Combinatorial Saturation Mutagenesis of the *Myceliophthora thermophila* Laccase T2 mutant: the Connection between the C-Terminal Plug and the Conserved $_{509}$VSG$_{511}$Tripeptide

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Abstract: A mutant laccase from the Ascomycete *Myceliophthora thermophila* has been submitted to iterative cycles of combinatorial saturation mutagenesis through *in vivo* overlap extension in *Saccharomyces cerevisiae*. Over 180,000 clones were explored, among which the S510G mutant revealed a direct interaction between the conserved $_{509}$VSG$_{511}$ tripeptide, located in the neighborhood of the T1 site, and the C-terminal plug. The $K_m^{O_2}$ value of the mutant increased 1.5-fold, and the electron transfer pathway between the reducing substrate and the T1 copper ion was altered, improving the catalytic efficiency towards non-phenolic and phenolic substrates by about 3- and 8-fold. Although the geometry at the T1 site was perturbed by the mutation, paradoxically the laccase redox potential was not significantly altered. Together, the results obtained in this study suggest that the $_{509}$VSG$_{511}$ tripeptide may play a hitherto unrecognized role in regulating the traffic of oxygen through the C-terminal plug, the latter blocking access to the T2/T3 copper cluster in the native enzyme.

Keywords: Ascomycete laccases, C-terminal plug, combinatorial saturation mutagenesis, redox potential, *Saccharomyces cerevisiae*.

INTRODUCTION

Laccases (EC 1.10.3.2) are blue multi-copper oxidases that catalyze the four-electron-reduction of O_2 to H_2O by one electron oxidation of the substrate [1,2]. The copper ion at the T1 site of the laccase sequesters one electron from the reducing substrate, transferring it to the trinuclear T2/T3 copper site where the molecular oxygen binds and is reduced to water [3]. Laccases from white-rot fungi are particularly outstanding biocatalysts due to their wide range of oxidising substrates. With the help of chemical or natural mediators, they can act on complex molecules such as the recalcitrant polycyclic aromatic hydrocarbons or lignocellulose material [4- 6].

Over the last decade, several residues in the neighbourhood of the catalytic copper ions have been subjected to site-directed mutagenesis to determine the parameters that define the catalytic activity and the redox potentials (Eº) of fungal laccases [7-9]. One consequence of these comprehensive structure-function studies has been the generation of a collection of mutants with structural perturbations at the T1 copper centre.

In recent years, combinatorial saturation mutagenesis has successfully been used to study structure-function relationships in many enzymes and to improve several enzymatic characteristics in the frame of laboratory evolution. Using this approach, codons are mutated to all other codons encoding the 20 naturally occurring amino acids, exploring all possible combinations of targeted amino acids to identify the optimal environment and geometry for any desired function. Iterative cycles of saturation mutagenesis can drive the discovery of new interactions that may not be revealed by conventional site directed mutagenesis [10].

The *Myceliophthora thermophila* laccase T2 mutant (MtLT2) expressed in *Saccharomyces cerevisiae* [11], constitutes an excellent scaffold to perform semi-rational approximations and *in vitro* evolution experiments [12-14]. In the *Myceliophothora thermophila* laccase, the non-coordinating axial ligand at position 513 seems to be involved in the Eº of the T1 site [7,8]. More significantly, the $_{509}$VSG$_{511}$ tripeptide in the vicinity of the reducing substrate binding site has been submitted to directed mutagenesis studies, although its role in the Ascomycete laccases remains uncertain [8].

In order to further understand the role of these key residues in the Ascomycete laccases, we have employed iterative combinatorial saturation mutagenesis to explore several regions of the MtLT2 gene. By determining the oxygen affinity at the T2/T3 copper cluster, coupled with an electrochemical and spectroscopic analyses, we evaluated whether the local biomolecular changes somehow affected intrinsic features of the selected variants. To the best of our knowledge, this is the first study where *semi-rational* tools (*i.e.* combinatorial saturation mutagenesis coupled to high-

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throughput screening protocols [10]) have been applied to thoroughly study the laccase.

EXPERIMENTAL PROCEDURES

The parental MtLT2 laccase gene was engineered as reported elsewhere [11]. The laccase from *Trametes hirsuta* was kindly donated by Prof. A. Yaropolov (Institute of Biochemistry, Moscow, Russia) and $K_4[Mo(CN)_8]$ was synthesized and purified according to a previously published method [15]. The mediators $K_4[W(CN)_8]$ and $K_4[Os(CN)_6]$ were kindly provided by Prof. Kenji Kano (Kyoto University, Japan). ABTS (2,2´-azino-bis(3-ethybenzthizoline-6-sulfonic acid)), 2,6 dimethoxyphenol, $K_4[Fe(CN)_6]$ and the *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). The *E. coli* XL2-blue competent cells and high-fidelity polymerase *Pfu*-Ultra were from Stratagene (La Jolla, CA) and the protease deficient *S. cerevisiae* strain BJ5465 was from LGCPromochem (Barcelona, Spain). The uracil independent and ampicillin resistance shuttle vector pJRoC30 was obtained from Novozymes (Davis, CA), while the zymoprep yeast plasmid miniprep kit, zymoclean gel DNA recovery kit, and the DNA clean and concentrator TM-5 kit were all from Zymo Research (Orange, CA). QIAprep spin miniprep kits were purchased from QIAGEN (West Sussex, UK) and the restriction enzymes *BamH*I and *Xho*I were from New England Biolabs (Hertfordshire, UK). All chemicals were of reagent-grade purity.

Culture Media

Minimal medium contained 100 ml 6.7% sterile yeast nitrogen base, 100 mL 19.2 g/L sterile yeast synthetic drop-out medium supplement without uracil, 100 mL sterile 20% raffinose, 700 ml *dd*H_2O and 1 mL 25 g/L chloramphenicol. YP medium contained 10 g yeast extract, 20 g peptone and *dd*H_2O to 650 mL. Expression medium contained 720 mL YP, 67 mL 1 M KH_2PO_4 pH 6.0 buffer, 10 µL 1 M $CuSO_4$, 111 mL 20% galactose, 1 mL 25 g/L chloramphenicol and *dd*H_2O to 1000 mL. YPAD solution contained 10 g yeast extract, 20 g peptone, 100 mL 20% sterile glucose, 100 mg adenine hemisulphate, 1 mL 25 g/L chloramphenicol and *dd*H_2O to 1000 ml. SC drop-out plates contained 6.7 g yeast nitrogen base, 100 mL 19.2 g/L sterile yeast synthetic drop-out medium supplement without uracil, 20 g bactoagar, 100 ml 20% sterile glucose, 1 mL 25 g/L chloramphenicol and *dd*H_2O to 1000 ml. The SC drop-out plates for the pre-screening also contained the following ingredients (final concentrations): $CuSO_4$ 10 µM, ABTS 200 µM, Buffer KH_2PO_4 pH 6.0, 60 mM and galactose 2 g/L instead of glucose.

