyme (Fig. 1). The mature peptides of these three enzymes are roughly equivalent in size (552, 488, and 542 amino acids for the yellow-poplar, *E. coli* and sycamore maple enzymes, respectively. The *E. coli* enzyme has a single potential glycosylation sites and is not glycosylated, while the sycamore maple LMCO has 17 potential glycosylation sites and is highly glycosylated (Takahashi and Hotta, 1985; LaFayette et al., 1995). The yellow-poplar LMCO sequence contains 14

putative glycosylation sites, but the amount of purified enzyme was insufficient to determine the glycosyl content of the heterologously expressed protein. It should be noted, however, that the zymogram gel system used in this study does not heat-denature proteins before running, and the migration rates of proteins in such a gel format cannot be strictly compared. As these observations were made with transgenic enzyme expressed heterologously in tobacco cells, it cannot be said for certain whether or not the yellow-poplar LMCO is normally localized intracellularly, or whether it is highly glycosylated when expressed in yellow-poplar xylem.

Sequence alignment and modeling of the Ltlacc2.2 LMCO identified a region of the protein that may be influential in conferring ferroxidase activity to these enzymes (Fig. 3). The loop containing residues 476-486 in the Ltlacc2.2 sequence is highly homologous to a region in the yeast Fet3 MCO (residues 409-414) that modeling has identified as a potential interaction site for iron (J.T. Hoopes and J.F.D. Dean, *unpublished*). Specifically, Fe2+ may be interacting specifically with an aspartyl residue in the sequence QDT*H, where the H is a liganding residue for the Type-1 Cu center and asterisks represent a short, variable stretch of amino acids. This residue is a target for site-directed mutagenesis studies to understand how these enzymes discriminate between substrates.

The possibility that plant LMCOs could function as components of iron uptake pumps provides an alternative rationale for their strong expression in lignifying vascular tissues (Fig. 4). Such pumps may be required in these tissues to reduce extracellular free iron to levels where it is not readily available to interact with the H_2O_2 that must be generated for peroxidase-catalyzed monolignol coupling (Harkin and Obst, 1973). The (Fenton) reaction between Fe^{2+} and H_2O_2 generates highly toxic reactive oxygen species, $OH\cdot$ and OH^-, particularly when reducing agents, such as ascorbate, are available to cycle iron back to Fe^{2+}. These radicals can cause severe damage to membranes, proteins and DNA, and given the close proximity of dividing meristematic cells in the cambium to lignifying cells, it seems reasonable that there should be mechanisms to keep free iron levels very low in these tissues.

The involvement of vascular tissue LMCOs in a fundamental metabolic process, such as iron metabolism, would mean that disrupting their expression via gene manipulation could lead to a wide variety of phenotypic outcomes in transgenic plants. Disrupted expression of LMCOs involved in iron metabolism could impact lignin levels by affecting heme biosynthesis and peroxidase assembly. However, overall poor growth and leaf chlorosis, as is often observed in plants grown under iron deficient conditions, could be expected in knockout mutants of such LMCOs. Such a phenotype has been seen in at least

one antisense laccase mutant generated in *Arabidopsis thaliana* (C. Halpin, *personal communication*). Further studies will be required to demonstrate a direct link between plant LMCOs and iron metabolism.

Methods

Tissue-culture:

Preparation of the plant expression vector in which the Lt*lacc*2.2 LMCO cDNA was placed under the control of a dual-enhanced CaMV 35S promoter was described previously (LaFayette et al., 1995). Transformations of *Nicotiana tabacum* L. Cv. Bright Yellow 2 (BY2) cells by micro-projectile bombardment using either the Lt*lacc*2.2 expression construct or the empty vector (pKYLX80) control were performed as previously described (LaFayette et al., 1995). For production of transgenic protein, suspension cultures of tobacco cell lines transformed with the LMCO expression construct or the empty vector control were grown in 2.8 L Fernbach flasks containing 500 mL of medium. The growth medium consisted of a commercial preparation of Murashige-Skoog salts (Life Technologies, Bethesada, MD) containing 88 mM sucrose, 2.7 mM potassium monophosphate, 343 μM kanamycin sulfate, 156 μM myo-inositol, 3 μM thiamine HCl, and 0.8 μM 2,4-D. The medium pH was

adjusted to 5.7, and cells were grown at 24 °C in the dark with shaking at 110 rpm. The lines were subcultured every seven days using a 1/10 (v/v) inoculation into fresh medium. Cells for enzyme extraction were harvested seven days after transfer to fresh medium.

Enzymes and reagents:

The laccase-like MCO from A. *pseudoplatanus* (Ap*lacc1.1*) was purified from suspension-cultured cells as previously described (Yoshida, 1883). The Ltlacc2.2 LMCO was concentrated and partially purified as follows. Suspension-cultured cells (900 g) were harvested by filtration and resuspended (1.6 $g \cdot mL^{-1}$) in chilled extraction buffer consisting of 50 mM sodium acetate (pH 6.0), 10 mM NaCl, 1 mM $CuSO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% (v/v) glycerol. Cells were homogenized, and insoluble debris was removed by centrifugation at 4 °C and 20,000 x G for 30 min. The supernatant was filtered through Miracloth (Calbiochem-Novabiochem Corp., San Diego, CA) and brought to 40% saturation with slow addition of solid ammonium sulfate. The solution was stirred overnight at 4 °C, and insoluble material was removed by centrifugation at 4 °C and 20,000 x g for 30 min. The supernatant was filtered through Miracloth, mixed with an equivalent volume of water, and PMSF was added to a final concentration of 1 mM. The

enzyme pool was then concentrated 140-fold against a 10 kDa molecular mass cutoff ultrafiltration membrane, and stored at –80 °C until use. Purification of the *E. coli yac*K gene product, an MCO having ferroxidase activity, was as described elsewhere (Kim et al., 2001). All other chemicals and reagents used in this study were of the highest purity commercially available and were used without further purification.

Protein and enzyme assays:

Protein was quantified using the ELS protein assay kit (Boehringer Mannheim, Indianapolis, IN) against BSA standards. Phenoloxidase activity for each LMCO was determined from the increase in absorbance at 420 nm due to the oxidation of 5 mM 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) in 100 mM sodium acetate buffer (pH 5.0). The molar absorptivity of oxidized ABTS (ε_{420}) was taken as 36 $mM^{-1} \cdot cm^{-1}$. Ferroxidase activity was determined using ferrous sulfate as the electron donor and 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a specific chelator to bind ferrous iron remaining at the end of the reaction. Reactions were carried out in disposable cuvettes containing 100 μM ferrous sulfate in 100 mM sodium acetate buffer (pH 5.0). The reactions were quenched by addition of ferrozine to a final concentration of 1.5 mM, and the rate of Fe^{2+}

oxidation was calculated from the decreased absorbance at 560 nm using a molar absorptivity of e_{560}= 25,400 $M^{-1} \cdot cm^{-1}$ for the Fe^{2+}-ferrozine complex.

Gel electrophoresis:

SDS-PAGE based zymograms were performed using gels prepared according to the protocol of Laemmli (Laemmli, 1970). Aliquots of recombinant laccases, loaded on the basis of equal amounts of phenoloxidase activity, were mixed 1/1 (v/v) with 2X Laemmli sample buffer containing no reducing agents, and loaded directly onto gels without heat denaturation. After electrophoresis, gels were stained for phenoloxidase activity using 1,8-diaminonapthalene as described previously (Hoopes and Dean, 2001). Ferroxidase zymograms were performed as described by Yuan et al. (Yuan et al., 1995) with modifications as follows. Ferroxidase activity was detected by soaking the gel in 100 mM sodium acetate buffer (pH 5.0) containing 5% (v/v) glycerol, and 1 mM $CuSO_4$ for 1 h. Gels were subsequently washed in 100 mM sodium acetate buffer (pH 5.0) containing 0.2 mM $FeSO_4$ for 1 h. Gels were then washed twice with deionized water to remove surface iron, and incubated at room temperature for 8 h in a dark humidifying chamber. Cleared zones representing ferroxidase activity were visualized by applying 1.5 mM ferrozine in water dropwise to the surface of the gel. Gels developed in

approximately 10 min and were immediately documented using an Alpha Innotech ChemImager 4000 imaging system and AlphaEase software (Alpha Innotech Corp., San Leandro, CA).

