Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile

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A *Myceliophthora thermophila* laccase and a *Rhizoctonia solani* laccase were mutated on a pentapeptide segment believed to be near the type-1 Cu site. The mutation L513F in *Myceliophthora* laccase and the mutation L470F in *Rhizoctonia* laccase took place at a position corresponding to the type-1 Cu axial methionine (M517) ligand in *Zucchini* ascorbate oxidase. The triple mutations V509L,S510E,G511A in *Myceliophthora* laccase and L466V,E467S,A468G in *Rhizoctonia* laccase involved a sequence segment whose homologue in ascorbate oxidase is flanked by the M517 and a type-1 Cu-ligating histidine (H512). The single mutation did not yield significant changes in the enzymic properties (including any significant increase in the redox potential of the type-1 Cu). In contrast, the triple mutation resulted in several significant changes. In comparison with the wild type, the *Rhizoctonia* and *Myceliophthora* laccase triple mutants had a phenol-oxidase activity whose pH optimum shifted 1 unit lower and higher, respectively. Although the redox potentials were not significantly altered, the K_m, k_{cat} and fluoride inhibition of the laccases were greatly changed by the mutations. The observed effects are interpreted as possible mutation-induced structural perturbations on the molecular recognition between the reducing substrate and laccase and on the electron transfer from the substrate to the type-1 Cu centre.

INTRODUCTION

Laccase is a representative 'blue' multi-copper oxidase found in various plant and fungal organisms (for recent reviews, see [1–12]). Based on a wide range of comparative studies (including sequence-homology analysis) carried out against Cu proteins with known crystal structures, it is believed that the Cu sites in laccase have co-ordinations similar to those found in *Zucchini* ascorbate oxidase (zAO) and human serum ceruloplasmin (hCP) [6,13,14]. Various models have been generated to correlate the Cu site structure and the molecular properties of laccase. In particular, it has been postulated that the co-ordination geometry and ligands of the type-1 (T1) Cu might determine the redox potential (E^0) of this site [8,15], that the presence of a phenylalanine at the position corresponding to that of the T1 Cu-ligating methionine in zAO (M157) and hCP (M690 and M1031) might be responsible for the high E^0 (0.8 V) observed in *Trametes* (*Polyporus* or *Coriolus*) *versicolor* laccase [6,9,16], and that exogeneous small molecules (such as O_2, H_2O, OH^- or F^-) are capable of binding to the type 2 (T2) Cu and inhibiting enzyme activity by regulating the internal electron transfer from the T1 Cu to the T2/type 3 (T3) Cu cluster [6,8–10]. However, these hypotheses have not been examined by site-directed mutagenesis, although the primary sequences of about 30 laccases are known.

Recently we studied several fungal laccases with regard to their redox and kinetic properties [17]. In an attempt to correlate the properties to the structures of these laccases, we noted that these laccases, including three with 'high E^0' of their T1 sites (0.7–0.8 V versus a normal hydrogen electrode) {from *Trametes* (*Polyporus pinsitus*) *villosa* (TvL; [18]), *Rhizoctonia solani* (RsL; [19]) and *Trametes* (*Polyporus*) *versicolor* (PvL; [9,16])} and two with 'low E^0' of their T1 sites (0.5 V) {from *Myceliophthora thermophila* (MtL; [20]) and *Scytalidium thermophilum* (StL; [21])}, differed in a pentapeptide segment believed to be located near the T1 site based on the sequence alignment with zAO (Table 1). First, all

Table 1 Sequence alignment between zAO, RsL and MtL

Underlined letters represent the mutated residues of this study.

Laccase	Sequence alignment												E^0 (V)
StL	^{506}H	C	H	I	A	W	H	V	S	G	G	L^{517}	0.51
MtL	^{502}H	C	H	I	A	W	H	V	S	G	G	L^{513}	0.47
RsL	^{459}H	C	H	I	D	W	H	L	E	A	G	L^{470}	0.71
TvL	^{452}H	C	H	I	D	F	H	L	E	A	G	F^{463}	0.78
PvL	^{451}H	C	H	I	D	F	H	L	E	A	G	F^{462}	0.79
zAO	^{506}H	C	H	I	E	P	H	L	H	M	G	M^{517}	0.34
	*	†	*				†					†	

*Ligand to T3 Cu.
†Ligand to T1 Cu.

Abbreviations used: RsL, recombinant *Rhizoctonia solani* laccase rsl4; RsL single mutant, L470F; RsL triple mutant, L466V,E467S,A468G; MtL, recombinant *Myceliophthora thermophila* laccase; MtL single mutant, L513F; MtL triple mutant, V509L,S510E,G511A; TvL, *Trametes villosa* (*Polyporus pinsitus*) laccase lcc-1; StL, *Scytalidium thermophilum* laccase; PvL, *Trametes* (*Polyporus*) *versicolor* laccase lcc-1; zAO, *Zucchini* ascorbate oxidase; hCP, human serum ceruloplasmin; wt, wild type; T1, type-1; T2, type-2; T3, type-3; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid); SGZ, syringaldazine; pH_{opt}, optimal pH; PEG, poly(ethylene glycol); the one-letter code for amino acids is used.

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three 'high E^0' laccases (TvL, RsL and PvL) had a leucine-glutamate-alanine tripeptide, rather than the valine-serine-glycine tripeptide found in both the 'low E^0' laccases (MtL and StL), at the position corresponding to 513LHM515 in zAO. These three amino acids are part of the T1 pocket with the H514 also serving as part of the substrate-binding pocket. Thus, based on sequence homology with zAO, the LEA/VSG segment is expected to be in the vicinity of substrate binding and the T1 Cu. The existence of the LEA segment seemed to 'correlate' with the 'high E^0', whereas the presence of VSG correlated with the 'low E^0'. Second, unlike the F found in both TvL and PvL, an L, was found in RsL at the position corresponding to the T1 Cu axial ligand (M517 in zAO). An L was also found in two other 'low E^0' laccases (MtL and StL), indicating that the F at this position might not be a prerequisite for the high E^0 as previously proposed [6,9,16]. Because the composition of the amino acid residues in the T1 pocket could affect properties of the T1 Cu [22–36], we continued to investigate whether the two sequence differences in the pentapeptide segment could induce the redox and kinetic variations observed for these laccases.