Combinatorial Saturation Mutagenesis by *In Vivo* Overlap Extension (IVOE) [12].

In each case, two separate PCR reactions (thermocycler Mycycler, Bio-Rad, USA) were carried out simultaneously to amplify the two DNA fragments that overlapped at the positions corresponding to the regions targeted for combinatorial mutagenesis of the laccase sequence. PCR reactions were performed in a final volume of 50 µL containing 0.25 µM of each primer, 100 ng of template, dNTPs (0.25 mM each), 3% dimethyl sulfoxide (DMSO) and 2.5 Units of Pfu-Ultra DNA polymerase. The PCR conditions were performed as follows: 95ºC for 2 min (1 cycle), 94 °C for 0.45 min, 55 °C for 0.45 min, 74 °C for 2 min (28 cycles), and 74 °C for 10 min (1 cycle). The PCR fragments were cleaned and concentrated (DNA clean and concentrator TM-5 Kit, Zymo Research), loaded on low-melting-point preparative agarose gels and purified using the Zymoclean gel DNA recovery kit (Zymo Research). The pJRoC30 plasmid containing MtLL1 gene (11) was linearized with the restriction enzymes XhoI and BamHI (New England Biolabs, UK) and the linearized vector was cleaned, concentrated and purified as described above for PCR fragments.

a) Combinatorial Saturation Mutagenesis of S510 and L513

The primers for 1st PCR were RMLNsense (5'-CCTCTA TACTTTAACGTCAAGG-3', binding to bp 5'-160-180-3' of pJRoC30) and 3CPOantisense (5´-GGTAGACGACGCC **SNN**GCCGCC**SNN**GACGTGCCAGGCGAT-3´, binding to bp 5´-1859-1899- 3´ of pJRoC30), and for the 2nd PCR the primers were 3CPOsense (5´-ATCGCCTGGCACGTC**NNS** GGCGGC**NNS**GGCGTCGTCTACC-3´, binding to bp 5´-1859-1899-3´ of pJRoC30) and RMLCantisense (5' GGG AGGGCGTGAATGTAAGC 3', binding to bp 5'-2139-21 58-3' of pJRoC30).

b) Combinatorial Saturation Mutagenesis of $_{509}VGG_{511}$

The primers for the 1st PCR were RMLNsense and TRIR-antisense (5'-GGTAGACGACGCCCAAGCC**SNNSNNSNN** GTGCCAGGCGATGTGGCAGTGG-3', binding to bp 5'-1849-1898-3' of pJRoC30), and for the 2nd PCR they were TRIR-sense (5'-CCACTGCCACATCGCCTGGCAC**NNSN NSNNS**GGCTTGGGCGTCGTCTACC-3', binding to bp 5'-1849-1898-3' of pJRoC30) and RMLCantisense.

c) Combinatorial Saturation Mutagenesis of Positions 558 and 559

Primers for the 1st PCR were RMLNsense and BOB-antisense (5'-CCTCCTCGACCCAGCGGCGCTT**SNNSNN** CGAGTCGGACTTGGGGTTGGGGTTGG-3', binding to bp 5'-1992-2045-3' of pJRoC30) and for the 2nd PCR, the primers were BOB-sense 5'-CCAACCCCAACCCCAAGTC CGACTCG**NNSNNS**AAGCGCCGCTGGGTCGAGGAGG-3´, bindinf to bp 5'-1992-2045-3' of pJRoC30) and RML Cantisense.

d) Combinatorial Saturation Mutagenesis of the C-Terminal Plug ($_{556}DSGL_{559}$)

Primers for the 1st PCR were RMLNsense and PLUG-antisense (5'-CCTCCTCGACCCAGCGGCGCTT**SNNSNN SNNSNN**GGACTTGGGGTTGGGGTTGG-3', binding to bp 5'-1992-2045-3'of pJRoC30) and the primers for the 2nd PCR were PLUG-sense 5'-CCAACCCCAACCCCAAGTCC **NNSNNSNNSNNS**AAGCGCCGCTGGGTCGAGGAGG-3', binding to bp 5'-1992-2045-3' of pJRoC30) and RMLCantisense.

The codons submitted to saturation mutagenesis are in bold and underlined, where **N** is (A+T+C+G) and **S** is (G+C).

In vivo overlap extension and cloning in *Saccharomyces cerevisiae*: PCR fragments (200 ng each) were mixed with the linearized vector (100 ng, 4:1 ratio PCR-product:vector) and transformed into competent yeast cells using the yeast transformation kit (Sigma, Madrid, Spain). Immediately

before thermal shock, 10% DMSO was added to the transformation mixture to improve transformation efficiency (close to 20,000 clones per transformation reaction). Transformed cells were plated in SC drop-out plates and incubated for 2 days at 30 °C. Colonies containing the whole autonomously replicating vector were submitted to the high-throughput screening (HTPS) assay.

HTPS Protocol

Screening in liquid format (in 96-well plates) was performed as published previously [12], using ABTS as the substrate and with the help of liquid handlers (Quadra96-320, Tomtec, Hamden, CT) and microplate readers (VersaMax, Molecular Devices, Sunnyvale, CA). When more than two positions were mutated, pre-screening was carried out in a solid format. SC-drop out plates for expression and detection of laccase activity against ABTS were plated with transformants and those colonies that developed a green *halo* were examined in the standard liquid screen.

DNA Sequencing

Plasmid-containing variant laccase genes were sequenced using an Applied Biosystems 377 automated fluorescent DNA sequencer at the Core Sequencing Facility of the "Instituto de Investigaciones Biomedicas, CSIC, Madrid." The primers used were as follows: forward IG88-S and mtlsq2 (5′-GAAGGGCACCAACCTGC-3′, binding to bp 5′-643-659-3′ of pJRoC30); reverse mtlsq3 (5′-CGCACGTAAAAGTCGTGG-3′, binding to bp 5′-1657-1673-3′of pJRoC30) and IG88-R.

O_2-Reduction Measurements

Parental and mutant proteins were produced in *S. cerevisiae* and purified exhaustively as reported elsewhere [12]. The O_2-reduction activity of the laccases was measured by mass spectrometry (Masstor 200 DX quadrupole, VG Quadrupoles Ltd.) in 10 mM acetate buffer pH 4.5, at 27 °C, and with 3.0 mM ABTS as the electron donor. The measuring cell had a volume of 10 mL and it was separated from the mass spectrometer by a Teflon membrane through which gasses from the solution diffuse [16]. The spectrometer response at certain mass values is proportional to the partial pressure of the gas in the cell solution. Although the principal peak of the spectrum was at mass 32, O_2-uptake was measured at mass 16 since there was less background signal. The solution saturated with pure O_2 was used for calibration and the background signal was estimated with the solution purged with pure Ar. O_2-consumption by the mass spectrometer (measured in the absence of laccase for each O_2 concentration) was subtracted from the activity measurements when 5-25 μg of laccase was added.

