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Does Tyrosine Protect *S. coelicolor* Laccase from Oxidative Degradation or Act as an Extended Catalytic Site?

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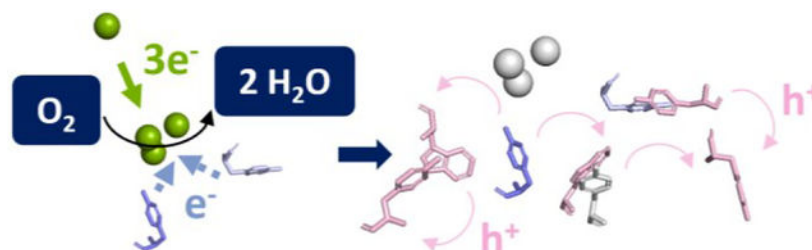
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Abstract

We have investigated the roles of tyrosine (Tyr) and tryptophan (Trp) residues in the four-electron reduction of oxygen catalyzed by *Streptomyces coelicolor* laccase (SLAC). During normal enzymatic turnover in laccases, reducing equivalents are delivered to a type 1 Cu center (Cu_{T1}) and then are transferred over 13 Å to a trinuclear Cu site (TNC: $(\text{Cu}_{\text{T3}})_2\text{Cu}_{\text{T2}}$) where O_2 reduction occurs. The TNC in SLAC is surrounded by a large cluster of Tyr and Trp residues that can provide reducing equivalents when the normal flow of electrons is disrupted. Prior studies by Canters and coworkers have shown that when O_2 reacts with a reduced SLAC variant lacking the Cu_{T1} center, a Tyr108^{*} radical near the TNC forms rapidly. We have found that the Tyr108^{*} radical is reduced 10 times faster than $\text{Cu}_{\text{T1}}^{2+}$ by excess ascorbate, possibly owing to radical transfer along Tyr/Trp chains.

Graphical Abstract



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Supporting Information

Protein expression and purification, metal content analysis, site-directed mutagenesis details, the use of electron transfer map eMap, steady-state Michaelis-Menten kinetics, and data from single oxygen turnover and EPR experiments are included in Supporting Information. The Supporting Information is available free of charge on the ACS Publications website.

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INTRODUCTION

The kinetically challenging task of reducing oxygen to water requires delivery of four electrons and four protons in a well-coordinated manner. As incomplete O₂ reduction often produces reactive oxygen species (ROS) that can be harmful to living cells, oxidases and oxygenases have evolved mechanisms to manage that risk by providing Tyr/Trp chains to remove high-potential holes from active-site regions.¹ A case in point is the protection mechanism associated with oxygen reduction by cytochrome *c* oxidase (CcO),^{2–4} an enzyme with two Cu active sites (Cu_A, Cu_B) and two hemes (heme *a*, heme *a*₃). Oxygen reduction proceeds at a coupled binuclear metal center (BNC, Cu_B-heme *a*₃) using electrons delivered from cytochrome *c* to the BNC via Cu_A and heme *a*. Molecular oxygen binds to the reduced BNC and formation of the next intermediate depends on the redox states of Cu_A and heme *a*.³ If O₂ binds to a fully reduced enzyme, the metal centers provide the four electrons to the bound O₂ molecule. If, however, O₂ binds to an enzyme in which only the BNC is reduced, the metal centers cannot provide the necessary reducing equivalents. In that case, heme *a*₃ provides 2 electrons, Cu_B delivers one, and the fourth electron is delivered by a nearby tyrosine residue (Tyr244, bovine numbering), thereby avoiding production of ROS by incomplete O₂ reduction.

In this study, we examine the role of Tyr/Trp chains in O₂ reduction catalysis of the small laccase from *Streptomyces coelicolor* (SLAC)⁵ belonging to the family of multicopper oxidases (MCOs) that efficiently utilize the thermodynamic potential of O₂ to oxidize a variety of organic and inorganic substrates. The enzymes contain four copper atoms: a type 1 Cu (Cu_{T1}, His, Met, Cys ligation); a trinuclear cluster (TNC) containing a binuclear Cu (Cu_{T3}, His₃ ligation); and a type 2 Cu (Cu_{T2}, His₂ ligation) (Figure 1).^{6–9} The SLAC Cu_{T1} center is reported to have a reduction potential of 375 mV *vs.* NHE.¹⁰ The active form of SLAC is a homotrimer, belonging to the family of two-domain laccases (2D-lacs) in which each monomer is comprised of two cupredoxin structural domains (Figure 1). This contrasts with the more common monomeric three-domain laccases (3D-lacs) found in fungi and bacteria.^{5,11–14} The TNC in 2D-lacs is constructed in the interface between the two monomers, highlighting the importance of proper protein folding to provide native coordination of Cu sites by His residues originating from two neighboring monomers. Three structural types (A, B, C) have been identified among the 2D-lacs, depending on the position(s) of Cu_{T1}.¹⁴ SLAC is a type B 2D-lac with Cu_{T1} located in the second of two cupredoxin domains.