We describe here a study of two RsL mutants and two MtL mutants in which a single or a triple mutation was made in the pentapeptide corresponding to the sequence segment 512HLHMGM517 in zAO (Table 1). The mutated position in the single mutants (L/F) corresponded to the T1 Cu axial ligand position (M517 in zAO) and the mutated tripeptide in the triple mutants (V/L, S/E, G/A) corresponded to the 513LHM515 in zAO. For the single mutation, we intended to study whether the replacement of the L by a bulkier, (π-)electron-rich F could increase the E^0 at the T1 site, as proposed previously [6,9,16]. We did not choose an M (which is found in zAO at the homologue position) to replace the L, because a decrease in E^0 would be expected based on a previous study in an azurin (in which the mutation of the T1 Cu-ligating M to an L led to a 0.1 V E^0 increase) [33]. For the triple mutation, we intended to study how the amino acid composition change and the associated electrostatic and/or steric effects near the Cu sites might alter the redox and kinetic properties of laccase. Our results showed that the L/F change did not significantly affect the properties (including E^0) and the kinetics of the laccases, whereas the change of the tripeptide segment had major effects on the K_m, k_{cat}, fluoride (F^-) inhibition and pH-activity profile of the laccases.

EXPERIMENTAL

Materials and enzyme assays

Chemicals used as buffers and substrates were commercial products of at least reagent grade. Britton and Robinson buffers (pH 2.7–11, made by mixing 0.1 M boric acid/0.1 M acetic acid/0.1 M phosphoric acid with 0.5 M NaOH to the desired pH) were used as buffers unless indicated otherwise. Recombinant RsL (isoform-4) and MtL were purified as reported previously [19,20]. The protocols for molecular biology experiments (including restriction digests, DNA ligations, gel electrophoresis and DNA preparations) were adapted from either the instructions of the manufacturer (e.g. Stratagene) or standard procedures.

All oligonucleotides were synthesized by an Applied Biosystems 294 DNA/RNA synthesizer. Nucleotide sequences were determined by an Applied Biosystems automatic DNA sequencer, Model 373A, version 1.2.0. EPR spectra were obtained using a Bruker ER 220-D-SRC spectrometer. All samples were run at 77 K in a liquid nitrogen finger Dewar. Spectrometer settings were as follows: power 13 dB (10 mW), time constant 500 ms, modulation amplitude 20 G, sweep width 1100 G and centre field of 3050 G. To obtain the ground-state spin Hamiltonian parameters, $g_{//}$ and $A_{//}$, spectra were simulated using the QCPE (Quantum Chemistry Program Exchange) computer program. The atomic absorption spectroscopy was performed on a Perkin-Elmer 2380 instrument. The spectrum of laccase was recorded on a Shimadzu UV160U spectrophotometer with 1 cm quartz cuvette. The absorption coefficient at 280 nm was determined by amino acid analysis.

Laccase inhibition by NaF was carried out as reported previously [37]. The E^0 of T1 Cu in laccase was measured as previously reported [17–20,38], by using the redox titrant couples $Fe(2,2'\text{-dipyridyl})_2Cl_3/Fe(2,2'\text{-dipyridyl})_2Cl_2$ and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)/$ABTS^+$ for RsL mutants, and the couple $K_3Fe(CN)_6/K_4Fe(CN)_6$ for MtL mutants. The photometric Cu titration was carried out by Felsenfeld's method [39]. Laccase-catalysed oxidation of syringaldazine (SGZ) or ABTS and kinetic analysis were performed as previously reported [17–20,38]. These two substrates were chosen to assay the activity of the mutants because of the two classes of the pH-activity profiles that they would possess. As proposed previously, two opposing effects, one generated by the redox potential difference (ΔE^0) between the reducing substrate and the T1 Cu (which would correlate with the electron-transfer rate and be favoured for a phenolic substrate by higher pH) and another generated by the binding of OH^- to the T2/T3 Cu or by the deprotonation of the T2 Cu-bound H_2O (which would inhibit the activity at higher pH), would contribute to the pH-activity profile of RsL and MtL. For a non-phenolic substrate (such as ABTS), the contribution of ΔE^0 would be minimal and the effect of the OH^- binding to the T2 Cu would result in a profile in which the activity decreases monotonically as pH increases; whereas for a phenolic substrate (such as SGZ), the contribution of ΔE^0 would be significant and, combined with the effect of the OH^- binding to the T2 Cu, would result in a bell-shaped profile in which the maximal activity would be found at pH 6–7 [37].

Fermentation

A protocol with Cu supplement was applied for the fermentation. An aliquot of a spore suspension (1 ml, approx. 10^9 spores) of an *Aspergillus oryzae* transformant expressing the laccase variant of interest was added aseptically to each of several 500 ml shake flasks containing 100 ml of sterile medium [Nutriose 725, 50 g/l/$MgSO_4 \cdot 7H_2O$, 2 g/l/KH_2PO_4, 10 g/l/K_2SO_4, 2 g/l/$CaCl_2 \cdot 2H_2O$, 0.5 g/l/citric acid, 2 g/l/yeast extract, 10 g/l/amg trace metals (so-called because of use in fermenting amyloglucosidase; $ZnSO_4 \cdot 7H_2O$, 14.3 g/l/$CuSO_4 \cdot 5H_2O$, 2.5 g/l/$NiCl_2 \cdot 6H_2O$, 0.5 g/l/$FeSO_4 \cdot 7H_2O$, 13.8 g/l/$MnSO_4 \cdot H_2O$, 8.5 g/l/citric acid, 3.0 g/l)/0.5 ml/l/urea, 2 g/l; made with tap water and adjusted to pH 6.0 before autoclaving] and incubated at 34 °C on a rotary shaker at 200 rev./min for about 18 h. This culture (200 ml) was transferred aseptically to a 15 litre fermentor containing 11 litres of the fermentor media [Nutriose 725, 30 g/l/$(NH_4)_2HPO_4$, 2 g/l/yeast extract, 10 g/l (sterilized separately)/$MgSO_4 \cdot 7H_2O$, 2 g/l/citric acid, 4 g/l/K_2SO_4, 3 g/l/$CaCl_2 \cdot 2H_2O$, 2 g/l/amg trace metals, 0.5 ml/l/pluronic acid, 2 ml/l; made with tap water and adjusted to pH 6.0 before autoclaving]. Supplemental Cu (as $CuCl_2$, $CuSO_4$ or other salts) was made as a 400× stock in water or a suitable buffer, filter sterilized, and added aseptically to the tank to a final concentration of 0.5 mM. The fermentor temperature was maintained at 32 °C by the circulation of cooling water through the fermentor jacket. Sterile air was sparged through the fermentor at a rate of 11 litres/min (1 v/v/min). The agitation rate