Electrochemical and Spectroscopic Characterization

Spectroelectrochemistry of purified laccases (the MtL variants as well as *Trametes hirsuta* laccase) was carried out using a micro-spectroelectrochemical cell with a gold capillary electrode as described elsewhere [17]. The potential of the gold capillary of the cell was controlled by a three-electrode potentiostat BAS LC-3E (Bioanalytical Systems, BAS, West Lafayette, IN, USA). In these measurements an $Ag|AgCl|KCl_{sat}$ reference electrode (BAS) and a platinum counter electrode were used. The absorbance spectra were monitored with on PC2000-UV-VIS, a miniature optic fiber spectrometer from Ocean Optics (Dunedin, FL, USA) with an effective range between 200 and 1100 nm. Pre-treatment of the gold capillary working electrode in the spectroelectrochemical cell was carried out by washing the cell capillary with a peroxide-sulfuric acid mixture and then rinsing with Millipore water.

The redox potential (Eº') of the T1 site of the enzyme was determined by mediated redox titration (MRT) using the spectroelectrochemical set-up described above. A complex mediator system containing four different mediators ($K_4[Fe(CN)_6]$, $K_4[W(CN)_8]$, $K_4[Os(CN)_6]$, and $K_4[Mo(CN)_8]$ with formal redox potentials of 430 mV, 520 mV, 640 mV, and 780 mV *vs* NHE, respectively) was used to perform the MRT in accordance with previously published methods [13,14,18]. Since the enzymes could be reversibly cycled between their fully oxidized and fully reduced states, titrations were carried out in both directions, from the fully oxidized to the fully reduced state of the enzymes (reductive titration) and vice versa (oxidative titration). However, significant fading of the blue color of both laccases (up to 20%) was observed even after a single titration cycle (oxidized laccase→reduced laccase→oxidized laccase). Thus, to calculate the Eº' the average points from both directions were used. It is important to note that the equilibration of the blue copper center at each potential applied was apparent from the stabilization of the absorbance at ~600 nm. Because the redox mediators used in our studies are transparent above 500 nm, the spectral changes at 600 nm were only attributed to the blue copper centers of laccases. It should be also emphasized that additional redox titration of the well-characterized *Trametes hirsuta* laccase was also performed as a control of the equilibration time, ratio between the redox mediators and the enzyme, buffer composition, solution pH, etc. Indeed, the calculated Eº'-value of the T1 site of *Trametes hirsuta* laccase (~780 mV *vs* NHE) was in excellent agreement with previously published data [19-21].

Cyclic voltammetry measurements: laccase-modified spectrographic graphite electrodes (SPGE) served as the working electrodes. The surface of the SPGE (type RW001, 3.05 diameter, 13% porosity) from Ringsdorff Werke GmbH (Bonn, Germany) was prepared by polishing on fine wet emery paper (Tufback Durite, P1200, Allar Co. Inc., Sterling Heights, MI, USA), and it was then rinsed thoroughly with Millipore water and allowed to dry. A volume of 10 μL of laccase solution was adsorbed by the electrode surface and after 15 min, the electrode was again rinsed with water. Cyclic voltammograms of the laccase-modified electrode were recorded using a CV-50W three-electrode potentiostat (BAS). The reference electrode was an $Hg_2|Hg_2Cl_2|KCl_{sat}$ electrode (SCE, 242 mV *vs* NHE) and platinum wire served as the counter electrode. The supporting electrolytes were 0.1 M citrate-phosphate buffers pH 4.0 and 7.0.

Electron paramagnetic resonance -EPR- analysis of the copper sites in purified laccases: EPR measurements were made with a Bruker ER200D instrument operating in the X-band (ν≈ 9.6 GHz) using a DPPH standard for frequency calibration (in a T-type double cavity). Samples in potassium phosphate buffer were introduced into a spectroscopic quartz probe cell. The spectra were recorded at 77 K, typically at: 19.5-milliwatt microwave power (no signal saturation was

apparent in independent experiments up to 40 milliwatts); 100 kHz modulation frequency; 2G modulation amplitude; 40 ms time constant; and 1 x 10^5 receiver gain.

Protein Modeling

The MtLT2 structural model constructed on the basis of the crystallographic structure of a homologous (75% sequence identity) laccase from *Melanocarpus albomyces* (PDB id: 1GW0) has been described previously [12]. Protein structures were analyzed with DeepView/Swiss-Pdb Viewer [22,23]

RESULTS AND DISCUSSION

Combinatorial Saturation Mutagenesis of L513 and S510

We initially applied combinatorial saturation mutagenesis to the L513 residue, the axial non-coordinating ligand thought to be essential for Eº at the T1 site, as well as to S510 from the $_{509}$VSG$_{511}$ tripeptide common to the low to medium Eº laccases. This mutagenesis was performed by *in vivo* overlap extension (IVOE), method recently developed in our laboratory [12]. This method takes advantage of the eukaryotic machinery of *Saccharomyces cerevisiae* to perform the splicing and recombination of a linearized plasmid with the mutated fragments in a single step, giving rise to an autonomously replicating plasmid. Accordingly, 50-54 bp overhangs were designed for the *in vivo* overlap in *S. cerevisiae*.

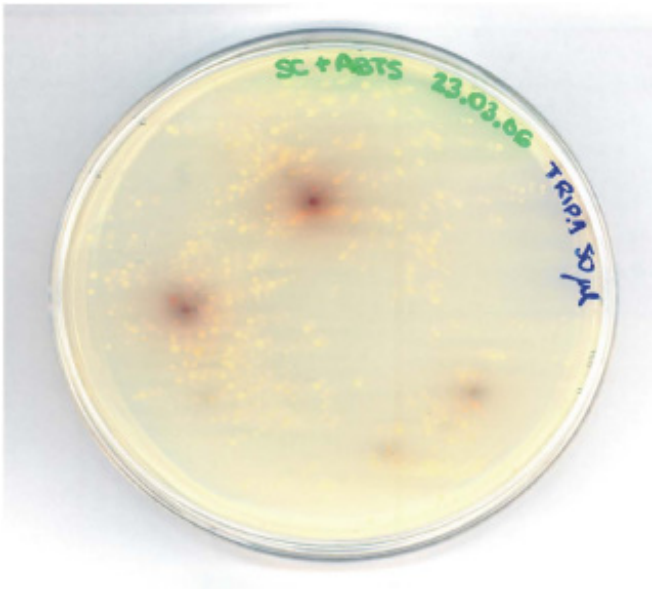
After exploring over 1700 clones to isolate an improved activity against ABTS, only one variant (mutant S510G) showed significantly better kinetics than the parental type (see below). When this mutant was sequenced, one synonymous mutation was detected, L513L (CTG/TTG), as well as the S510G mutation (TCG/GGG). It is important to point out that the latter cannot be achieved by single base substitution (i.e. conventional error-prone PCR) since it is dependent on two consecutive nucleotide changes. Regarding the axial ligand L513, our results suggest that this strictly conserved residue has already achieved the highest level of adaptation in MtLT2 and any change to the remaining 19 natural amino acids would not improve the catalytic capacity. This result is in good agreement with previous studies where the axial ligand of *Myceliophthora thermophila* laccase was mutated by site-directed mutagenesis (L513F mutant) without showing significant change in the turnover of phenolic and non-phenolic substrates [8, 9].