Sequence analysis and modeling:

The mature sequences of the Aplacc1.1 (GenBank U12757), Ltlacc2.2 (GenBank U73104), and the *S. cerevisiae* Fet3 (GenBank L25090) proteins were aligned using the online ClustalW tool (Higgins et al., 1994) available through the Baylor College of Medicine server (dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html). The membrane-anchoring domain (115 amino acids) at the carboxyl-terminus of the Fet3 protein was removed prior to alignment. Box-shading to highlight conserved residues, as shown in Fig. 2, was performed using GeneDoc, ver 2.5 (www.psc.edu/biomed/genedoc/). The mature protein sequence of Ltlacc2.2 was aligned to the protein sequence of the laccase from *C. cinereus*, for which a crystal structure has been reported (Ducros et al., 1998), using the GAP alignment function of the GCG Wisconsin Package, ver. 10.2 (Pharmacopeia, Inc., Burlington, MA). The GAP initiation penalty was set at 12, but all other parameters were maintained at default values. Variations in gap elongation penalty did not seem to significantly alter the alignments. The

resulting alignment then used to generate a draft structural model for Ltlacc2.2 using the SWISS-MODEL server accessible through the Swiss-Pdb Viewer (www.expasy.ch/spdbv/). The resulting model was hand-refined at failure points, based on minimization of threading energy and number of identical residues, and resubmitted until a stable full-length model was identified.

Acknowledgements

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Figure 1

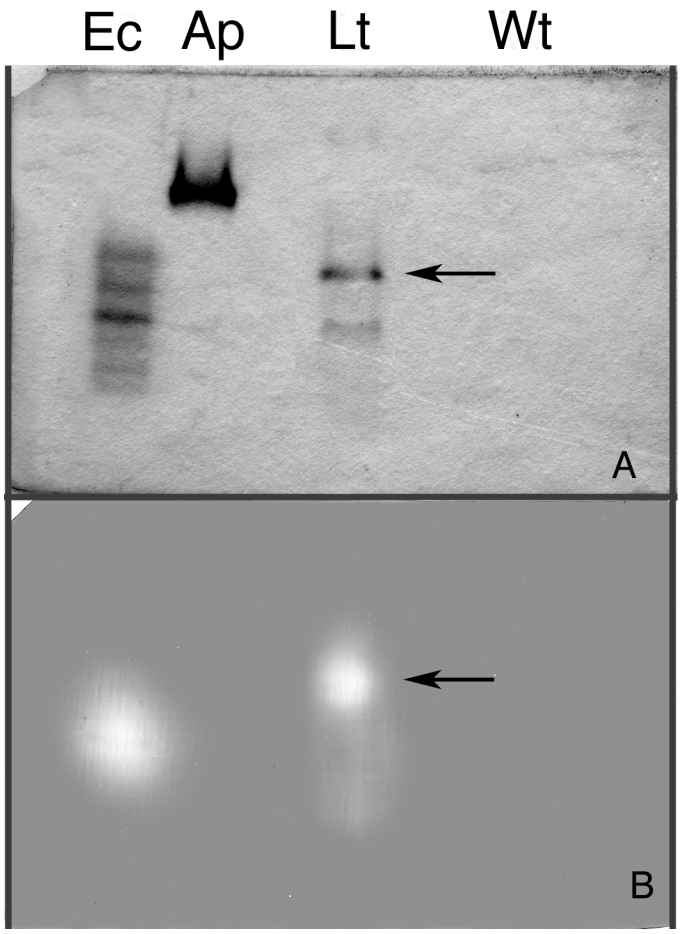

Figure 1. Activity staining of multicopper oxidases separated using SDS-PAGE. For phenoloxidase staining with 1,8-diaminonaphthalene (panel A), lanes Ec and Ap were loaded with equivalent amounts of ABTS-oxidizing activity from the *yac*K protein and Aplacc1.1 enzyme pools, respectively. Lane Lt was loaded with 200µg total protein from partially purified extracts of suspension-cultured tobacco cells transformed with the Lt*lacc*2.2 gene, while lane Wt was loaded with 200 µg total protein prepared in an identical fashion from tobacco cells transformed with the control vector. For detecting ferroxidase activity (panel B), the amount loaded for each sample was 5x that loaded for phenoloxidase staining. Arrows denote the position of the transgenic yellow-poplar enzyme.

Figure 2

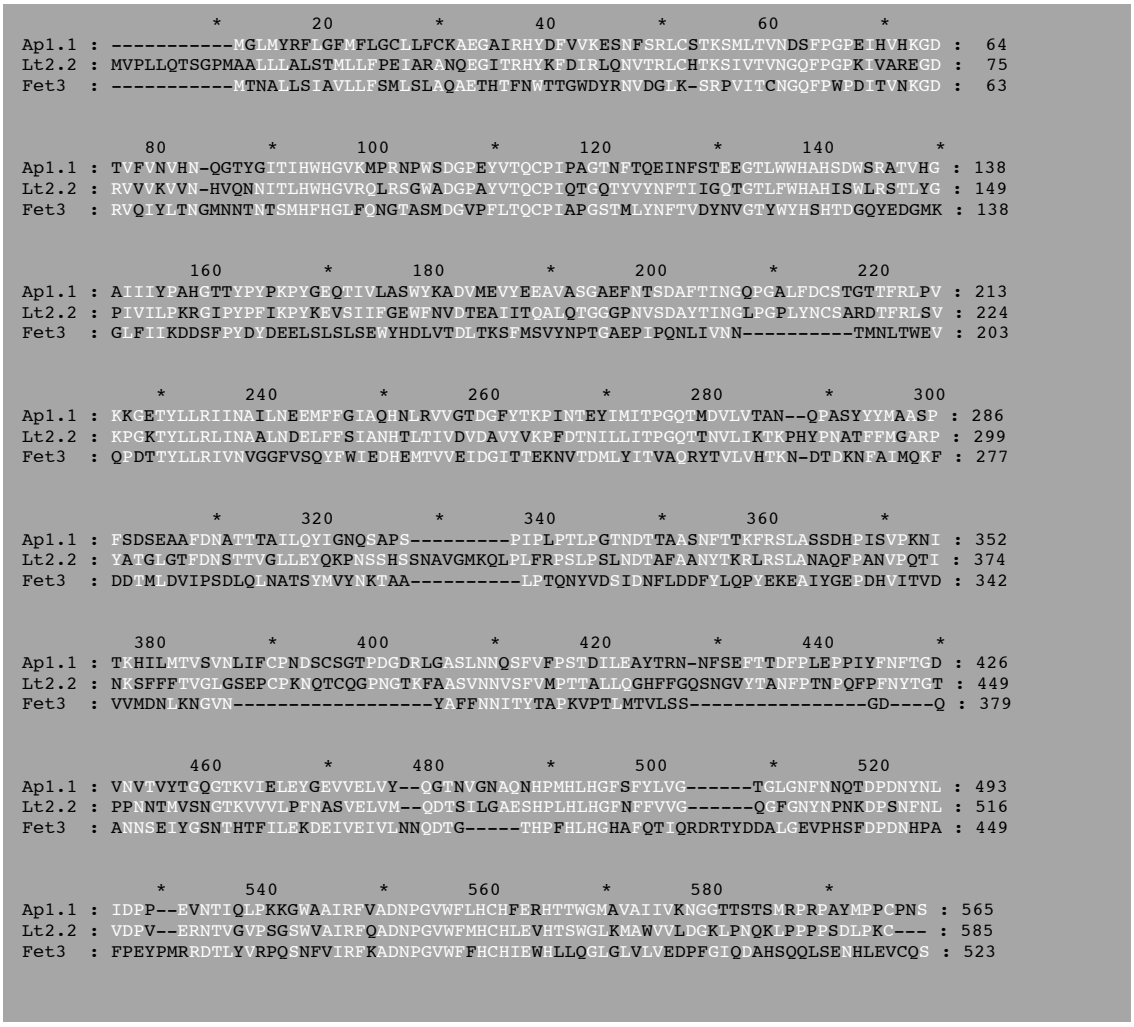

Figure 2. Amino acid sequences of two plant laccases aligned with the yeast Fet3 protein. ClustalW was used to align the complete protein sequences of the Ap*lacc1.1* and Lt*lacc2.2* gene products, as well as a Fet3 protein sequence from which the carboxyl-terminal 113 amino acid transmembrane domain had been deleted. Amino acids shaded in white are strongly conserved in LMCOs