was maintained at 700 revs./min. The pH of the fermentation medium was kept at 7.0 by the automatic addition of 5 M NaOH or 1.7 M H_3PO_4. Sterile feed (Nutriose 725, 270 g/l/urea, 30 g/l, filter sterilized separately/yeast extract, 15 g/l/pluronic acid, 2 ml/l) was added to the fermentor by peristaltic pump. The approximate feed rate profile during the fermentation was: 0–24 h, 0 g/h; 24–72 h: 2 g/h; 72–end: 5 g/h. Fermentations were typically run for 140–180 h, at which time the mycelia were separated from the broth with Miracloth.

Preparation of MtL mutants

The scheme for constructing a mutant MtL expression vector was similar to that published previously [20]. Briefly, a small DNA fragment containing the 3′-terminus of the *lcc-1* coding region (including stop codon) was generated by PCR using pRaMB5 as a template for *Pfu* polymerase with the primers (forward) 5′-dGTCGTCTACCTCGAGCGCGCC-3′ and (reverse) 5′-dGTCATCTAGACGCTCACGCCTTGACCAGCCA-3′, digested with *Xba*I plus *Xho*I, purified by agarose gel electrophoresis, then mixed in a three-part ligation reaction with an *Asp*718I–*Xho*I segment (1286 bp) of the *lcc-1* gene from pRaMB5 and pUC518 (a derivative of pUC118 containing additional restriction sites for *Bgl*II, *Cla*I, *Xho*I and *Nsi*I in the polylinker), which had been cleaved with *Asp*718I–*Xba*I. The resulting plasmid, pInt2.22, which contained approx. 1.5 kb of the *lcc-1* coding region, was extended from an internal *Asp*718I site through the stop codon that was followed immediately by an *Xba*I site. A single-stranded DNA template of pInt2.22 was prepared and used as a template for oligonucleotide-directed mutagenesis with the primer 5′-dGTAGACGACGCCGAAGCCGCCCGAGAC-3′ (for the L513F mutant). The mutant was identified by hybridization with radiolabelled oligonucleotide primer and verified by DNA sequence analysis. The next step in the construction of the expression vector involved two other plasmids. A derivative of pMWR3-SAN was constructed by replacing the *Apa*I–*Xba*I TAKA-amylase terminator fragment with a small linker (5′-CGGTACCGTCTAGAGTC-GCGATGCATC-3′ and 3′-CCGGGCCATGGCAGATCTCAGCGCTACGTAGGATC-5′), which introduced *Asp*718I, *Xba*I and *Nru*I cloning sites and inactivated the *Xba*I site of pMWR3-SAN. The resulting new vector was digested with *Swa*I and *Asp*718I and mixed in a three-part ligation with an 853 bp *Bsm*I–*Asp*718I fragment comprising the 5′ end of the *lcc-1* coding region and synthetic DNA adapter containing the translation initiation region (5′-ATGATGAAGTCCTTCATCAGCGCCGCGACGCTTTTGGTGGG-3′ and 3′-TACTACTTCAGGAAGTAGTCGCGGCGCTGCGAAAACCAC-5′) to generate the plasmid pInt1. Another plasmid pUC::AMGterm was constructed to contain a 597 bp *Aspergillus niger glaA* terminator DNA piece [isolated by PCR using pHD414 as a template with the primers (forward) 5′-dGGGTCTAGAGGTGACTGACACCTGGCGGT-3′ and (reverse) 5′-dTGACCCGGGAACTGGCCCCGACATTCCAGC-3′, which introduced *Xba*I and *Sma*I sites at the 5′ and 3′ ends of the terminator respectively, then cleaved with *Xba*I plus *Sma*I and subcloned into pUC118].

Finally, a 1.5 kb fragment containing the wild-type (wt) or mutant *lcc*-1 gene sequence was excised from pInt2.22 by digestion with *Asp*718I and *Xba*I, purified by agarose gel electrophoresis, and mixed in a three-part ligation with *Asp*718I- and *Nru*I-digested pInt1, plus a 597 bp *Xba*I–*Sma*I-digested *glaA* terminator fragment from pUC::AMGterm to produce an expression vector (pRaMB17T for the wt MtL or pRaMB17M for the L513F mutant; both re-sequenced to verify the mutations). The transformation of an *A. oryzae* was similar to that reported previously [20]. Briefly, equal amounts (approx. 5 μg each) of MtL expression vector and pToC90 (containing *A. nidulans amd*S gene) were added to approx. 10^6 protoplasts of *A. oryzae* HowB711 in suspension. Transformants were selected on Cove medium containing 1 M sucrose, 10 mM acetamide (as the sole nitrogen source) and 20 mM CsCl (as a background growth inhibitor). The transformants selected in this way were subsequently screened for the ability to produce MtL on Cove medium containing 1 mM ABTS. Positive transformants were purified twice through conidiospores and grown for 4–5 days at 37 °C in shake-flask cultures containing 25 ml of ASPO4 medium [$CaCl_2$, 1 g/l/$MgSO_4.7H_2O$, 1 g/l/KH_2PO_4, 5 g/l/citric acid, 2 g/l/urea, 1 g/l/$(NH_4)_2SO_4$, 2 g/l/yeast extract, 2 g/l/amg trace metals, 0.5 ml/l/malto dextrin, 20 g/l]. Cultures producing high levels of extracellular laccase activity, as assayed by MtL activity and SDS/PAGE, were fermented in a laboratory-scale fermentor.