Combinatorial Saturation Mutagenesis of $_{509}$VGG$_{511}$

It should be noted that the amino acid composition of the $_{509}$VSG$_{511}$ sequence might be important for the catalytic behavior of Ascomycete laccases (including the Eº'-value of the T1 site). However, while the rates were drastically reduced when the $_{509}$VSG$_{511}$/$_{509}$LEA$_{511}$ triple mutant was studied previously (from 3800 min^{-1} to 530 min^{-1} with ABTS as substrate), the redox potential was not significantly altered [8]. Hence, how these residues influence the activity of the fungal laccase remains uncertain.

Using the S510G mutant as our point of departure, all the possible changes in the $_{509}$VGG$_{511}$ tripeptide were explored. A statistical analysis of the libraries generated by NNG/C randomisation implies that when 2 codons are randomized, 400 variants must be screened at the amino acid level rather than >3000 variants at the DNA level. Multiple saturation mutagenesis (3 or more) generates libraries with large numbers of variants that cannot really be explored by conventional high-throughput methods (*i.e.* liquid microcultures in 96 well plates). Therefore, a solid-format pre-screening was used to discriminate the clones that exhibited weaker activity than the parental clone (Fig. **1a**).

(**A**)



(**B**)

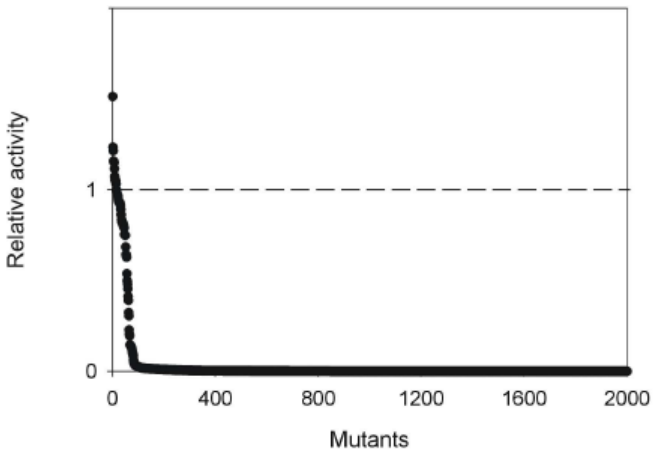


Fig. (1). (**A**) Solid format pre-screening using SC-drop out plates with galactose instead of glucose for laccase expression and ABTS to detect activity (see Materials and Methods for details). Clones with an intense green-purple halo were picked and further characterized. (**B**) Landscape of the library constructed by saturation mutagenesis at positions 558 and 559. Activity against ABTS oxidation is plotted in decreasing order and the dotted line shows the activity of the parental type in the assay.

After pre-screening over 20,000 clones, only ~2% of the library (400 clones) displayed activity close to that of the parental S510G mutant. These clones were further submitted to a standard liquid screen and the selected variants were sequenced and re-incorporated into the S510G parental mutation. These data indicate that in accordance with earlier studies, the *M. thermophila* laccase structure does not toler-

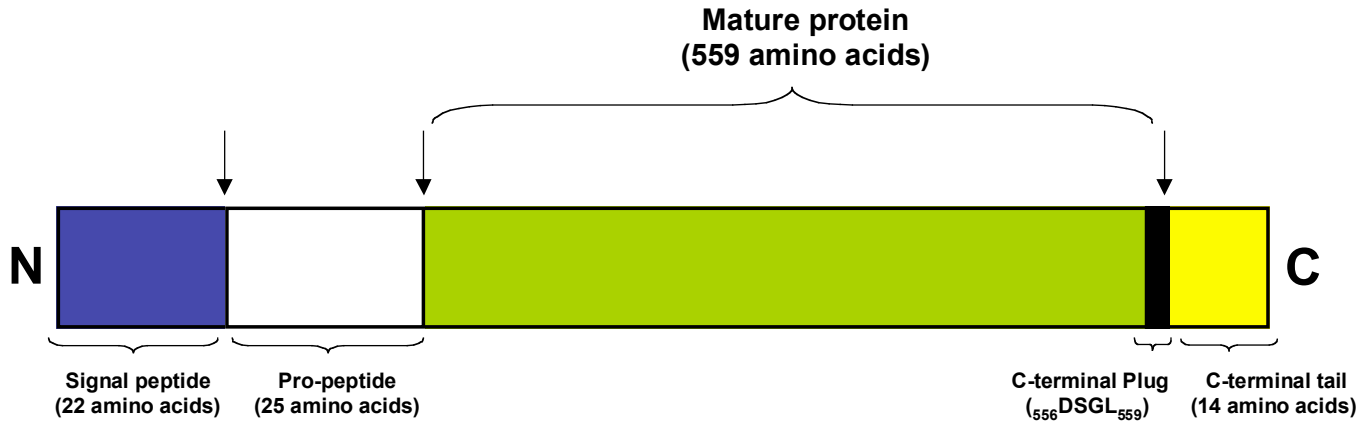


Fig. (2). *Myceliophthora thermophila* laccase gene. The arrows indicate the cleavage positions.

ate mutations at positions 509 and 511 of $_{509}$VSG$_{511}$ tripeptide without a loss of catalytic performance [8]. Indeed, the selection of S510G as the only positive mutation from the combinatorial saturated mutated library corroborates the significance of this position for enzyme activity.

Combinatorial Saturation Mutagenesis of the C-Terminal Plug $_{556}$DSGL$_{559}$

At this point it is worth noting that the *Myceliophthora thermophila* laccase gene is a complex scaffold for directed evolutionary studies or for *semi*-rational approximations. During its maturation and cellular secretion, the protein undergoes 3 processing events to generate the mature 559 amino acid protein: i) cleavage of the leading signal sequence (22 residues); ii) cleavage of the pro-leader (25 residues); iii) cleave of the C-terminal tail (14 residues: Fig. **2**). The C-terminal tail seems to fulfill an important role during protein folding and it is finally processed by the *Kex*2 protease in the Golgi of heterologous hosts [13]. As a consequence, the C-terminus of the mature protein ends in a 4 residue plug ($_{556}$DSGL$_{559}$) that blocks the access of oxygen and water to the trinuclear T2/T3 copper cluster [24]. The closure of this entrance affects the function of Ascomycete laccases and it is thought that a yet unknown interaction regulates the conformational changes that eventually allow the transit of oxygen to the T2/T3 site.