Figure 3

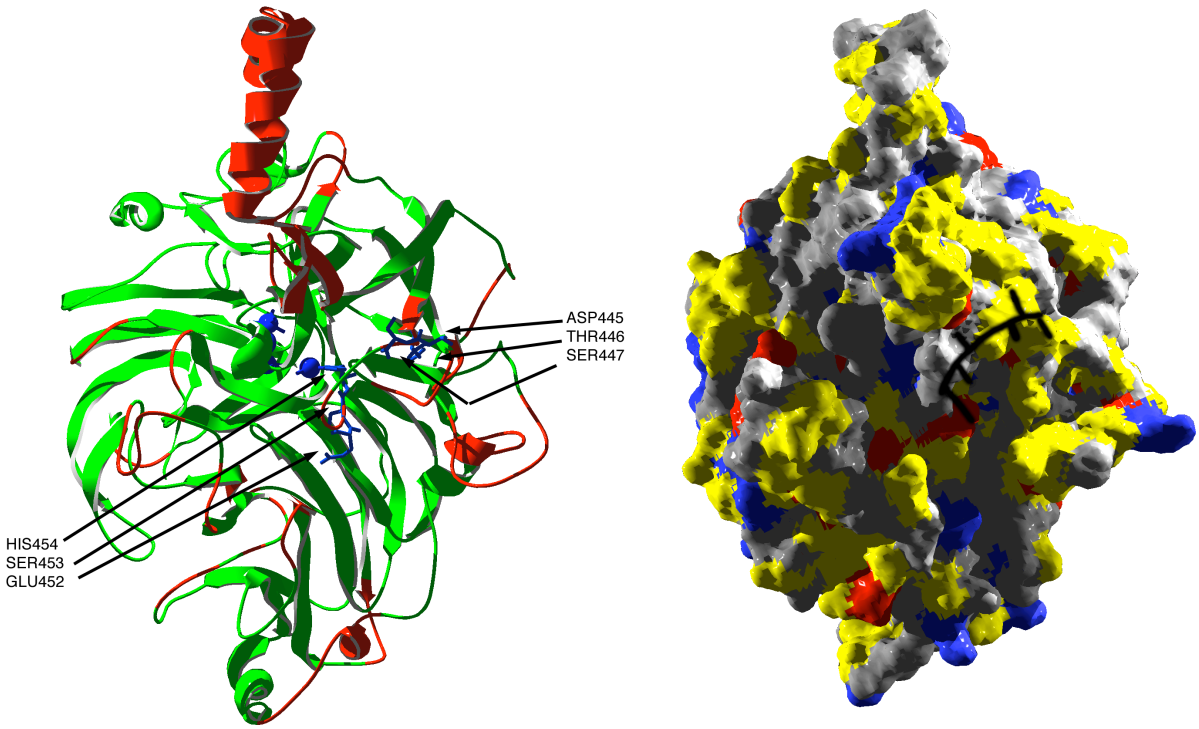

Figure 3. Structural models for the Ltlacc2.2 LMCO protein. A strand representation of the model noting critical amino acid residues near the Type-1 copper center is depicted on the left. The panel on the right shows a molecular surface of the model with neutral, polar, acid, and basic amino acids represented by gray, yellow, red and blue respectively. The curved black line indicates the approximate position of the residues labeled in the strand representation.

Figure 4

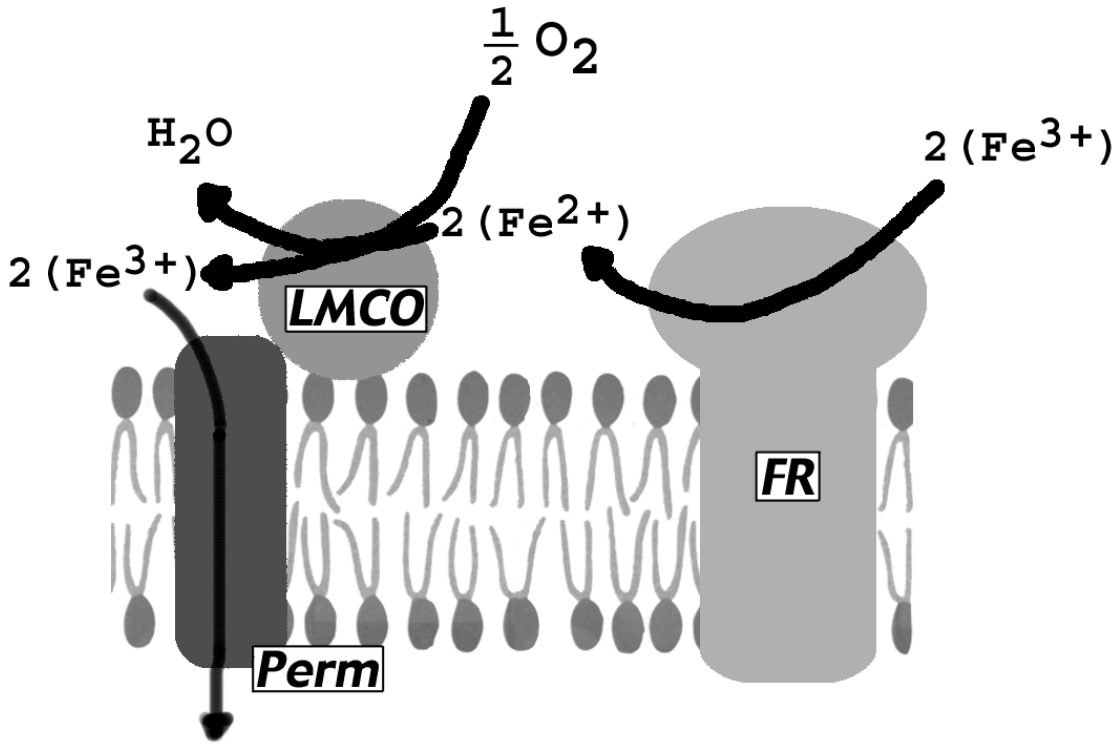

Figure 4. Generalized model for an LMCO-dependent iron uptake system in plants. In a system analogous to that described for *S. cereviseae* (Messerschmidt, 1997), iron reduced to its ferrous state via the action of ferrireductases (FR), is oxidized back to its trivalent, ferric form by the action of an LMCO. The marginally soluble ferric ion is then immediately passed through the membrane with assistance from a trivalent metal-specific, LMCO-associated permease (Perm).

CHAPTER 5

Homology Modeling of the Fet3 Multicopper Oxidase Identifies Active Site Residues Likely Involved in Ferroxidase Activity[1]

[1]Hoopes and Dean To be sumitted to *FEBS Letters*

1. Introduction

Multicopper oxidases (MCOs) have so far been described in organisms from all kingdoms but Archea (Hofer and Schlosser, 1999 Alexandre, 2000 #868 de Silva, 1997 #678), although recent genome annotation identifying an MCO (AAL63794) in *Pyrobaculum aerophilum* suggests that MCOs are likely to be broadly distributed in this kingdom as well. The best-studied member of this class of enzymes is "laccase," which is readily isolated from the sap of the Japanese lacquer tree (*Rhus vernicifera*). The plant enzyme, laccase, is characterized by a single polypeptide chain that organizes a highly conserved catalytic center containing four copper atoms organized so as to represent the three known types of organic copper electromagnetic centers. This fundamental structure is echoed in enzymes from across the phylogenetic kingdoms, and we have proposed the terminology "laccase-like multicopper oxidase" (LMCO) to describe other members of the family that do not employ as natural substrates the alkylcatechols (laccols) used by the enzyme in *R. vernicifera* sap (Hoopes and Dean, 2004).