To construct a plasmid for the V509L,S510E,G511A MtL triple mutant, the MtL gene from pRaMB17T (0.6 mg/ml) was amplified by PCR using the forward primer MTLTA, 5′-ggg*atttaaat*ATGAAGTCCTTCATCAGCGCC-3′, and reverse primer MTLTB, 5′-ggg*ttaattaa*<u>TTA</u>CGCCTTGACCAGCCACTCGCC-3′ (the lower-case letters indicating polynucleotide adapter sequences not present in the laccase gene; the italic letters indicating the restriction sites for *Swa*I in MTLTA and *Pac*I in MTLTB; the underlined letters showing the change of wt UGA stop codon to UAA). The resultant PCR fragment was cut with *Swa*I and *Pac*I and cloned into *Swa*I- and *Pac*I-cut plasmid pBANe6 to generate plasmid pJeRS31. A site-directed mutagenesis was performed on pJeRS31 with a MORPH Site-Specific Plasmid DNA Mutagenesis Kit (5 Prime $\geqslant$ 3 Prime; Boulder, CO, U.S.A.). The oligonucleotide 5′-ATACACAACTGGATGATGAAGTCCTTCATCAGCG-3′ was used to remove the *SwaI* site between the NA2 promoter and the initiator M of the MtL gene and to generate plasmid pBANe22 (for the V509L,S510E, G511A triple mutant). pBANe22 was transformed into the protoplasts of an *A. oryzae* strain JaL227 by incubating the protoplasts (2×10^7/ml) and DNA (5 μg) in 100 μl solution gently on ice for 30 min, followed by adding 250 μl poly(ethylene glycol) (PEG) solution [60 % PEG/10 mM $CaCl_2$/10 mM Tris (pH 7.5)] and incubating at 37 °C for 20 min. COVE Top Agar (7 ml; COVE salts/0.8 M sucrose/15 mM CsCl/10 mM acetamide/1 % low-melt agarose) was added prior to plating on COVE plates, which were incubated at 37 °C for 7 days. Transformants were spore-isolated and tested for laccase production using an ABTS assay. The top producer was grown in a 2 litre fermentor with a protocol similar to that described previously [20].

The purification of the recombinant laccases were similar to that described previously [20]. Briefly, the broth was first washed/concentrated by an Amicon Spiral Concentrator (on an S1Y100 membrane), then subjected to Q-Sepharose chromatography [Pharmacia XK-26 column, 120 ml gel; equilibrating/washing buffer, 10 mM Tris/HCl (pH 7.6); eluting buffer, 10 mM Tris/HCl/2 M NaCl (pH 7.6)]. The active fractions were subjected to a Mono-Q chromatography (Pharmacia 16/10 Hi-Load column, the same equilibrating/eluting buffers) and most of MtL activity was found in the pass-through fraction. Recovery yields of 33–65 % and 32–122-fold purification were obtained.

Preparation of RsL mutants

Site-specific mutations were introduced into the *rsl4* gene of the expression plasmid pJiWa59 [19], using the overlap extension PCR method together with the primers 5′-CATTGACTGGCACGTGTCGGGTGGGCTCGCACTTG-3′ (for the L466V, E467S,A468G triple mutant) and 5′-CTTGGAGGCTGGGT-

TCGCACTTGTC-3′ (for the L470F mutant). For each mutation, a 505 nt *Sac*1/*Nor*1 fragment was generated by PCR and used to replace the homologous fragment in pJiWa59 (5′-CATTGACTGGCACTTGGAGGCTGGGCTCGCACTTG-3′ or 5′-CTTGGAGGCTGGGCTCGCACTTGTC-3′). Both vectors were re-sequenced to verify the mutations. *A. oryzae* HowB711 was transformed using 8 μg of RsL vector plus 2 μg of pToC90 via a standard PEG-mediated protocol, and transformants were selected on minimal plates with 10 mM acetamide and 1 M sucrose and scored for laccase activity on minimal media with 10 mM acetamide and 1 g/l ABTS. Selected spores were spore-purified twice, fermented in 125 ml shake flasks (15 ml of MY51 medium) and the transformants giving the highest activity were further fermented in a laboratory-scale fermentor with a protocol similar to that reported previously [19]. Expressed RsL mutants were then purified from the culture broth with a protocol similar to that applied for wt RsL [19], comprised of ultrafiltration (on S1Y100 membrane) and chromatography on Q-Sepharose [equilibrating buffer, 10 mM Tris/HCl (pH 7.6); eluting buffer, equilibrating buffer plus 2 M NaCl; most of RsL activity fractions found in the pass-through fraction], SP-Sepharose [equilibrating buffer, 10 mM Mes (pH 5.5); eluting buffer, equilibrating buffer plus 1 M NaCl] and Superdex 200 [Pharmacia; equilibrating–eluting buffer, 10 mM Mes (pH 5.5)/0.2 M NaCl]. Recovery yields of 2–5 % and 150–280-fold purification were obtained.

RESULTS AND DISCUSSION

Effects of the single (L/F) mutation

The mutants exhibited chromatographic elution patterns similar to their wt counterparts. The purified mutants had the characteristic blue colour of laccase and showed typical laccase properties (Table 2). It was noted that the ratio of the absorbance at 330 nm to the absorbance at 600 nm in the RsL mutant (0.70) was lower than that in wt RsL (0.98), whereas the ratio in the MtL mutant (1.6) was higher than that in wt MtL (1.1). Both the atomic absorption and the photometric Cu titration yielded a Cu/subunit value of approx. 3 for the RsL mutant and approx. 3–4 for the MtL mutant.

At the position corresponding to M517 in zAO, both RsL and MtL have an L residue, which would not be expected to coordinate the Cu atom. The high E^0 of RsL indicated that an F at the position might not be a pre-requisite for a laccase to have a high E^0 ($\gg$ 0.5 V), as proposed previously [6,9,16]. To minimize effects from other structural differences (such as global protein stabilization and dielectric effects) that could arise when different laccases (such as RsL and TvL or MtL) are compared, we made a point mutation in the same laccase to replace the L in RsL (a representative 'high E^0' laccase) and in MtL (a representative 'low E^0' laccase) by a bulkier, (π)-electron-rich F, in an attempt to focus on the effect of the amino acid residue at this position. Due to its close vicinity to the T1 Cu, the substitution of L by F could have an impact on the electronic and geometric structure of the T1 site. To assess this possibility, we took EPR spectra to determine the ground-state spin Hamiltonian parameters. Our results showed that the mutation did not lead to any significant effect on $g_{\|}$ or $A_{\|}$ (Figure 1, Table 2), indicating that the L to F mutation did not alter the electronic and geometric structure of the T1 Cu.

