Targeted Mutations in a *Trametes villosa* Laccase

AXIAL PERTURBATIONS OF THE T1 COPPER*

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†The abbreviations used are: T1, type 1 copper; T2, type 2 copper; T3, type 3 copper; TVL, recombinant *T. villosa* (Polypora pisatus) laccase (isozyme 1); LsL, recombinant *R. solani* laccase; MtL, recombinant *M. thermophila* laccase; CcL, recombinant *C. cinereus* laccase; PvlL, *P. versicolor* laccase; zAO, *Zucchini* ascorbate oxidase; RvL, *R. vernicifera* laccase; Pp, poplar plastocyanin; wt, wild type; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); SO4, syringaldazine; E°, single-electron redox potential (referred to the normal hydrogen electrode) at T1 site; pHopt, optimal pH; G, gauss; MES, 4-morpholineethanesulfonic acid.

*Laccase (p-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) is a copper-containing oxidase that couples the oxidation of substrate (usually diphenols, aryl diamines, or amino phenols) with the reduction of dioxygen to water (1–4). Sequence comparisons, crystal structure analyses, and spectroscopy indicate that all multicopper oxidases contain at least one type 1 copper center, one type 2 (T2) copper center, and one type 3 (T3) copper center (3–7). These copper sites are defined by their spectroscopic properties. The T1, or "blue," copper is characterized by an intense (ε = 5000 M⁻¹ cm⁻¹) absorption band around 600 nm and an unusually small (<100 × 10⁻⁴ cm⁻¹) parallel hyperfine coupling in EPR. The T2, or normal, copper site does not exhibit a strong feature in the visible absorption spectrum, but has a parallel hyperfine coupling > 160 × 10⁻⁴ cm⁻¹. The T3, or coupled binuclear, copper center consists of two copper atoms connected by a hydroxide bridge. This bridge provides a strong superexchange pathway and therefore mediates antiferromagnetic coupling. The T3 center is EPR-silent, but is characterized by an absorption band around 330 nm (ε = 5000 M⁻¹ cm⁻¹). The T2 and T3 sites form a trinuclear copper cluster that is the site for O₂ reduction (5, 8, 9). Laccase is the simplest of the multicopper oxidases, containing one of each type of copper site for a total of 4 copper atoms. Based on a wide range of comparative studies, including sequence homology and crystal structure analysis, the copper site coordination is very similar among the multicopper oxidases. The main difference is in the coordination sphere of the T1 copper site. The typical T1 site, such as that found in the blue copper protein plastocyanin (Pc) and the multi-copper protein *Zucchini* ascorbate oxidase (zAO), contains two histidines (His), a cysteine (Cys) that forms a short S-Cu bond, and a methionine (Met) that forms a long S-Cu bond. These four ligands bind T1 copper in a distorted tetrahedral coordination geometry (Fig. 1, A and B). In contrast, most fungal laccases with known primary sequence have either a leucine (Leu) or phenylalanine (Phe) at the position corresponding to the axial Met ligand (3, 5). Neither Leu nor Phe would be expected to coordinate to the copper that would render a tri-coordinate T1 site. The recent crystal structure of *Coprinus cinereus* laccase (CcL) confirms this and shows that the T1 site has only three ligands (two His and one Cys) in a trigonal planar geometry (Fig. 1C) (7). An interesting question is how this difference in geometry is manifested in terms of differences in the electronic structure and the electron transfer function of the T1 site. The redox potential (E°) of fungal laccases ranges from 0.48 V to 0.78 V, whereas Pc and zAO have E° in the range of 0.3 to 0.4 V (3). It has been proposed that the lack of an axial ligand might be responsible for the high E° observed in some fungal laccases (10–12), as it is expected that the elimination of the axial Met donor interaction would preferentially stabilize the reduced Cu(I) state. A number of mutagenesis studies (13–15) have been performed on the T1 copper site in azurin, and it was found that the nature of the axial ligand can influence E° to some extent. Mutation of Met-121 was found to tune the E° over a range of ~0.105 V to 0.135 V with respect to the E° of the wild-type (wt) azurin at pH 7.0.

The present study utilized site-directed mutagenesis to examine how the composition of the amino acid residues in the vicinity of the T1 copper affect the structure and E° of this copper in a *Trametes villosa* laccase (TvL) (16). We were also interested in determining how the mutations would impact the enzymatic properties, kcat, Km, and the pH dependence of en-
zymatic activity. TvL has a primary structure and redox/enzymatic properties very similar to the Trametes (Polyergus or Coriolus) versicolor laccase (PvL), an enzyme that has been extensively studied and is regarded as a representative high $E^o$ laccase (1–4). We mutated Phe-463, which corresponds to the axial Met-517 in zAO, and two other residues (Glu-460 and Ala-461), corresponding to the His-514 and Met-515 in zAO (Table I). Our results showed that the F463M mutation led to significant changes in the T1 copper site, decreased the $E^o$, and altered $k_{cat}$ and $K_m$.