In their catalytic reaction, LMCOs abstract a single electron from each molecule of donor substrate (oxidized substrate) and transfer a total of four electrons to diatomic oxygen (reduced substrate) to produce a fully reduced

product, water (Messerschmidt, 1993; Solomon et al., 1996). Oxygen intermediates of the reaction remain tightly bound in the active site through the end of the catalytic cycle. In general, LMCOs have the capacity to use many different compounds as donor substrates *in vitro*, but with exception of the Fet3 protein from *Saccharomyces cerevisiae* and the laccase from *R. vernicifera*, most physiological substrates for particular LMCOs remain unknown. Thus, even in the case of lignin degradation by fungi, it is remains unclear as to whether fungal LMCOs act directly on the lignin polymer or oxidize lignin substructures via redox intermediates (Eggert et al., 1995; Li et al., 1999).

The *S. cerevisiae* Fet3 protein functions as a ferroxidase using Fe^{2+} as the *in vivo* electron donor (Askwith et al., 1994; de Silva et al., 1995). The enzyme works in conjunction with an integral membrane permease, Ftr1, and the heterodimeric complex acts as a high-affinity iron uptake pump critical to survival of this organism under low-iron conditions (Askwith et al., 1996; de Silva et al., 1997; Askwith and Kaplan, 1998). Evidence suggests that similar iron uptake systems are widely distributed in eukaryotes, with ceruloplasmin, and its homolog, hephaestin, representing copper-dependent ferroxidases critical to iron metabolism in mammals (Vulpe et al., 1999; Li et al., 2003). Whereas ceruloplasmin and hephaestin are structurally more complex than typical LMCOs, with each containing more than the four catalytically reactive

cooper ions that define LMCOs, the Fet3 protein sequence is unmistakably that of an LMCO (Askwith et al., 1994). The physical and spectral properties of purified Fet3 protein are also fairly typical of an LMCO (Hassett et al., 1998; Machonkin et al., 2001). Thus, structure-function studies of the Fet3 enzyme should be broadly applicable to understanding how copper-dependent ferroxidases of the LMCO class function in a wide variety of organisms.

Structural studies of LMCOs date back to 1895 when Bertrand first suggested the *Rhus* laccase was a metalloenzyme (Bertrand, 1895), and determination of a high-resolution crystallographic structure for ascorbate oxidase was a breakthrough for structure-function studies of LMCOs (Messerschmidt and Huber, 1990; Messerschmidt et al., 1992). Although not a true LMCO by virtue of its dimeric structure, ascorbate oxidase was sufficiently similar to provide a critical stepping-stone for creating the first useful structural models of LMCOs (Patti et al., 1999). Recently, high-resolution crystal structures for LMCOs from C. *cinereus* and other organisms have provided paths to the development of better structural models for LMCOs, including the Fet3 protein (Ducros et al., 1997; Ducros et al., 1998; Roberts et al., 2001). Using these new structures in two different computational approaches, we developed an improved structural model for the Fet3 LMCO

that may help to explain some ambiguities in results from site-directed mutagenesis studies of this enzyme.

Materials and Methods

Amino Acid Alignments:

Mature amino acid sequences for the *S. cerevisiae* Fet3 protein, C. *cinereus* LMCO, and the zucchini (*Cucurbita pepo*) ascorbate oxidase were obtained from GenBank (accessions AAA64929, 1A65A, and P37064, respectively). The amino acid sequence for the mature Fet3 protein was truncated at Lys-522 since sequence beyond this point encompassed a membrane-spanning anchor domain that showed no homology to ascorbate oxidase or other LMCOs, making it unsuitable for homology modeling.

A multiple sequence alignment of amino acids for these proteins was generated using the PILEUP program in the GCG software suite (GCG Sequence Analysis Software Package Version 3.2, Genetics Computer Group, a subsidiary of Pharmacopeia, Inc., Madison, WI), and the alignment was adjusted manually. Separate GAP alignments of the Fet3 protein with each of the potential templates were also generated for comparison. The alignment between the Fet3 protein and the C. *cinereus* LMCO was judged best on the

basis of overall identity/similarity score, and was consequently used as the primary template on which the models were developed.

The alignment used as the starting point for generating the models was that generated using the GAP alignment. To optimize this alignment, the best GAP initiation penalty was determined by plotting the percent identity score against changes in the gap penalty over the range of 0 to 20 in increments of 2. The smallest gap penalty at which the resultant curve flattened out ($f'(x)=0$) was 12, and this value was considered the optimal gap initiation penalty for the analysis. All other parameters were left at their default settings as variations in the gap elongation penalty did not seem to improve the alignment scores.

Model Generation:

The *Coprinus* LMCO crystal structure (PDB 1A65) was used as the primary threading template for initial model generation via the Swiss-PDB Viewer 3.6b. Zucchini ascorbate oxidase was tested as a secondary template. First, the two templates were structurally aligned in Swiss-PDB viewer, and then the target sequence was manually aligned to the primary template based on results from the optimized GAP alignment. Threading requests for the aligned sequences were then submitted via the Swiss-PDB Viewer to SWISS-MODEL, an automated homology-modeling server (Guex et al., 1999). The returned

alignments were hand-refined at failure points to minimize threading energies and maximize the number of identical residues, then resubmitted to the server until a stable, full-length model was generated.

This initial threading model was subjected to further refinement using SYBYL 6.6 (Tripos Inc., St. Louis, MO). Energy minimization was performed using the Powell method in conjunction with the Tripos Force Field and the Kollman all-atom charge set. The field gradient was set at 0.025 kcal/mol*A for 100 iterations, and all other parameters were left at default settings. Bonds were adjusted using the "Shake Bonds and Angles" function, and model was minimized again using a gradient of 0.05 kcal/mol*A.

A second, independent model based on the same alignment and template submitted to SWISS-MODEL was generated using the MODELLER homology-modeling program (Sali and Blundell, 1993).

Insertion of Copper Atoms:

The SWISS-MODEL system does not work with heteroatoms. Consequently, copper ions had to be added manually to the energy-minimized structure using coordinates from the PDB 1A65 file for copper atoms in the *Coprinus* LMCO. Using default parameters for copper found in the file "metals.tpd" supplied by Tripos, the energy of the resultant structure was minimized again. Energy minimization was performed as described above,

except no charge set was used. In addition, the region around the Type-1 copper site (15 Å sphere) was minimized separately before the protein energy was minimized globally.

Identification of Potential Glycosylation Sites:

Aspargine residues having the potential to be modified by N-glycosylation were identified of the basis of amino acid sequence motifs using PAWS 8.1.1 (Genomic Solutions, Ann Arbor, MI), and those residues predicted by SYBYL to lie on or within one amino acid residue of the surface of the Fet3 protein, based on a 30% exposure potential, were considered the most likely sites for glycosyl modification.

Visualization:

Structural images were created using the Mega Raytrace function in POV-Ray (http://www.povray.org) to convert the ray trace files created by Swiss-PDB Viewer to raster images. Figures were subsequently assembled using Adobe PhotoShop 5.5 and Adobe Illustrator 6.0 (Adobe Systems, Inc., San Jose, CA).

Results and Discussion

Considerations for homology modeling of the Fet3 protein:

Homology modeling is capable of reliable, predictive structures having an accuracy of 1 Å RMS deviation (Sanchez and Sali, 1997), but this degree of accuracy requires high identity between template and target sequences. Commonly in such cases the template is altered to resemble the target through a sequential series of amino acid replacements followed by energy minimization routines. However, for situations in which the template and target are more distantly related, other methods, though not capable of such high accuracy, often produce more useful models. In either case, the primary determinant for model reliability remains the degree of homology between template and target. In general, an average amino acid identity of about 30% is required in order to generate an acceptable structure for large biopolymers. However, homology is often distributed unevenly along the polypeptide chain, and even predicted structures for highly homologous proteins must be considered carefully with respect to domains having minimal homology (Broughton, 1997).