It has been proposed that in blue Cu proteins the nature of the axial ligand affects E^0; stronger axial ligands help stabilize Cu(II), thereby lowering the E^0, whereas weaker axial ligands tend to destabilize Cu(II), thus raising the E^0. Several site-directed mutageneses of simple blue (T1) Cu proteins have shown that detectable effects (including -30 to $+100$ mV changes in E^0) could be induced when the T1 Cu axial ligand (M) was mutated to various other amino acids [30–36]. Thus, the nature of this axial amino acid residue has been shown to tune the E^0, at least to some extent. In our study, mutating the non-ligating L at this axial position to F did not result in a change in the E^0 of the T1 site. This result can be easily understood recognizing that neither L nor F would be expected to coordinate the T1 Cu and therefore would not directly donate charge to the Cu. Direct charge donation is not the only contribution to E^0; the protein environment can also affect E^0 through site dielectric, H-bonding, charged residues, etc. [40]. Because the L-to-F mutation does not alter E^0, it appears that they contribute similarly toward the dielectric associated with the protein environment.

For the single mutation, essentially no change was observed in the enzymic properties of the laccases. When SGZ and ABTS were used as the substrate, K_m and k_{cat} varied by less than 3-fold from the wt enzyme (Table 3). Figures 2 and 3 illustrate how K_m, k_{cat} and k_{cat}/K_m varied with pH when ABTS and SGZ were used as the substrate. Figure 4 shows the pH profiles of the NaF inhibition, as quantified by I_{50}, the [NaF] at which 50 % of the initial laccase activity was observed. Overall, pH profiles of both RsL and MtL single mutants were similar to those of their wt counterparts. The observed K_m and k_{cat} indicate that the effect of the L/F mutation on the kinetics of the substrate oxidation is minor. Because the geometric and electronic structure and E^0 of the the T1 Cu did not change (see above) and sequence comparisons with zAO indicate that the L/F mutation is probably not located at a position that could have close contact with the substrate binding [13], the single mutation was expected to have minimal impact on the binding and oxidation of a reducing substrate in the T1 pocket.

Table 2 Properties of RsL and MtL mutants

Molecular mass (MM; kDa) was estimated on SDS/PAGE. The observed smears were due to heterogenous glycosylation of the laccases by the host. λ_{max} values are shown with corresponding absorption coefficient (ϵ) values in parentheses. Units: λ_{max}, nm; ϵ, $mM^{-1} \cdot cm^{-1}$; $A_{//}$, cm^{-1}. For wt RsL, wt MtL, and MtL mutants, $g_{//}$ and $A_{//}$ were obtained by simulating the experimental EPR spectrum. For RsL mutants, $g_{//}$ and $A_{//}$ of the T1 Cu were obtained by inspection. The T2 parameters were difficult to obtain because of low sample concentration and impurity at low field.

	MM	λ_{max} (ϵ)	T1 $g_{\|}$	T1 $A_{\|}$	T2 $g_{\|}$	T2 $A_{\|}$
RsL wt	70–85	276 (66), 602 (4.7)	2.208	84×10^{-4}	2.265	160×10^{-4}
RsL single mutant	70–90	276 (63), 600 (3.7)	2.208	84×10^{-4}	–	–
RsL triple mutant	70–90	276 (63), 600 (4.8)	2.208	84×10^{-4}	–	–
MtL wt	85–90	276 (134), 589 (4.6)	2.201	87×10^{-4}	2.247	185×10^{-4}
MtL single mutant	70–90	280 (134), 600 (3.8)	2.201	87×10^{-4}	2.248	183×10^{-4}
MtL triple mutant	70–90	276 (134), 600 (2.9)	2.192	90×10^{-4}	2.247	175×10^{-4}

Effects of the triple mutations

The mutants exhibited chromatographic elution patterns similar to the wt enzymes and had the typical laccase optical properties (Table 2). The ratio of the absorbance at 330 nm to the absorbance at 600 nm in the RsL mutant (0.35) was lower than that in wt RsL; whereas the ratio in the MtL mutant (1.9) was higher than that in wt MtL. Both the atomic absorption and the photometric Cu titration yielded a Cu/subunit value of approx. 3 for the RsL mutant and approx. 4–5 for the MtL mutant. The triple mutation caused a change in the maximal absorbance

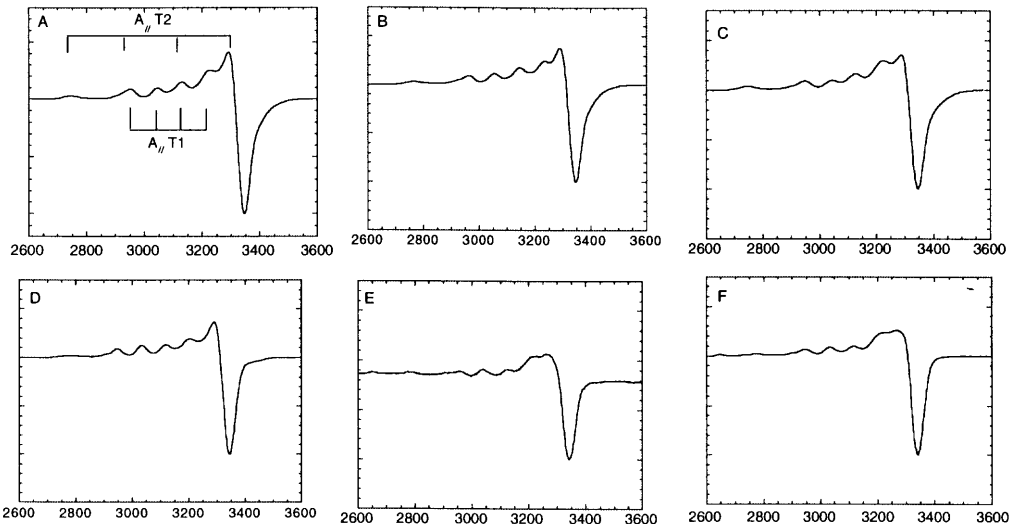

Figure 1 EPR spectra of the laccases

(**A**) MtL wt, (**B**) MtL single mutant, (**C**) MtL triple mutant, (**D**) RsL wt, (**E**) RsL single mutant, and (**F**) RsL triple mutant. Spectra were taken at 77 K with a power of 13 dB (10 mW) and a frequency of 9.501 GHz.