We constructed a reliable molecular model to map the S510G mutation in the laccase structure (see Materials and Methods). The S510G substitution interrupts the interaction established by a hydrogen bond between S510 and D556 that contributes to the C-terminal plug, affecting the association between these two regions (Fig. **3**). A more detailed inspection of the molecular model revealed a complex network of interactions through hydrogen bonds at the C-terminal plug, not only with S510 but also with other surrounding residues (S142, I382 and V509: Table **1**, Fig. **3**).

As mentioned above, the C-terminal plug is followed by a C-terminal tail of 14 residues that is processed in the mature protein (Fig. **2**). Partial alignment of the amino acid sequences of MtLT2, the S510G mutant and other known Ascomycete laccases indicates that the C-terminal plug may or may not be followed by cleavable extensions (Fig. **4**), and no such conservation was found among Basidiomycete laccases. The processing of the C-terminal tail along with the

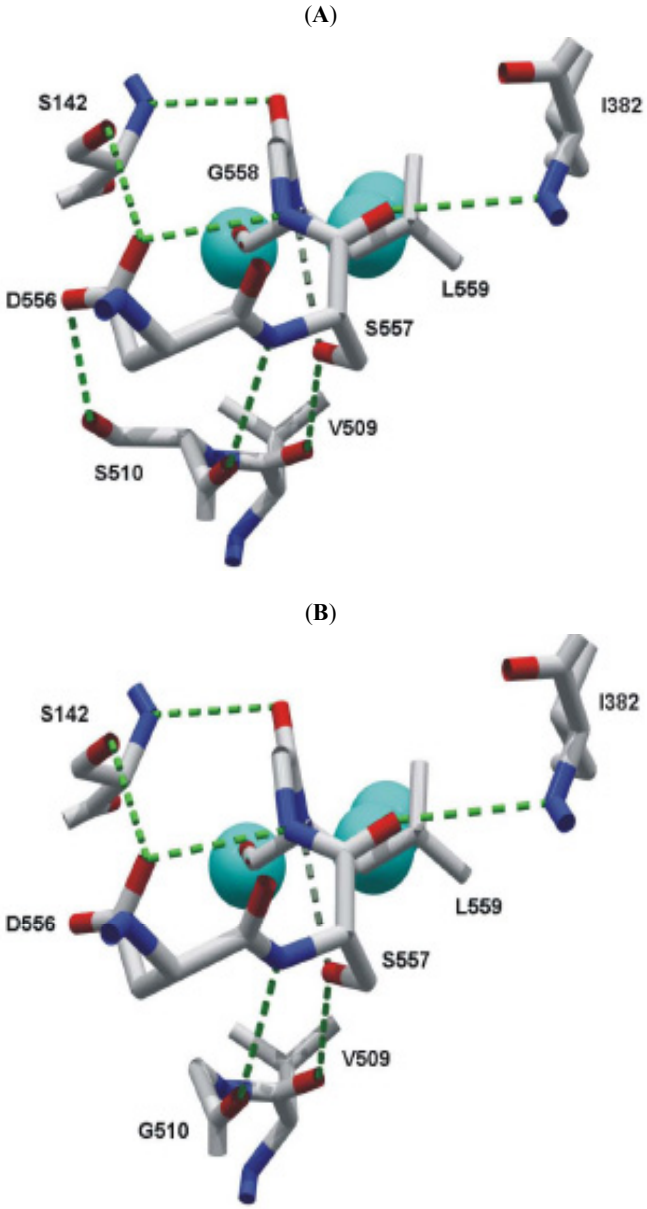


Fig. (3). Interactions of the C-terminal plug ($_{556}$DSGL$_{559}$) with the neighboring residues in (**A**) MtLT2 and (**B**) mutant S510G. In the S510G mutant, the hydrogen bond between positions 510 and 556 no longer exists. The blue spheres represent the copper atoms.

Table 1. Hydrogen Bond Interactions of the MtLT2 C-Terminal Tetrapeptide (DSGL)

C-Terminal Plug	Interactions			
D556	S142	S510	G558	
S557	I382	V509	S510	L559
G558	S142	D556		
L559	S557			

increase in the activity upon mutation at position 510 (see below) strongly suggests that the C-terminal plug plays an important structural role in Ascomycete laccases, as suggested previously [24]. This information encouraged us to explore the C-terminal plug in the S510G mutant by combinatorial saturation mutagenesis. Since position 559 is the least conserved position in the plug (Ile/Leu/Val depending on the organism, see Fig. (**4**)), the L559 and its neighboring G558 residue were the first to be submitted to combinatorial saturation mutagenesis. Accordingly, 96% of the clones obtained displayed less than 10% of the parental activity, which indicates that the amino acids targeted were essential for the activity of the laccase (Fig. **1b**). Indeed, only 0.7 % of the 2,000 clones screened showed similar or slightly better activity than that of the parental structure. Selected variants were sequenced, purified and characterized, and among the most significant nucleotide changes, we found the variants: 2D8 ($G_{(GGC)}558N_{(AAC)}$; $L_{(CTC)}559N_{(AAC)}$, with a 1.0 fold-improvement), 8C1 ($G_{(GGC)}558R_{(AGG)}$; $L_{(CTC)}559K_{(AAG)}$, with a 0.5-fold-improvement); 18D9 ($G_{(GGC)}558P_{(CCC)}$; $L_{(CTC)}559P_{(CCC)}$, with suppressed activity); and 7D7 ($G_{(GGC)}558G_{(GGG)}$; $L_{(CTC)}559G_{(GGG)}$, with suppressed activity). All these variants displayed lower kinetics than the parent type for phenolic and non-phenolic substrates (data not shown).

Finally, when the whole C-terminal plug $_{556}$DSGL$_{559}$ was submitted to combinatorial saturation mutagenesis, 160,000 clones were pre-screened of which only 1.93% were active. Once again, after screening in the liquid format none of these clones displayed significant improvements in activity.

Effect of the S510G Mutation on Laccase Function

After exploring over 180,000 clones generated by iterative cycles of combinatorial saturation mutagenesis of two structurally relevant related regions, the $_{509}$VSG$_{511}$ tripeptide and the $_{556}$DSGL$_{559}$ C-terminal plug, only the S510G mutation showed a clear effect on laccase activity. The S510G and MtLT2 mutant (which contains the original Ser510) were characterized biochemically, demonstrating a reduction in the thermostability of the S510G mutant and alterations to the pH profile in the acidic region (Fig. **5**). When the catalytic efficiency against phenolic and non-phenolic substrates was assessed, the activity of the S510G mutant on non-phenolic and phenolic substrates improved 3.3 and 8 fold, respectively (Table **2**). The dramatic reduction in the K_m for 2,6 dimethoxyphenol indicates that the S510G mutation might allow a better fit of the phenolic structures into the pocket of the T1 site.