Although homology between the *Coprinus* laccase and the Fet3 protein predicted using the default parameters in GAP alignment fell short of what is generally needed to ensure reliable prediction of structure (Sanchez and Sali,

1997), several of the primary and tertiary structural features that characterize LMCOs were useful in strengthening homology models for the Fet3 protein. The first of these was the primary sequence around the amino acid residues that provide ligands for copper (Messerschmidt and Huber, 1990). Figure 1 indicates the relative positions of the invariant histidines residues that make up the four copper-binding domains characteristic of LMCOs. Not only are the histidines residues themselves invarient, but their spacing within each binding domain is invarient as well. The sequential order of four domains is conserved in all LMCOs and their relative spacing on the polypeptide chain is very similar as well. In fact, alignment of over 50 LMCO sequences (*data not shown*) found no more than a ±1 residue variation in the number of intervening amino acids between domains 1 and 2, or between domains 3 and 4. Although, the region between domains 2 and 3 typically displayed a great deal of variation in length and composition (Figure 2A, green boxes), the high degree of conservation for the four copper-ligand domains allowed them to act as reliable anchors for LMCO alignment and structure.

It should be noted here, perhaps, that the four copper-ligand domains do not correspond to the four different electromagnetic copper centers of LMCOs. Copper binding between these domains is mixed, with each domain interacting with more than one copper center. Indeed, this configuration is

thought to provide the probable "through-bond" routes for electron transfer between the Type-I copper center and the trinuclear copper site (Messerschmidt, 1997). That being said, it should also be noted that some evidence suggests that copper atoms in the trinuclear center may actually switch between the Type-II and Type-III state during the catalytic cycle (Fraterrigo et al., 1999). Thus, strict designations as to which of the three copper atoms in the structural model for the trinuclear center is responsible for specific spectroscopic characteristics of the enzyme cannot be made.

Components of tertiary structure, such as the conserved three-domain ß-sheet "scaffold" first seen in plastocyanin (Messerschmidt, 1997), provide further aid in the modeling of LMCO structures. Although not predicted from primary sequence comparisons between ascorbate oxidase and plastocyanin, crystal structures clearly show this scaffold structure in both proteins (Messerschmidt et al., 1992). The two most striking regions of the scaffold are Domain 2, which comprises one six-stranded and one five-stranded ß-sheet, and Domain 3, which comprises a ß-barrel structure consisting of two five-stranded ß-sheets (Messerschmidt et al., 1992). Sequence alignments between ascorbate oxidase monomers and LMCOs suggested that the three-domain scaffolding should be conserved in all LMCOs (Messerschmidt and Huber, 1990), and crystal structures for the C. *cinereus* LMCO bore this out (Figure

2A) (Ducros et al., 1998). In all of the MCO crystal structures the copper atoms in the catalytic core are held at the interface of the three scaffold domains.

Fet3 protein model based on ascorbate oxidase:

The Protein Data Bank (PDB) currently contains crystal structures for three proteins that could serve as appropriate templates for homology modeling of LMCOs: ascorbate oxidase, fungal laccase, and the *Escherichia coli* enzyme encoded by the *yacK/cue*O gene (Messerschmidt et al., 1992; Ducros et al., 1998; Roberts et al., 2001). The structure for ascorbate oxidase was the first of these available, and a homology model for the Fet3 protein based on the ascorbate oxidase crystal structure was first proposed by di Patti et al. (Patti et al., 1999). Unfortunately, several aspects of the ascorbate oxidase crystal structure, most of which stem from the dimeric nature of the protein, make this structure less than perfect as a basis for homology modeling of LMCO (Kawahara et al., 1985). Highlighted in Figure 3 (panel B) are three contact zones mediating interactions between the subunits. One of these contact zones is very close to a stretch of α-helix that interacts with the enzyme's Type-1 copper center, and modeling suggests that protein-protein interactions in this contact zone require polypeptide conformations that are

energetically unfavorable in monomeric LMCOs. Not shown in the figure is a structural copper atom that lies at the interface between the monomers and is bound to amino acid residues in each subunit. This Cu^{2+} has the same valence as the Fet3 protein substrate, Fe^{2+}, and the binding site for this structural metal is again close to the ascorbate oxidase Type-1 copper center. Since the Type-1 center is the site for Fe2+ oxidation in the Fet3 protein, structures evolved for binding the structural copper in ascorbate oxidase could easily be mistaken for substrate recognition sites (Fe^{2+} binding sites) in the Fet3 protein model. Finally, terminal protrusions from each subunit of ascorbate oxidase (Figure 3B, red highlights at top and bottom), although unlikely to have significant impact on modeled active structure, and the tendency to drive these regions in LMCO models into energetically unfavorable conformations.

The differences between crystal structures for the ascorbate oxidase monomer and the C*oprinus* LMCO are shown in a subtractive ribbon view (Figure 2C). Regions of the two enzymes that overlap with high structural homology have been omitted from this overlay, while regions of considerable difference are shown as green and blue ribbons for ascorbate oxidase and the LMCO, respectively. Not surprisingly, the ascorbate oxidase and LMCO structures match up very well for scaffold domains in the protein interiors, while most of the discrepancies are associated with surface loops. Unfortunately, it is these

surface loops that are of greatest interest with respect to binding sites that might confer substrate specificity or interactions with other proteins, such as ferric permeases.

As a specific example of how such differences can lead to misleading models, in the Fet3 protein model based on the ascorbate oxidase crystal structure [Patti, 1999 #901] Glu-164 in the Fet3 protein sequence GAEPI (Figure 3, first red box in each alignment) is predicted in the model to be positioned near Trp-163 of ascorbate oxidase, which is a residue involved recognition and binding of ascorbate in the ascorbate oxidase active site. (Note that the amino acid numbering system used by di Patti et al. included the Fet3 protein signal sequence so the Glu residue in question is designated Glu-185 in their publications.) On the basis of this model it was proposed that Glu-164 was involved in active site recognition of Fe^{2+} by the Fet3 ferroxidase, even though the residue is located at least 14 Å from the Type-I copper center. While 14 Å is plausibly close for a substrate the size of ascorbate, it seems less reasonable for a substrate like Fe^{2+}, which has a diameter of less than 2 Å.

This model of Fet3 protein structure provided the foundation for two subsequent studies that used site-directed mutagenesis to probe the function of Glu-164 (Patti et al., 2000; Patti et al., 2001). Although the authors of those studies contend that their data support Glu-164 as important for Fe^{2+} binding in

the active site, their conclusions are equivocal. In the initial study (Patti et al., 2000), K_m and V_{max} for the mutagenized Fet3 protein were assessed in partially purified extracts, which is not considered appropriate for accurate analysis of enzyme kinetics due to potential interference from other cellular materials. Beyond this, the observation of reduced in V_{max} in the modified enzyme is disturbing since changes in K_m due to alterations in iron binding should have no impact on V_{max}. Interestingly, while V_{max} for Fe^{2+} oxidation varied, the V_{max} for *p*-phenylenediamine (*p*PDA) oxidation did not change. The redox potential of *p*PDA is lower than that of iron and these results suggest that, in fact, the redox potential of the Type-1 active site may have been changed by modification of Glu-164. Unfortunately, the more recently published study (Patti et al., 2001) sheds no further light on the subject since it is primarily an *in vivo* analysis of the impact these mutations have on survival of yeast under different culturing conditions. Mutations leading to deficiencies in the enzyme oxidative potential will be no more able to rescue an iron-challenged cell lines as those that specifically impact in iron binding.

On the other hand, it is certainly the case that there may not be a way to change the Fet3 protein K_m for Fe^{2+} without altering other kinetic parameters of the enzyme. Tyr-354 was also highlighted in the model as a potential Fe^{2+} ligand (Patti et al., 1999). Unfortunately, the authors did not point out that

similar tyrosine residues appear in the ascorbate oxidase and *Coprinus* LMCO crystal structures. This would seem to suggest that this Try residue is intrinsic to MCOs and is not specific to those having ferroxidase activity.