**EXPERIMENTAL PROCEDURES**

Materials, Methods, and Enzyme Assays—Chemicals used as buffers and substrates were commercial products of at least reagent grade. Britton and Robinson buffer (pH 2.7–11, made by mixing 0.1 M boric acid, 0.1 M acetic acid, and 0.1 M phosphoric acid with 0.5 M NaOH to desired pH) was used for the pH profile. Recombinant TvL (isoform-1) was purified as reported previously (16). The protocols for molecular biology experiments (including restriction digest, DNA ligations, gel electrophoresis, and DNA preparations) were adapted from either the instruction of the manufacturer or standard procedures. Oligonucleotides were synthesized by a specified DNA/RNA synthesizer. Nucleotide sequences were determined by an Applied Biosystems model 394 DNA/RNA sequencer. Laccase-cat and laccase-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and kinetic analysis were performed (for A461E/F463L mutant), and 5 $d$-dGACTTC-

![Figure 1](image1.png)

**FIG. 1.** Comparison of the ligation of the T1 copper in Pc (Protein Data Bank code 1PLC) (A), zAO (Protein Data Bank code 1AOZ) (B), and CcL (Protein Data Bank code 1A65) (C). In Pc, the Cu-S458 bond length is 2.82 Å; in zAO, the Cu-S514 bond length is 2.90 Å; in CcL, the distance between the copper and L462 is ~3.5 Å.

**FIG. 2.** UV-visible spectra of TvL. A, wt; B, F463L mutant; C, F463M mutant. The $E$ for the 330–800 nm region has been magnified 16× to enable viewing.

| Laccase | Sequence alignment | $E^o$ |
|--------|-------------------|-------|
| TvL    | $^{452}$HCHDFHPLEAGF$^{463}$ | 0.78  |
| PvL    | $^{452}$HCHDFHPLEAGF$^{462}$ | 0.79  |
| ReL    | $^{452}$HCHDPWLEAGF$^{470}$ | 0.71  |
| MlL    | $^{502}$HCHAIHNVGSL$^{513}$ | 0.47  |
| CcL    | $^{452}$HCHIEPHLNGM$^{462}$  | 0.55  |
| zAO    | $^{502}$HCHIEPHLNGM$^{517}$ | 0.34  |

**TABLE I**

Underlined letters represent the mutated residues of this study. *1, ligand to T3 copper; †, ligand to T1 copper.

RESULTS AND DISCUSSION

**F463L Mutant and A461E/F463L Double Mutant**—The purified mutants each had an UV-visible spectrum quite similar to the wt TvL (Fig. 2, Table II). The mutant enzymes had ~4 copper/protein and ~50% paramagnetism. The EPR spectrum of the F463L mutant was obtained and showed a slight increase in $g_2$ and decrease in $A$ at the T1 site and a small change in $g_1$ of the T2 copper site (Fig. 3, Table II). However, these changes were minor, indicating that the F463L mutation did not significantly alter the electronic structure of the T1 site. In 10 mM MES, pH 5.5, F463L exhibited an $E^o$/T1 copper) that was only 0.05 V lower than that of the wt TvL (Table III). This suggests that changing the non-coordinating, bulky, π electron-rich Phe to Leu did not have a dramatic impact on the T1 copper site. This result is consistent with a previous study in which mutation of Leu to Phe in Rbizoctonia solani and Myce- liophthora thermophila laccases did not result in alteration of the EPR parameters and $E^o$ for their T1 copper (18). Table III and Fig. 4 summarize the SOZ and ABTS oxidase activities of both the mutants. $K_m$, $k_{cat}$, and optimal pH (pHopt) were not significantly altered in the T1 copper single or the double mutant. The lack of a significant effect of these mutations on the electronic properties of the T1 copper is consistent with the lack of an impact on the kinetic parameters of the enzyme. Previous studies have shown that, under steady state conditions, the rate-limiting step in the catalytic cycle is most likely the oxidation of the substrate by transfer of an electron from the...
substrate to the T1 copper (22). If the electronic structure of the T1 copper were significantly altered by a mutation-induced perturbation of the site, it would seem likely that the rate of substrate oxidation would also be affected.