A new method to measure the kinetics of O_2-reduction catalyzed by laccases was set up and an enzymatic reaction cell connected to a mass spectrometer was used to directly estimate the oxygen consumption in the presence of the ABTS substrate. In this way, the rate of O_2-uptake in the reactor cell can be measured at different concentrations of the gas and the $K_m^{O_2}$ of laccases determined. The k_{cat} values measured for the parental and mutant enzymes in this assay follow the same trend as those measured by the colorimetric assays (Table **2**). Interestingly, the $K_m^{O_2}$ values were increased by the mutation (Table **2**), highlighting the possible role of the C-terminal plug in regulating the entry and exit of O_2 and H_2O from the T2/T3 cluster through a channel, in agreement with previous theories about the function of the

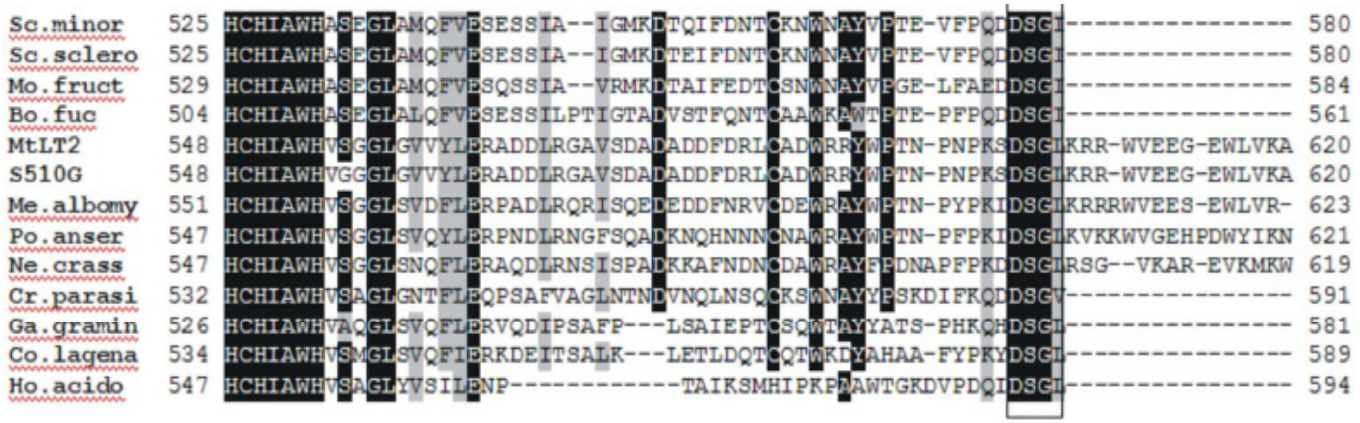


Fig. (4). Partial alignment of the amino acid sequences of MtLT2 (with the original Ser in position 510) and the S510G mutant with other known Ascomycete laccases with the same kind of C-terminal end (with or without C-terminal extension). The sequences were aligned using the Clustal W multiple-sequence alignment program and the numbers refer to the amino acid sequence. The numbering of the MtLT2 and S510G mutant sequence includes the preproleader signal sequence and the C-terminal plug is boxed. Sc. minor, *Sclerotinia minor* Lacc2 (ABM21603); Sc. sclero, *Sclerotinia sclerotiorum* Lacc2 (ABM21604); Mo. fruct., *Monilinia fructigena* Lacc2 (ABM21605); Bo. fuc, *Botryotinia fuckeliana* Lacc1 (Q12570); Me. albomy, *Melanocarpus albomyces* laccase (Q70KY3); Po. anser, *Podospora anserina* laccase (P78722); Ne. crass, *Neurospora crassa* laccase (AAA33591); Cr. parasi, *Cryphonectria parasitica* laccase (Q03966); Ga. gramin, *Gaeumannomyces graminis* var. *tritici* laccase (CAD10749); Co. lagena, *Colletotrichum lagenarium* laccase (BAB32575); Ho. acido, *Hortaea acidophila* Lacc1 (AAY33970).