Fet3 protein model based on Coprinus LMCO:

Gap alignments show somewhat greater homology between the Fet3 protein and *Coprinus* LMCO sequences (31% identity) than between the Fet3 protein and ascorbate oxidase (28% identity), suggesting that the *Coprinus* LMCO crystal structure might provide a superior template for modeling. As previously noted in Figure 2C, there are large differences in the surface loop structures of ascorbate oxidase and the LMCO right in the vicinity of the active site and the sequence containing Glu-164. The structure much more compact and densely packed in the *Coprinus* LMCO crystal, reflecting the lack of binding points for dimerization. Our Fet3 model based on the LMCO structure consequently places Glu-164 much further from the active site, at the interface of Domains 1 and 3 of the scaffold (Figure 4A). This positioning suggests that mutations of this residue could disrupt contact between Domains 1 and 3 with consequent impact on the Type-1 copper center.

Another group recently published work based on a Fet3 protein structure model developed from the *Coprinus* LMCO crystal structure (Wang et

al., 2003). Although the paper contained no description of how they developed their model, the authors claimed that as di Patti et al. (Patti et al., 1999) had seen for the ascorbate oxidase-based model, their LMCO-based model predicted that Glu-164 (Glu-185 using their numbering) was in a position to participate in iron binding. Spectroscopic studies of the mutagenized enzyme noted a normal signal for the Type-1 copper center, suggesting that these mutations had not caused significant perturbation. However, the kinetic data was still somewhat troubling as these mutations still produced substantial variations in both K_m and V_{max} for both iron and phenolic substrates, which suggested a broader affect than just alteration of iron binding. Our suspicion is that these researchers used the model that came directly from SWISS-MODEL, which we noted left some loop regions, including one containing Glu-164, exposed to solution. In an effort at independent verification that our energy-minimization of the SWISS-MODEL structure for the Fet3 protein was reasonable, the *Coprinus* LMCO was used as a template for modeling Fet3 with the Modeller homology-modeling package. The model generated by this package confirmed our energy-minimized positioning of the loop containing Glu-164.

By virtue of their orientation to the Type-1 copper center, three stretches of sequence in the LMCO crystal structure were identified as potentially

involved in substrate binding (Figure 3A, blue boxes; Figure 4D, black ribbons). A short sequence in one of these stretches, NQDTG (Figure 3A, blue box 2, red ellipse), contained Asp-388, another substrate-binding site candidate originally considered by di Patti et al. (Patti et al., 1999). Mutagenesis of this residue (Asp-409 in their paper) resulted in a two-fold increase in the K_m of the Fet3 protein for Fe^{2+} (Patti et al., 2001) compared to the six-fold increase in K_m reported for the Glu-164 mutants. His-392, which provides a ligand for the Type-1 copper center, is immediately adjacent to this sequence, and appears to act as the direct conduit for entry of electrons from the substrate to the copper center. Depicted in Figure 4B, our model suggests that several residues in this sequence (Thr-389, Asp-388, and Thr-391) could participate in iron binding, with each contributing a small amount of the total binding energy.

Site-directed mutagenesis was also used by Askwith and Kaplan (Askwith and Kaplan, 1998) in an attempt to identify critical residues in the Fet3 protein. Two basic approaches were used in an effort to alter iron oxidase activity. In one approach, Leu-494 was modified in an effort to reduce the redox potential of the Type-I copper center. This was similar to work performed using a fungal laccase (Xu et al., 1999), and in each case the results were more or less the same, activity remained unchanged or was lost completely. In

the second approach, Askwith and Kaplan tried to alter substrate-binding by focusing on Fet3 amino acid residues shown by alignment with the Fio1 protein (a Fet3 homolog from *S. pombe*) to be possible iron binding sites. Mutagenesis of these residues (Glu-227, Asp-228, and Glu-330) did not produce the desired results. This result could be predicted from our model since none of these residues is predicted to be close to the active site.

Interaction with the Ftr1 Permease:

The Fet3 protein is thought to associate *in vivo* with a permease, Ftr1, and these two proteins together constitute the high affinity iron transport system in yeast (Stearman et al., 1996). From their structural model of the Fet3 protein, di Patti et al. (di Patti et al., 1999) proposed that a region rich in acidic amino acid residues was likely involved in the interaction with the Ftr1 permease and also facilitated movement of Fe^{3+} between the two proteins. According to our model these residues fall on a long coil that connects Domains 1 and 2 of the scaffold. This region, while showing considerable variability amongst LMCOs in public databases, does tend generally contain a high percentage of negatively charged residues. For example, ascorbate oxidase has three aspartate residues in this region (Asp-318, Asp-319, and Asp-321), while the *Coprinus* LMCO has four aspartate residues in this loop,

although they are not as closely spaced as those in ascorbate oxidase. However, neither of these enzymes is known to associate with other proteins through this domain, and there is some indication that these charged amino acids may be involved in adhesion of this loop to the surface of Domains 1 and 2, which would help to stabilize these structures as a whole.

While our model neither supports nor conflicts with the assertion that some of these residues are involved in Ftr1 association and/or Fe^{3+} guidance, it suggests to us that a different region could also be major player in Ftr1 interactions. The final alternative template for homology modeling of LMCOs is the crystal structure of the *yacK/cue*O gene product (Figure 4E). This structure is striking for several reasons. First, despite the initial strangeness of its sequence compared to other LMCOs, it does contain the distinctive three-domain scaffold seen in the other two structures. Second, there is a large α-helical structure that occludes the Type-1 copper center, and this 14-amino acid helix contains seven Met residues. The *yacK/cue*O gene product is involved in a protective response to copper in *E. coli*. Since copper binding and uptake is mediated through Met residues in certain transport proteins (Puig et al., 2002), it seems likely that the spatial relationship between the Met-rich helix and the enzyme active site is significant to its response to copper. Previous work in our lab has shown that addition of copper greatly increases the oxidative activity

of the *yacK/cue*O enzyme for many substrates, including iron (Kim et al., 2001). This activation by copper is likely mediated by the Met-rich helix, which is oriented to bring additional copper ions into close proximity to the active site.

Comparison of our Fet3 protein model (based on the C*oprinus* LMCO) with the *yacK/cue*O protein structure drew attention to a helical structure that is near the active site in the model. As depicted in Figure 4A, the Fet3 protein model has an _-helix-containing loop that extends into solution at the right of the Figure The same structure is magnified in Figure 4D, and is depicted in black in Figure 4F (labeled ß). This loop is rich in acidic amino acids, with four aspartate and two glutamate residues out of the total 23 residues in the loop. In our model, this loop is highly flexible and there is virtually no energy barrier to folding it over to place the helix in close proximity to the Type-1 copper center, similar to the helix seen in *yacK/cue*O enzyme. Using the same reasoned as di Patti et al. (di Patti et al., 1999), we believe that the acidic residues in this helix help shuttle Fe^{2+} to the active site and may assist in transfer of Fe^{3+} to the awaiting permease. The change in charge on the iron could drive a conformational change that allows the helical arm to swing back and forth between the site of oxidation and the loading site on the permease. Such a mechanism would minimize opportunities for the oxidized iron to react with

water to form insoluble ferric hydroxide. Thus, this helical loop would appear to be an attractive, if previously overlooked, target for site-directed mutagenesis experiments to probe Fet3 protein function with respect to both iron oxidation and interactions with the Ftr1 permease.

Figure 1

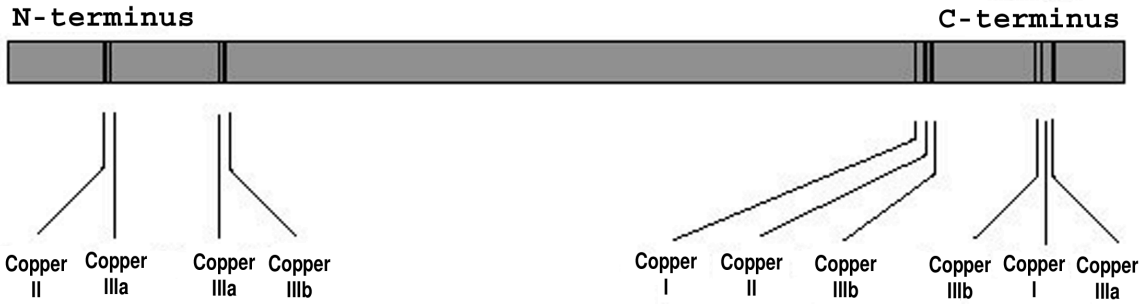

Figure 1. Copper ligands in LMCOs. Schematic showing the relative positioning of invariant histidines residues along a generalized LMCO polypeptide. Roman numerals indicate which electromagnetic type copper center (Type-1, -2, or –3) is liganded to that particular histidine. Designations of IIIa or IIIb indicate ligands for the two different copper atoms that make up the Type-3 center.