*F463M Mutant*—The transformation and expression of the F463M mutant was comparable to that of the wt TvL. The expressed mutant exhibited a chromatographic elution pattern similar to that of the wt TvL. Copper content measurements revealed a stoichiometry number close to 4 copper/protein for the wt and mutant. The purified protein had an UV-visible spectral similarity to Pc (Fig. 2, Table II). The F463M mutant was comparable to that of the wt TvL. The Cu(II) over the reduced Cu(I) site, thus leading to a lower kcat (Table III). The decrease in the kcat can be explained by an increase in $k_{cat}$ of substrate oxidation that can be explained by an increase in $k_{cat}$

The EPR spectra of wt TvL showed a shoulder around 750 nm, indicating that the mutation perturbed the T1 copper. The transformation and expression of the F463M mutant was similar to that of the wt TvL. The T2 copper remained unaltered. These changes are consistent with the T1 copper coordination also including the axial Met (3, 4, 17). Thus, other factors such as hydrogen bonding, solvent accessibility, and orientation of local dipoles clearly play an important role in tuning the $E^\circ$ (4, 23).

The pH profiles of the $E^\circ$ for the wt and F463M mutant are depicted in Fig. 5. The profile of both enzymes changed very little between pH 4 and 8 but showed an increase at pH > 8. This increase was more pronounced in the F463M mutant. It has been reported that the $E^\circ$ of Pc and azurin increases as the pH decreases, due to the titration of a T1 copper-ligating H in the reduced site (24). The $E^\circ$ (T1) of RvL also increases when pH decreases (25). Thus for TvL, the pH effect on $E^\circ$ was in the opposite direction. Fig. 5 also shows the pH profile of the $\lambda_{max}$ change was observed in either the wt or F463M mutant over pH 2 through 8 but at pH > 8, a significant decrease in $\lambda_{max}$ was observed. This change in $\lambda_{max}$ indicates a pH-dependent structural change of the T1 copper site. While further spectroscopic studies are needed to elucidate the origin of this change, it may be noteworthy that the decrease in $\lambda_{max}$ coincides with the increase in $E^\circ$.

The F463M mutation led to a 5-fold increase in $K_m$ for the SGZ substrate and a 38-fold increase in $K_m$ for the ABTS substrate (at pH 5.5). The mutation also resulted in a 2-fold increase in $k_{cat}$ (Table III). For an electron-transfer reaction (such as the one between the substrate and the T1 copper), its rate ($k_{ET}$) is determined by thermodynamic driving force ($E^\circ$ difference between donor and acceptor), electronic coupling (orbital overlap between donor and acceptor), and Frank-Condon barrier (reorganization energy associated with electron transfer) (26). The parameter $k_{cat}$ is reflective of the rate-limiting step that was shown to be the substrate oxidation under steady state conditions (22). While a number of steps may contribute to $k_{cat}$, the fact that $k_{cat}$ was strongly correlated with the difference in $E^\circ$ between the substrate and the T1 copper for a series of substrates suggests that $k_{ET}$ is a major component of $k_{cat}$. Our data illustrate an increase in $k_{cat}$ of substrate oxidation that can be explained by an increase in $k_{ET}$

In 10 mM MES, pH 5.5, the mutant exhibited an $E^\circ$ of 0.1 V lower than that of the wt TvL (Table III). It has been proposed that the additional Met ligand would stabilize the oxidized Cu(II) over the reduced Cu(I) site, thus leading to a lower $E^\circ$ (3, 10, 11). Our EPR and absorption data strongly suggested that the introduction of Met significantly perturbed the T1 site and that Met probably coordinated to the T1 copper based on the spectral similarities to Pc. The decrease in the $E^\circ$ was also consistent with this interpretation. The extent of the $E^\circ$ change (0.1 V) agrees with studies on the T1 copper site in azurin in which the reverse mutation was carried out; replacement of Met by a non-ligating Leu led to a 0.1-V increase in $E^\circ$ (13, 14). These observations support the hypothesis that ligation of the axial amino acid can tune the $E^\circ$ to some extent. However, it is important to note that the $E^\circ$ of 0.68 V observed for the F463M mutant is still 0.1–0.4 V higher than that of zaO, human serum ceruloplasmin, and Myrothecium verrucaria bilirubin oxidase, whose T1 copper coordination also includes the axial Met (3, 4, 17). Thus, other factors such as hydrogen bonding, solvent accessibility, and orientation of local dipoles clearly play an important role in tuning the $E^\circ$ (4, 23).