Table 3 Redox potential and substrate specificity of the mutants

Measured in 10 mM Mes, pH 5.5.

		SGZ		ABTS	
	E^0 (V)	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})
RsL wt	0.73 ± 0.02	28 ± 4	550 ± 40	52 ± 6	2500 ± 100
RsL single mutant	0.72 ± 0.02	35 ± 4	255 ± 11	75 ± 13	760 ± 30
RsL triple mutant	0.74 ± 0.02	3.9 ± 0.3	1.1 ± 0.1	60 ± 4	20 ± 1
MtL wt	0.48 ± 0.01	1.4 ± 0.2	4500 ± 200	110 ± 20	3800 ± 300
MtL single mutant	0.50 ± 0.02	1.8 ± 0.2	3000 ± 100	43 ± 3	1800 ± 100
MtL triple mutant	0.47 ± 0.02*	0.9 ± 0.1	360 ± 20	11 ± 2	530 ± 20

* In 10 mM potassium phosphate (pH 6) a value of 0.489 ± 0.004 V was observed.

wavelength (λ_{max}) of the T1 Cu, particularly in MtL at alkaline pH (Figure 5B). The origin of the pH dependence of the T1 λ_{max} in the wt enzyme is not understood and needs to be further investigated.

Among the fungal laccases with known E^0, the 'high E^0' TvL, RsL and PvL have an LEA tripeptide, in contrast with the VSG found in the 'low E^0' MtL and StL. From sequence alignment, these three amino acids correspond to the tripeptide 513LHM515 in zAO (Table 1), which is part of the T1 substrate-binding pocket [6,13]. Owing to its location, the negative charge on the E of the LEA tripeptide could affect various properties of the T1 Cu. However, this possible correlation between the nature of this tripeptide and E^0 was not found in the triple mutations of this study: switching LEA with VSG and *vice versa* did not significantly affect the E^0 of RsL or MtL (Table 3). In order to evaluate the effect of the triple mutation on the geometric and electronic structure of the T1 site, we studied the mutants by EPR. The ground-state spin Hamiltonian parameters, $g_{//}$ and $A_{//}$ (Figure 1, Table 2) were obtained by simulating the experimental spectra. These parameters were unaffected by the mutation indicating that the T1 and T2 sites remained unaltered. This is consistant with the lack of an effect of the mutation on the E^0 of the T1 site.

In terms of laccase kinetics, significant changes were found with the triple mutants. As can be seen from Table 3, the triple mutation resulted in a significant decrease in k_{cat} for both MtL and RsL when SGZ and ABTS were used as the substrate. The parameter k_{cat} is reflective of the rate-limiting step at pH 5.5, which has been shown to be electron transfer from the substrate to the T1 Cu [38]. The observed decrease in k_{cat} indicates that this electron-transfer step must have been affected by the triple mutation. According to Marcus and Sutin's theory [41], the rate of electron transfer is related to the activation energy ($\Delta G^{\neq}$) or Franck–Condon Barrier to electron transfer and the electron transmission coefficient (κ_{el}), i.e. the normalized probability that the electron will be transferred. The Franck–Condon Barrier is dependent on the free energy change (ΔG^0) for the electron transfer reaction (i.e. the difference in redox potential of the donor and acceptor) and the reorganization energy. It has been shown above that the triple mutation did not alter the E^0 of the T1 Cu and therefore the ΔG^0 ($= -F\Delta E^0$) between the substrate and the T1 Cu did not change. In addition, the mutation would not be expected to have an impact on the reorganization energy and thus the observed change in rate cannot be due to a change in the Franck–Condon Barrier. Alternatively, the mutation could have an effect on κ_{el}, which is dependent on the electronic-coupling matrix element (H_{DA}). H_{DA} is a function of the overlap of donor and acceptor orbitals through the protein and thus reflects the pathway for electron transfer. From sequence comparisons with zAO, the three mutated amino acids lie between the proposed substrate binding site and the T1 Cu, therefore it is likely that this mutation could affect the electron transfer pathway. The mutated amino acids would introduce electrostatic and steric changes to the nearby protein matrix, which could alter the overlap between donor and acceptor orbitals. The triple mutation also resulted in a decrease in K_m for both MtL and RsL when SGZ was the substrate and for MtL when ABTS was the substrate (Table 3). This decrease in K_m reflects a change in substrate binding that is best explained by a change in molecular recognition between laccase and substrate, which can be ascribed

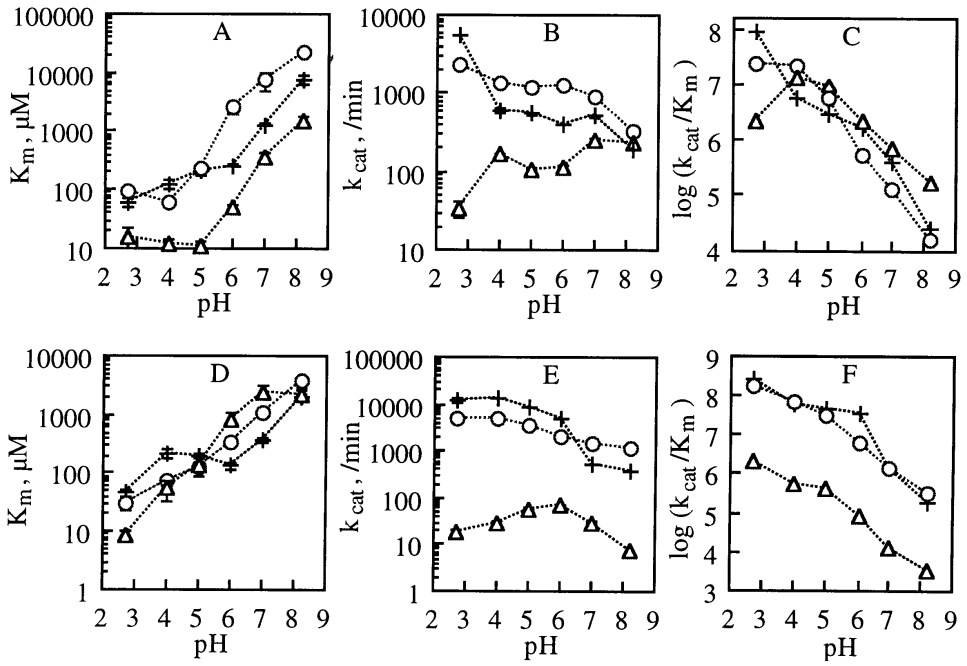

Figure 2 The pH profiles of K_m, k_{cat} and log(k_{cat}/K_m) for the non-phenolic substrate ABTS