Figure 2

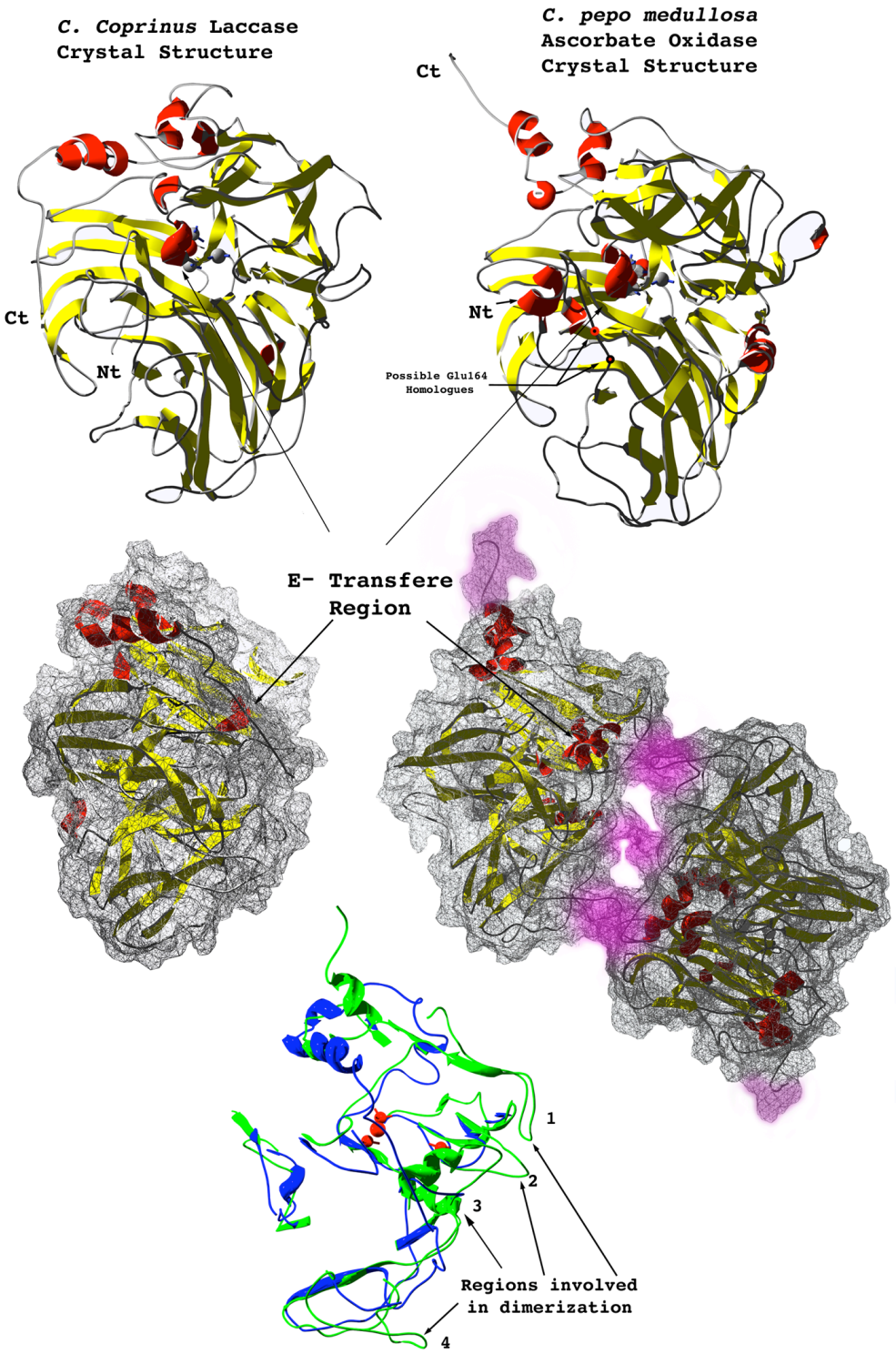
C. Coprinus Laccase
Crystal Structure
C. pepo medullosa
Ascorbate Oxidase
Crystal Structure
Ct
Nt
Ct
Nt
Possible Glu164
Homologues
E- Transfere
Region
1
2
3
4
Regions involved
in dimerization

Figure 2. Comparison of crystal structures for C. *cinereus* LMCO and ascorbate oxidase. Ribbons projections of the two crystal structures in PDBV are shown at the top. Amino- and carboxyl-termini are labeled Nt and Ct. respectively. In the middle are surface models for each protein structure, and in the case of ascorbate oxidase the second subunit of the dimer has been added. The surface models for each structure have been rotated slightly from the positions in the ribbon diagram. In each case, the point of view has been turned 45° counter-clockwise on the Z-axis and 15° downward on the Y-axis. For orientation purposes, arrows point to a short, conserved helix in each enzyme lies between the tri-nuclear and Type-1 copper centers. Areas of contact in the ascorbate oxidase dimer, as well as extended C-terminus, are highlighted in red. A subtractive overlay view of the ascorbate oxidase monomer (green) and the LMCO (blue) is shown in panel C. The orientation the polypeptides is the same as in the surface model projections, and areas of having structural deviations of <1Å have been removed.

Figure 3

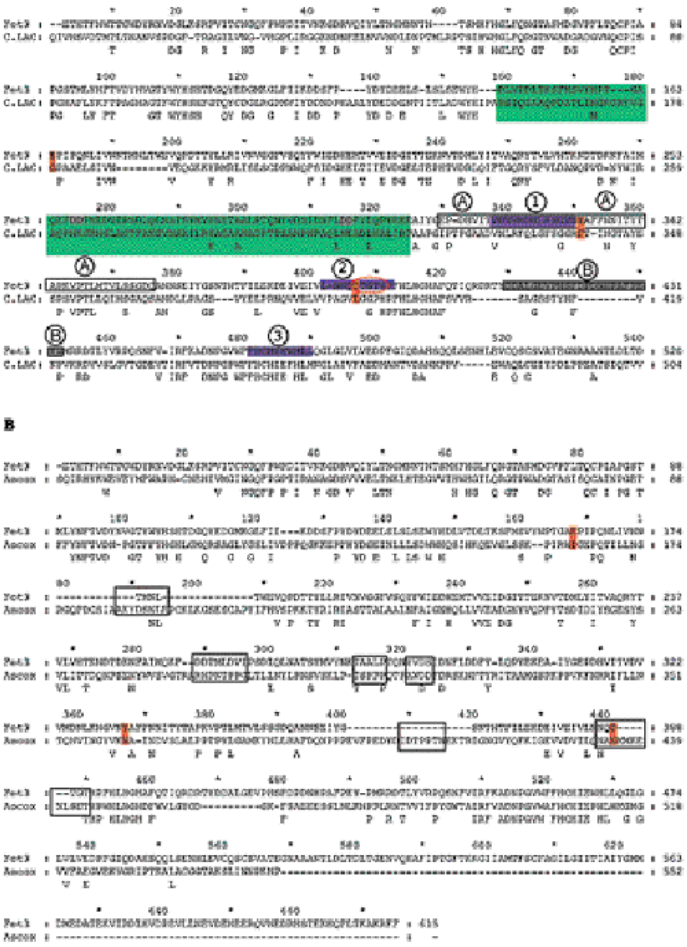

Figure 3. Amino acid alignments of the Fet3 protein with *Coprinus* LMCO (A) and ascorbate oxidase (B). GAP was used to align the two pairs of sequences, and the trimmed Fet3 protein sequence was used to determine numbering in the Figure Regions of high variability, determined from comparison of over 50 LMCO sequences, are boxed in green. Three domains that are proximal to the Type-1 copper center and have potential to be involved n substrate interaction are shown in blue boxes. Regions that might contribute to loading and unloading of iron from the active site, as proposed by di Patti et al. are in the open box labeled with a circled A, while those proposed in this study are in the gray shaded box labeled with a circled B. Amino acids proposed to be essential to iron binding by di Patti et al. are shown in red, while amino acids suggested by our model to be involved in iron oxidation are circled in red