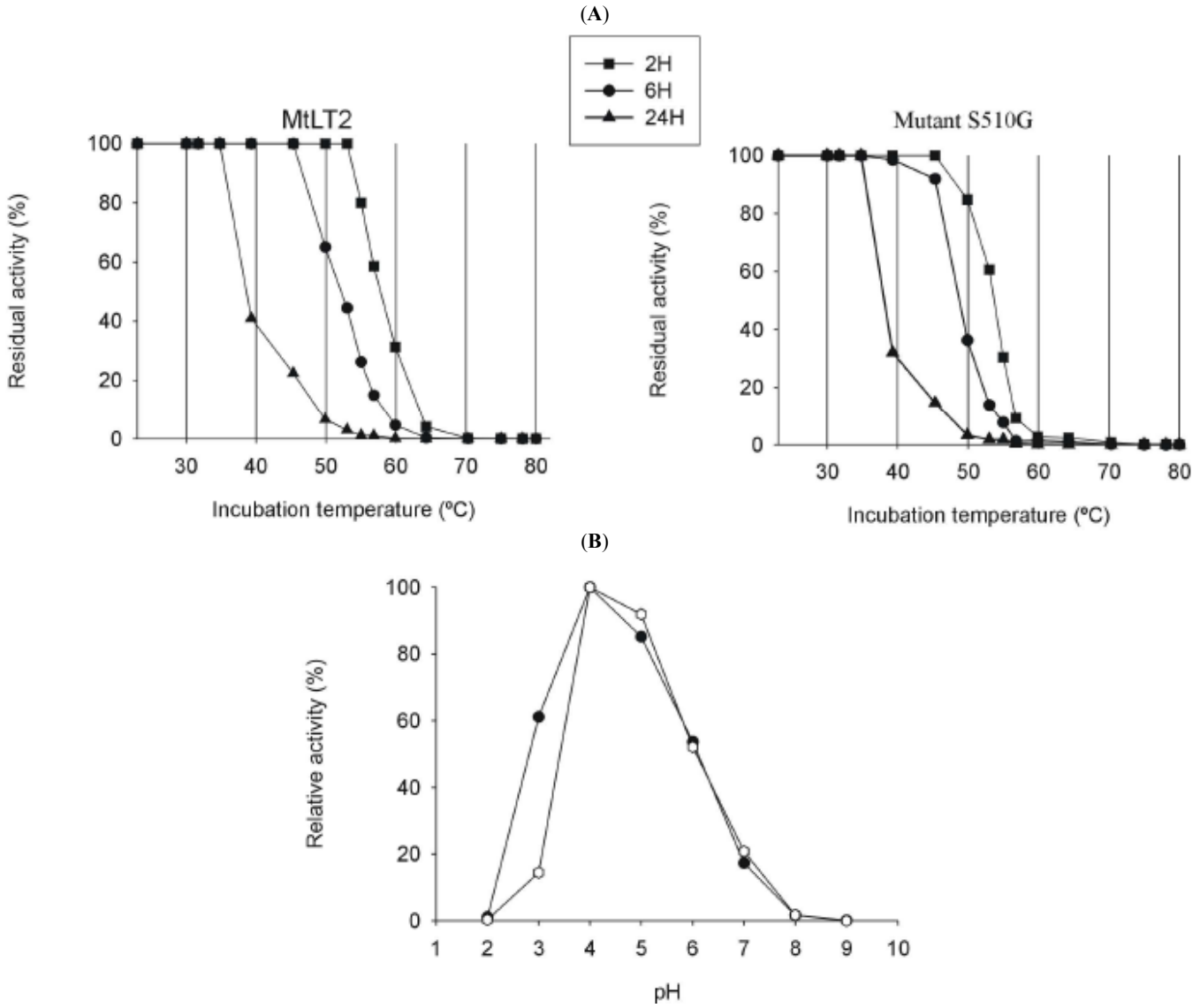


Fig. (5). (**A**) Thermostability of MtLT2 and mutant S510G in 60 µL of 10 mM Britton and Robinson buffer (pH 6.0) containing 0.6 U/mL of laccase incubated at different temperatures. Aliquots of 10 µL were removed at different time points (0, 2, 6 and 24 h) and assayed for residual activity at room temperature with 190 µl of 0.5 mM ABTS in 20 mM sodium acetate buffer (pH 4.5). The activity was normalized to the initial activity at room temperature before incubation, and each point was measured in triplicate. (**B**) pH activity profiles of MtLT2 (black circles) and the S510G mutant (white circles) measured in 100 mM Britton and Robinson buffer at different pH values with 0.5 mM ABTS as the substrate. Laccase activity is normalized to the optimum activity value. Each point represents the average of three independent experiments.

C-terminal plug [24]. According to our molecular model in which there is 75% sequence identity with the laccase from *Melanocarpus albomyces*, disruption of the interaction between S510 and D556 (Fig. **3**) provokes a widening of the channel that leads to the trinuclear copper cluster (Fig. **6**). Indeed, the more relaxed structure produced by the mutation seems to affect the tightly regulated traffic of oxygen and water through the channel to the T2/T3 cluster, with the concomitant increase in the $K_m^{O_2}$. Laccases catalyze one-electron substrate oxidation coupled to the four-electron reduction of oxygen. It is well known that laccases operate as a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen to two molecules of water [1]. Therefore, it seems plausible that synchronization between the binding of reducing substrates at the T1 site and the molecular oxygen at the trinuclear copper cluster is highly dependent on the connection between the plug and the $_{509}$VSG$_{511}$ tripeptide.

Spectroscopic and Electrochemical Studies of the S510G Mutant

To further evaluate the effect of the S510G mutation, the geometric and electronic structure of the coppers at the T1 and T2/T3 sites in the MtLT2 and S510G mutant were analyzed by EPR. A comparison of the $g_{\|}$ and $A_{\|}$ parameters of the parallel component of the T1 copper signal (Fig. **7**) revealed that the mutation induced subtle yet noticeable changes in the chemical environment of this site (Table **3**). On the other hand, only the outermost parallel hyperfine component of the T2 copper could be resolved in the spectra,

Table 2. Kinetic Parameters for Different Substrates (Phenolic and Non-Phenolic) and Molecular Oxygen Consumption

Variant	Catalytic Constants of ABTS Oxidation[1]				Catalytic Constants of 2,6 DMP[1] Oxidation[2]				Catalytic Constants of O_2 Consumption[2]	
	k_{cat} (s^{-1})	K_M (μM)	k_{cat} / K_M ($s^{-1}M^{-1}$)	Improvement (Fold)	k_{cat} (s^{-1})	K_M (μM)	k_{cat} / K_M ($s^{-1}M^{-1}$)	Improvement (Fold)	K_M (μM)	k_{cat} (s^{-1})
MtLT2	28.3	120	2.3×10^5	1	25.8	175	1.4×10^5	1	160	39.2
Mutant S510G	96.7	125	7.7×10^5	3.3	65.9	56	11.7×10^5	8.3	240	49.8

[1]The activities were assayed in 100 mM acetate buffer (pH 4.5) at 22 °C. $\varepsilon_{418nm\ for\ ABTS^{\bullet+}} = 36{,}000\ M^{-1}cm^{-1}$; $\varepsilon_{469\ nm\ for\ 2,6DMP} = 27{,}500\ M^{-1}cm^{-1}$.
[2]Activity assayed in 100 mM acetate buffer (pH 4.5) at 27 °C with 3.0 mM ABTS.

Table 3. Spectroscopy and Electrochemical Features

Variant	Mutations	*E°′T2/T3 Cluster (mV, pH 7.0); E°′, CV, mV/pH	**E°′ T1 Site (mV, pH 7.0)	EPR at the T1 site	
				$g_{\|}$	$A_{\|}$ (cm^{-1})
MtLT2	Variant with 12 mutations accumulated from a previous *in vitro* evolution experiment [11]	400; 50	690	2.212	8.0×10^{-3}
Mutant S510G	S510G	390; 55	700	2.207	7.7×10^{-3}

* Values extracted from cyclic voltammetry experiments; **Values extracted from spectroelectrochemical titrations.

which does not permit definitive conclusions to be drawn regarding the eventual modifications produced by mutations at this site.

The E°'-values of the T1 site and the T2/T3 copper cluster of the enzyme were estimated (Table **3**) and accordingly, the redox potential of the T1 site was not significantly altered by the mutation (690 mV ± 10 mV). This result is in agreement with previously published electrochemical studies of MtL, where the triple mutant $_{509}VGG_{511}/_{509}LEA_{511}$ did not significantly alter the redox potential at the T1 center despite the drastic changes in laccase kinetics [8].

When adsorbed on spectrographic graphite electrodes, both MtLT2 and S510G mutants largely decreased the overvoltage needed for the electroreduction of molecular oxygen. These results are in excellent agreement with the published data for many laccases of different origins [13,14,18]. Moreover, well-pronounced low potential Faradaic processes on CVs could be seen and values for both mutants are presented in Table **3**. As previously suggested, these Faradaic processes can be attributed to the redox transformation of the portion of the laccase connected to the electrode surface *via* its T2/T3 copper clusters [13,14,20,21]. The redox transformations of the T2/T3 cluster were almost reversible (the difference between anodic and cathodic peaks did not exceed 20 mV) and they were highly pH dependent (50 – 60 mV/pH, Table **3**).