Laccase studied: (**A**–**C**) 6–60 μM wt MtL (+), 46–330 nM MtL single mutant (○), 50–67 nM MtL triple mutant (Δ); (**D**–**F**) 26 nM wt RsL (+), 96 nM RsL single mutant (○), 0.3 μM RsL triple mutant (Δ). The unit of k_{cat}/K_m in (**C**) and (**F**) is $M^{-1} \cdot min^{-1}$. At pH 2.7, a substrate inhibition was observed for the RsL single mutant (at [ABTS] > 0.1 mM) and the MtL triple mutant (at [ABTS] > 1 mM). Because of this substrate inhibition and the instability of MtL triple mutant at pH 2.7, the K_m and k_{cat} values of the mutant shown in (**A**) and (**B**) could have larger uncertainty than the K_m and k_{cat} values obtained at other pHs.

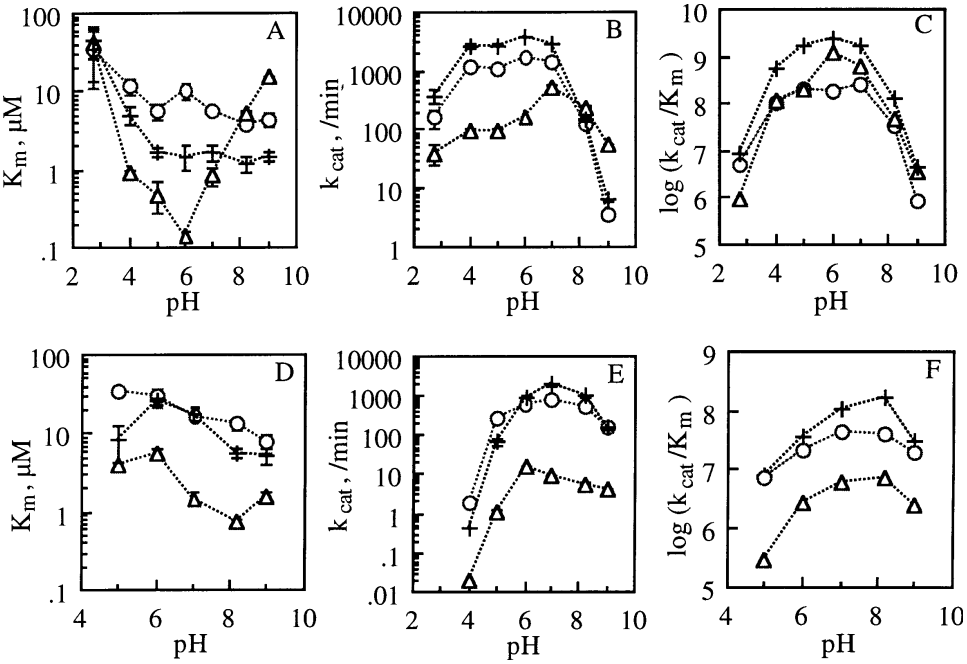

Figure 3 The pH profiles of K_m, k_{cat} and log(k_{cat}/K_m) for the phenolic substrate SGZ

Laccase studied: (**A**–**C**) 1.1–11 nM wt MtL (+), 11–110 nM MtL single mutant (○), 0.01–2.5 μM MtL triple mutant (Δ); (**D**–**F**) 0.23–2.3 μM wt RsL (+), 0.28 μM RsL single mutant (○), 0.75 μM RsL triple mutant (Δ). The unit of k_{cat}/K_m in (**C**) and (**F**) is $M^{-1} \cdot min^{-1}$.

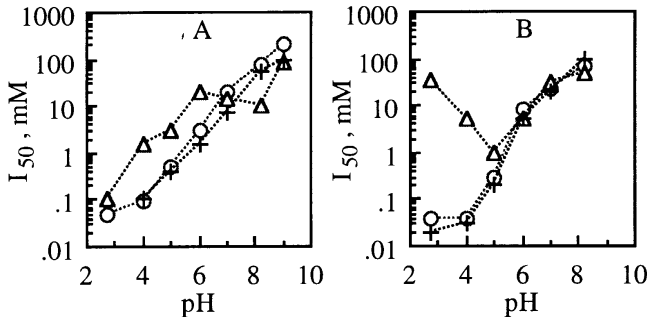

Figure 4 The inhibition of the laccases by NaF as a function of pH

The activity was based on the initial absorbance change rate at 405 nm with 1 mM ABTS. Laccase studied: (**A**) wt MtL (+), 0.17 μM; MtL single mutant (○), 0.03 μM for pH 2.7–7.0 and 0.3 μM for pH 8.2; MtL triple mutant (Δ), 0.25 μM for pH 2.7 and 0.05 μM for pH 4.0–8.2; (**B**) wt RsL (+), 0.02 μM for pH 2.7–7.0 and 0.23 μM for pH 8.2; RsL single mutant (○), 0.22 μM for pH 4.0–7.0 and 0.73 μM for pH 2.7 and pH 8.2; RsL triple mutant (Δ), 2.2 μM for pH 2.7–7.0 and 11 μM for pH 8.2.