Figure 4

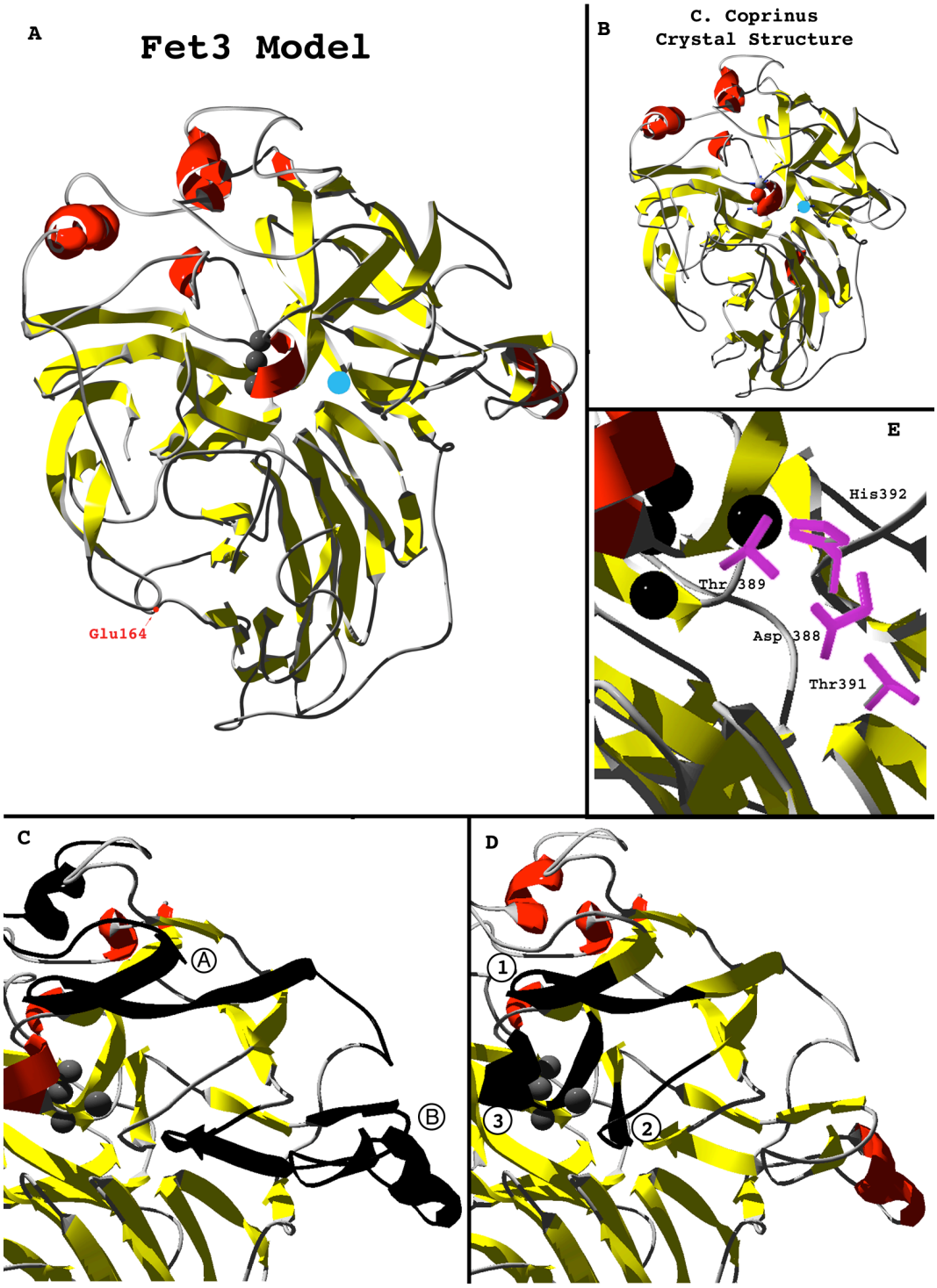
A
Fet3 Model
Glu164
B
C. Coprinus
Crystal Structure
E
His392
Thr389
Asp388
Thr391
C
A
B
D
1
2
3

Figure 4. Energy-minimized model of the Fet3 protein (Panel A). Glu-164, the amino acid residue predicted by di Patti et al. to be involved in iron binding near the Type-1 copper center, is shown in red at some distance from the active site. Similarly oriented ribbon projections of the C*oprinus* LMCO and the *E. coli yacK/cue*O crystal structures are depicted in panels C and E, respectively. An enlargement of the Type-1 copper site in the Fet3 protein model is shown in panel B, along with side-chain structures for three amino acid residues proposed to be involved in substrate binding. Panel D shows a lower magnification of the same region with the three loops most likely to interact with substrates colored in black. Panel F shows the same region, but the structures colored in black denote the acidic residue-rich domains proposed by di Patti et al. [3] (å), or in this study (ß) to be involved in iron movement into and out of the active site.

CHAPTER 6

Conclusion/Discussion:

Truth is said to be stranger than fiction. This assertion is perhaps true of one in a hundred occurrences. What seems to be true far more often is that things in nature are never as simple as they look and rarely as neat and tidy as one would like.

For many years researchers have argued over the role of LMCOs in lignin deposition and/or lignin degradation. At various times LMCOs have been the favored enzymes for each of these processes, while at other times no physiological function has been ascribed to them.

In chapter 3 it was demonstrated that *Acer* cell cultures secreted multiple LMCOs, likely distinct gene products. It is my belief that this is the case with all plant systems as data indicates a large number of LMCO genes in plant genomes. One has to ask the question why would there be these multiple LMCO genes if there were not multiple physiological functions mediated by LMCOs.

In chapter 4 it was demonstrated that a recombinant LMCO cloned from *Liriodendron tulipifera* was capable of oxidizing iron. Furthermore, it was suggested that the function of this LMCO could be to reduce iron levels in lignifying tissue via an iron pump system not unlike that found in yeast.

In chapter 5 molecular modeling was used to show that substrate specificity could arise from a handful of changes in a small region of LMCO primary sequence. In particular, three loop regions that occur in roughly the same position is all LMCO sequences are well positioned to confer substrate specificity.

It has long been know that LMCOs have the ability to oxidize a wide range of substrates. Work by Davin showed that that an LMCO associated with a "helper" (dirigent) protein was able to direct stereo-specific polymerization of monolignols *in vitro* (Davin et al., 1997). More recently, metals, such as iron, manganese and copper, have been added to the list of potential LMCO substrates. All of these data taken together point to LMCOs as "engines" that provide oxidative power for a vast array of physiological functions. Other proteins associated physically with the LMCO may control the specific outcomes of these reactions, and these physical interactions could be modulated by a handful of amino acids located on the active face of the protein. On of the difficulties researchers face in trying to determine the

physiological functions of LMCOs is that when we strip this "engine" of its protein regulators it can run wild, oxidizing anything that can get close to the active site and has a redox potential within an appropriate range.

One might ask why biological systems would make such wide use of an oxidative system that could so easily run amok. In contrast, peroxidases can carry out an even wider array of reactions than LMCOs, and they are easier to control by elimination of hydrogen peroxide. However, heme is a complex functional group requiring many enzymes for its synthesis. In addition, heme can persist after the enzyme it is incorporated into breaks down, and the released, free heme can catalyze many deleterious reactions. This contrasts with LMCO breakdown, which only releases a small amount of free Cu that is likely quickly scavenged. Also, there are, no doubt, numerous situations where environmental, physiological or cellular conditions will not permit hydrogen peroxide generation, yet oxygen is present.

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