In accordance with the mechanism by which the laccase acts and given that the reduction of the T1 site is known to be the rate-limiting step, the increase observed in k_{cat} (Table **2**) indicates that the electron transfer step but not the free energy change must have been affected by the mutation. According to the Marcus theory [25], the rate of electron transfer is related to the activation energy ($\Delta G^{\neq}$) and the electron transmission coefficient (κ_{el}). The $\Delta G^{\neq}$ 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 because $\Delta G^0 = -nF\Delta E^0$) and the reorganization energy. Because the E°'-values for the T1 site are similar, it is highly likely that the main parameter affected by the mutation is the electron transmission coefficient (κ_{el}, *i.e.* the normalized probability that the electron will be transferred (25)), which is dependent on the electronic coupling matrix element (H_{DA}). The H_{DA} is a function of the overlap of donor and acceptor orbitals through the protein and thus, it reflects the pathway for electron transfer. Hence, the significant change of k_{cat} was due to the effect of this mutation on the electron transfer pathway between the electron donor (reducing substrate) and the T1 Cu ion (8). By contrast, neither the reduction of oxygen at the T2/T3 copper cluster ($k \sim 2 \cdot 10^6\ M^{-1}\ s^{-1}$) nor the intraprotein electron transfer are rate-limiting steps (> 1000 s^{-1}) (3),(26). Thus, only a dramatic change of the T2/T3 cluster or the His-Cys-His pathway between the T1 and the T2/T3 cluster could be visualized catalytically and in accordance with our spectroelectrochemical data, no such changes were obtained. Indeed, the redox potentials of the cluster were very similar in both MtLT2 and the S510G mutant (400 mV ± 10 mV), while the changes in the absorbance spectra in the region of the T3 site (~330 nm) were correlated with differences in oxygen binding inside the cluster, which usually did not significantly affect the overall catalytic properties of laccases (19).

Combinatorial saturation mutagenesis is a valuable tool to improve certain features of enzymes and to perform "*semi-rational*" studies. Here, we have discovered an important relationship between the C-terminal plug and the conserved $_{509}VSG_{511}$ tripeptide of laccases, which largely depends on the interaction between S510 and D556. Hence, after exploring over 180,000 clones, we identified improved variants that exclusively incorporated the S510G mutation. In these S510G mutants, the synchronization between the binding of the substrate at the T1 site and the entrance of O_2 into the T2/T3 cluster was altered. Therefore the catalytic constants for both the reducing substrate and O_2 are perturbed, which directly affects the electron transmission coefficient between the reducing substrate and the T1 site. Al-

though the redox potentials were not significantly altered by the mutation, the geometry of Cu at the T1 site varied slightly, opening a window for further experiments. The redox potentials of fungal laccases range from +465 mV to +800 mV (21). The combination of *in vitro* evolution experiments in defined regions, along with the circular permutation of fungal laccases, will permit critical residues to be discovered that can be further explored by combinatorial saturation mutagenesis (currently ongoing). It is highly likely that this kind of approach will ultimately unmask the elements that determine the redox potential of laccases in the not so distant future (27).

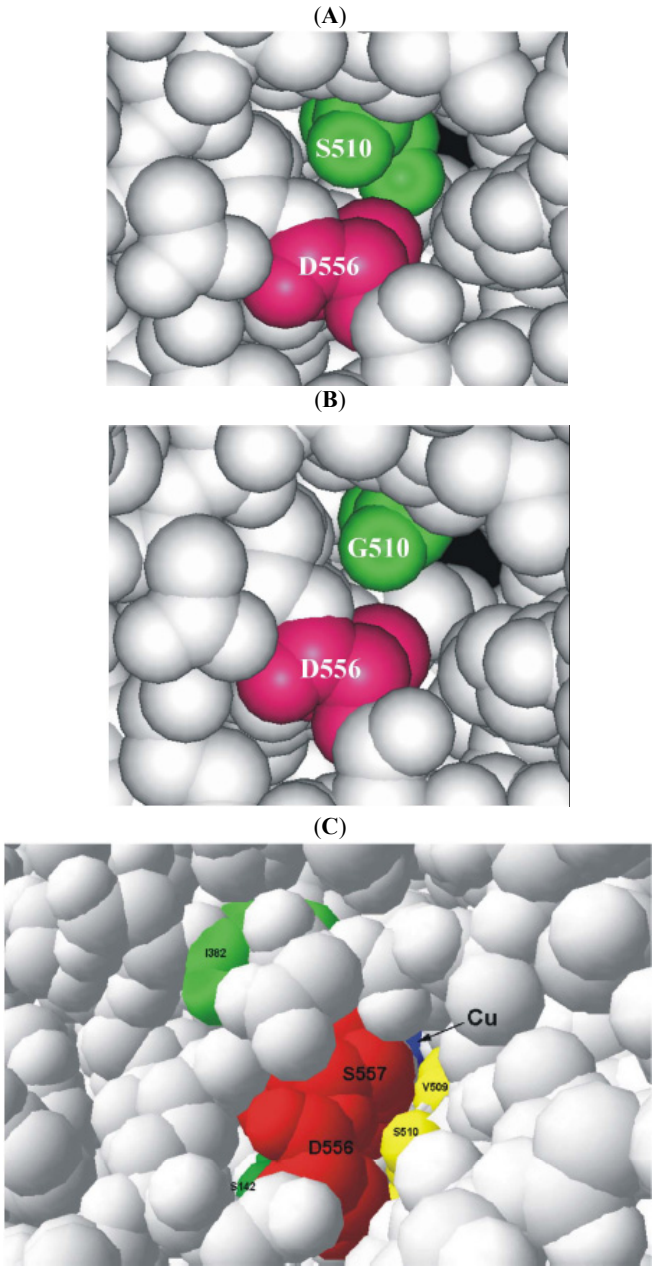


Fig. (6). The tunnel accessing the trinuclear copper cluster T2/T3. Mutation at position 510 widens the tunnel by impeding the interaction between the $_{509}$VSG$_{511}$ tripeptide and Asp556 in the C-terminal plug, (**A**) before and (**B**) after mutation. (**C**) View of several residues that interact with the C-terminal plug (red) showing the copper cluster in blue.

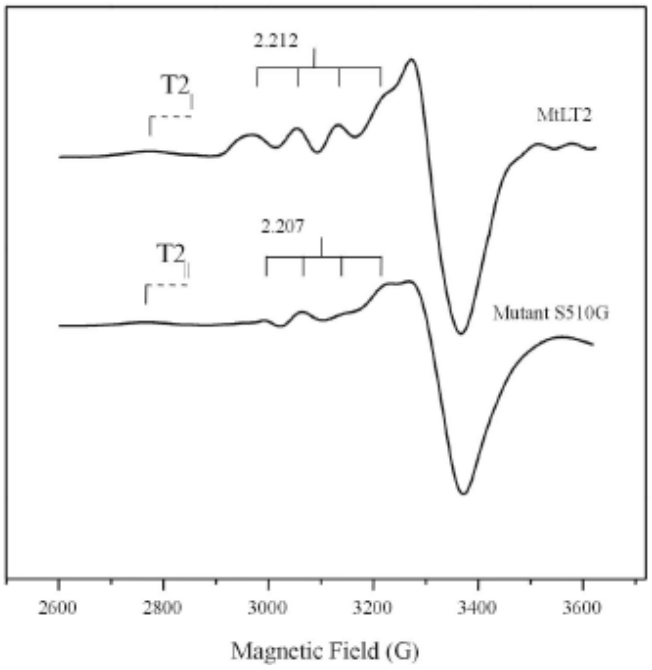


Fig. (7). EPR spectra of MtLT2 and mutant S510G recorded at 77 K.

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