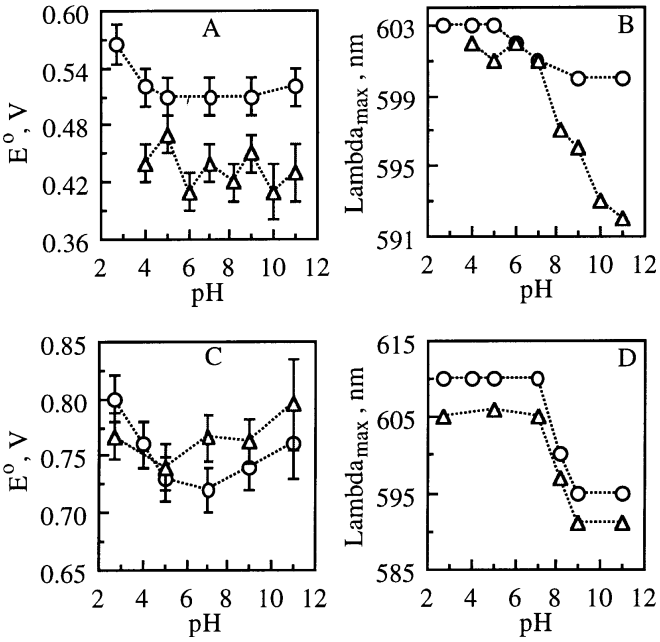

Figure 5 The pH profiles for the spectral and redox properties of the laccases

(**A**,**C**) Dependence of E^0 on pH. (**B**,**D**) Dependence of λ_{max} on pH. Laccase studied: (**A**,**B**) MtL; (**C**,**D**) RsL. Symbols: (○), wt; (Δ), triple mutant. Buffer: Britton and Robinson buffer. At pH 2.7, a slow but steady loss of the blue absorbance band was observed for the MtL mutant, indicating potential instability at this pH, and accurate E^0 and λ_{max} measurements were not obtained.

to electrostatic and steric changes in the substrate-binding pocket induced by the mutation.

The pH-dependent variations in E^0 for both the RsL triple mutant (from pH 2.7 to 11) and the MtL triple mutant (from pH 4 to 11) were similar to those of the wt enzymes (Figure 5), indicating that the redox potential difference [$\Delta E^0 = E^0$(laccase T1 Cu) − E^0(substrate)] was not significantly affected by the mutation. The variations in K_m, k_{cat} and k_{cat}/K_m with pH are illustrated in Figure 2 (for ABTS as the substrate) and Figure 3 (for SGZ as the substrate). When ABTS was used as the substrate, both the RsL and MtL triple mutants showed pH − k_{cat} and pH − (k_{cat}/K_m) profiles similar to those of the wt RsL and MtL. The pH-activity profile is dependent on (i) the ΔE^0 and (ii) at high pH, deprotonation of the H_2O molecule bound at the T2 Cu [37]. The similarity between the pH profiles indicates that neither of these factors was significantly altered by the mutation. However, the mutation had a noticeable effect on the pH − k_{cat} profile of the SGZ oxidase activity: the triple mutation in RsL resulted in an optimal pH (pH_{opt}) shift in the acidic direction, whereas the 'reverse' triple mutation in MtL resulted in an opposite pH_{opt} shift (in the alkaline direction) (Figures 3B and 3E). This pH_{opt} shift might be attributed to an altered protonation equilibrium of the phenolic substrate in the T1 substrate-binding pocket of the mutants. If SZG bound as an anion, then its interaction with the mutated E or S could affect the pH_{opt} as observed. The increase in K_m above pH 6 was observed for SGZ as a substrate and only in the triple mutant, which also exhibited a change in the λ_{max} of the T1 Cu at high pH (Figure 5B, see above). This effect would not be observed for ABTS because there is no protonation equilibrium in this substrate.

Anions such as OH^-, F^-, N^{3-} and CN^- have been shown to bind to the T2/T3 trinuclear cluster and inhibit protein activity [6,8–10]. Binding of F^- to the T2 Cu has been shown to decrease the potentials of the trinuclear cluster, thus decreasing the thermodynamic driving force ΔG^0 for internal electron transfer from the T1 to the trinuclear cluster. As the driving force decreases, the rate of internal electron transfer would decrease and could eventually become lower than the rate of electron transfer from the substrate to the T1 Cu (the rate-determining step of the unperturbed enzyme at low pH when H_2O is bound to the T2 Cu). Thus, internal electron transfer would become rate limiting. Figure 4 shows the pH profiles of the NaF inhibition, as measured by the ABTS oxidase activity of the laccases. Both the MtL and RsL (as well as MtL and RsL single mutants) showed similar pH dependence of I_{50}, indicating similar F^- and OH^- inhibitory interactions with the T2/T3 cluster [37]. However, the triple mutants exhibit a different pH dependence of I_{50} than the wt enzymes. At low pH the triple mutants required a higher concentration of NaF to achieve 50 % inhibition. These data can be understood by considering that the triple mutation in both MtL and RsL resulted in a decrease in k_{cat} when ABTS was used as the substrate. Because electron transfer from the substrate to the T1 Cu is the rate-limiting step, this decrease in k_{cat} in the triple mutant indicates that this step has become even slower than in the wt enzyme. This would mean that the rate of internal electron transfer (which appears to be unaffected by the mutation, see above) would have to decrease by more in the triple mutant than in the wt in order to become the rate-limiting step, which would require a higher concentration of NaF. This behaviour is observed in both the MtL and RsL triple mutants at low pH. At high pH (pH $\geqslant$ 7 for MtL and $\geqslant$ 6 for RsL) the H_2O, which is bound to the T2 Cu, is deprotonated and the resulting OH^- competes with F^-. Both OH^- and F^- inhibit enzyme activity and thus at higher pHs the rate of internal electron transfer is already decreased due to the T2-Cu-bound OH^-.

Concluding remarks

Although site-directed mutagenesis has been applied to simple blue Cu proteins (possessing one T1 Cu site) in an effort to elucidate their electron-transfer mechanism [22–34], a similar approach for multi-Cu blue oxidases (including laccase) had yet to

be reported. We describe here the first such study in which RsL, a laccase with high-T1 Cu E^0, and MtL, a laccase with low-T1 Cu E^0, were subjected to mutagenesis targeting a pentapeptide segment believed to be located near the T1 Cu site in laccase. Our results demonstrate that the replacement of an L by an F at the position corresponding to the T1 Cu axial ligand (M517 in zAO) does not significantly affect the properties (including E^0) of the T1 Cu. In contrast, the change on a tripeptide that is likely to be part of the T1 substrate-binding pocket had major effects on the laccases. An overall consideration of the effects on the spectrum, redox potential, kinetics, anion inhibition and pH dependence suggest a scheme in which the substrate-binding pocket and the electron-transfer path from the substrate to the T1 Cu were affected by the triple mutation. Our results indicate that it may be possible to regulate laccase catalysis by targeted protein engineering. The present work is still in its initial phase; a better understanding of the structure–function correlation for laccase (and other multi-Cu oxidases) awaits future studies and will be complemented by the availability of the three-dimensional structure of laccase [42].

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