The pH profiles of the $E^\circ$ for the wt and F463M mutant are depicted in Fig. 5. The profile of both enzymes changed very little between pH 4 and 8 but showed an increase at pH > 8. This increase was more pronounced in the F463M mutant. It has been reported that the $E^\circ$ of Pc and azurin increases as the pH decreases, due to the titration of a T1 copper-ligating H in the reduced site (24). The $E^\circ$ (T1) of RvL also increases when pH decreases (25). Thus for TvL, the pH effect on $E^\circ$ was in the opposite direction. Fig. 5 also shows the pH profile of the $\lambda_{max}$ change was observed in either the wt or F463M mutant over pH 2 through 8 but at pH > 8, a significant decrease in $\lambda_{max}$ was observed. This change in $\lambda_{max}$ indicates a pH-dependent structural change of the T1 copper site. While further spectroscopic studies are needed to elucidate the origin of this change, it may be noteworthy that the decrease in $\lambda_{max}$ coincides with the increase in $E^\circ$.

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The decreased $E^\circ$ of the T1 copper in the F463M mutant would lead to a decreased thermodynamic driving force and thus a lower $k_{ET}$. Thus, in order to account for the increase in $k_{cat}$, the mutation must have affected the electronic coupling and/or the reorganization energy. The EPR spectrum of F463M provides initial evidence that the mutation caused a change in the ground state of the T1 copper. This could result in a change in the orbital overlap between the donor (substrate) and acceptor (the half occupied highest molecular orbital of the T1 copper).

There is some evidence from ENDOR that the spin density on the imidazole nitrogen ligands of different T1 copper sites can vary, depending on the nature of the ground state (27). This
Redox potential and substrate specificity of the mutants

Redox potential and substrate specificity were measured in 10 mM MES, pH 5.5. ND, not determined.

|       | E°  | SGZ  |
|-------|-----|------|
|       | V   | k<sub>cat</sub> | μM |,min<sup>−1</sup> | μM | min<sup>−1</sup> |
| wt    | 0.79 ± 0.01 | 3.9 ± 0.1 | 3000 ± 100 | 58 ± 8 | 2700 ± 100 |
| F463L | 0.74 ± 0.01 | 4.6 ± 0.8 | 3900 ± 200 | 54 ± 8 | 3500 ± 200 |
| A461EF463L | ND | 6.9 ± 0.4 | 2900 ± 100 | 66 ± 7 | 2600 ± 100 |
| F463M | 0.68 ± 0.02 | 19 ± 4 | 5300 ± 200 | 2200 ± 200 | 4700 ± 200 |

**Fig. 4.** The pH activity profile for substrate SGZ (A) and ABTS (B). Laccase used: wt (○); F463L (×); F463M (Δ). Substrate concentration: SGZ, 19 μM; ABTS, 2 mM.

**Fig. 5.** The pH profiles of E° (A) and λ<sub>max</sub> (B) of wt (○) and the F463M mutant (Δ).

difference could influence the orbital overlap between the donor and acceptor. It is also possible that the mutation altered the Frank-Condon barrier to electron transfer. Evaluation of electronic structure differences between wt and F463M, which would relate to this difference in reactivity, is presently under way.

The F463M mutation led to a shift in pH<sub>opt</sub> for the oxidation of SGZ but not ABTS (Fig. 4). It has been shown that oxidation of a phenolic substrate depends on whether it is protonated; the deprotonated phenol has a lower redox potential and therefore is more easily oxidized (19). The fact that the pH dependence of activity was only altered when SGZ, a phenolic substrate, was used but not when ABTS, a non-phenolic substrate, was used indicates that the mutation must have caused a slight change in the substrate-binding pocket. This is also consistent with the change in K<sub>m</sub> and could perturb the protonation equilibrium of the phenolic substrate.

**Concluding Remarks**—Understanding how the E° of copper sites in proteins is regulated and how E° and geometric and electronic structure perturbations influence the electron transfer function of a protein is one of the major challenges in the field of metallo-biochemistry. Various theories have been proposed to explain how two seemingly similar T1 copper sites could have different E° and different reactivity (1–4, 17). For these comparisons, it is important to obtain a detailed description of the electronic structure of the copper in a similar envi-ronment. Our results provide initial evidence that a more “classic” blue copper site can be created in fungal laccase through site-directed mutagenesis and that the resulting electronic structure appears to be intermediate between the classic Pe T1 copper site and that of the wt fungal laccase, which has no axial Met. This leads to a lowering of E° in the same protein environment consistent with the axial Met stabilizing the oxidized, Cu(II) state more than the reduced, Cu(I) state. Detailed spectral studies are currently being pursued to understand the effects of the associated electronic structure changes on the reactivity of these enzymes.

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