In the canonical MCO reaction mechanism, four-electron oxygen reduction proceeds at the TNC with reducing equivalents delivered from substrates via Cu_{T1}.^{6–8,15,16} Investigations of SLAC by Canters and coworkers revealed that a TNC-proximal tyrosine (Tyr108) is oxidized during catalysis.^{17,18} Interestingly, these investigators suggested that the radical intermediate, Tyr108[•], might protect the enzyme from oxidative degradation.¹⁷ It is known from sequence alignments of 3,323 type B 2D-lacs that Tyr108 is highly conserved (>90%) among *Streptomyces* enzymes, but not among the broader class of type B proteins (14%).¹⁹ A Tyr107 residue in six-domain human ceruloplasmin (Cp) occupies a position analogous to that of Tyr108 in SLAC, and an intermediate with a spectroscopic signature reminiscent of a Tyr radical has been observed upon aerobic oxidation of this enzyme.^{17,20–22} Investigations

of human Cp provide compelling evidence that Tyr107 protects the protein from damage when O₂ reacts with the partially reduced enzyme, reminiscent of the role played by Tyr244 in CcO.²² Of relevance here is our suggestion of a similar role for chains of tyrosine and tryptophan residues in enzymes that generate high-potential reactive intermediates.^{1,23,24} As Tyr108 is buried in the interior of SLAC, reduction of the radical intermediate at this site would require intraprotein electron transfer from Cu_{T1} or hole hopping through Tyr/Trp chains to a reductant at the surface. To explore these possibilities, we have investigated Tyr radical generation in wild-type SLAC as well as in three mutants designed to disrupt Tyr/Trp hole hopping pathways.

MATERIALS AND METHODS

Protein expression and purification.

The gene for *Streptomyces coelicolor* small laccase encodes 343 amino acids, but in matured form the enzyme lacks residues 1–45.¹² A custom pET28b(+) vector containing genes encoding a His₆-tag and thrombin-cleavage site (HHHHHHSSGLVPRGSH) followed by a truncated version (tSLAC) of the protein (45–343 residues) was purchased from Biomatik Corporation (www.biomatik.com). The plasmid was first transformed into Novagen NovaBlue cells grown overnight at 37 °C. Single colonies were chosen for inoculation in media supplemented with kanamycin antibiotic. After cell growth, plasmids were extracted, purified, sequenced, and then transformed into BL21 (DE3) cells for overexpression. Protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the cell media. At the same time, 0.6 mM of CuSO₄ was added to cell culture to induce SLAC metalation during folding.³⁰ Cells were harvested by centrifugation, resuspended in phosphate buffer, and their contents released by sonication. The lysate was separated by centrifugation, filtered, then subjected to two stages of affinity chromatography and one of ion-exchange. Mass spectrometry indicated a mass of 32256 Da (WT), which corresponds to the His₆-SLAC protein without the final three residues (EPH). All other SLAC variants (W284F, W132F, and Y108F) were prepared in a similar fashion. Additional details are available in Supporting Information (SI:1. Materials and Methods).

Copper analysis.

A modified bicinchoninic acid (BCA) assay was used to analyze copper content in purified samples. The protein was denatured in 10 M urea, acidified to pH 3.5, and left overnight at room temperature. The copper ions liberated from the protein were reduced with L-ascorbic acid and BCA reagent was added to the solution to chelate Cu⁺ ions. The copper concentration was assessed by Cu-BCA absorption at 568 nm. SLAC samples were found to contain approximately 3.9 copper atoms per monomer. Additional details are available (SI: Materials and Methods).

Single oxygen turnover (UV-vis) experiments.

Solutions of SLAC variants in 10 mM phosphate buffer pH 7.5 were deoxygenated using evacuation/backfill cycles on a Schlenk line using Ar as inert gas. In a separate flask, a 10 mM solution of ascorbic acid was deoxygenated in the same way. The experiment was initiated by addition of 100 μL of deoxygenated ascorbic acid solution to an air-tight cuvette

containing *ca.* 2 mL of SLAC sample. Subsequently, UV-vis spectra were recorded until complete SLAC reduction, indicated by loss of Cu_{T1}²⁺ (580 nm peak) absorption. The single-oxygen turnover experiment was initiated by the addition of *ca.* 250 μ L of oxygenated (270 μ M) phosphate buffer (initial reagent concentrations are given in Table S3). UV-vis spectra were recorded every 2 s (up to 40 min) until the sample was completely re-reduced. Recorded spectra were baseline-corrected by fitting the background blank spectrum to a 5th-degree polynomial and subtracting it from the measured spectrum to remove the background signal. Component analysis was used to detect and assign 408, 424, and 583 nm features by fitting Gaussian curves to the peaks.

EPR spectroscopy.

SLAC (WT, Y108F) solutions were first concentrated to 150 μ L to reach a final concentration of *ca.* 0.5 mM (Y108F) or 1.2 mM (WT) in 10 mM phosphate buffer with addition of 100 mM NaCl at pH 7.5 and *ca.* 15% glycerol. Protein samples transferred to an O₂-free glovebox were reduced by addition of ascorbate solution (*ca.* 30 μ L) that was 16:1 reductant: Cu. The solution lost color after *ca.* 5–10 min, indicating reduced Cu_{T1}. Protein solutions were frozen (liquid nitrogen) right after removal from the glovebox.

CW X-band EPR spectra of WT SLAC in the oxidized and initial ascorbate-reduced state were collected using a laboratory-built spectrometer (microwave bridge, ER041MR, Bruker; lock-in amplifier, SR810, Stanford Research Systems; microwave counter, 53181 A, Agilent Technologies) equipped with a Bruker SHQ resonator. Spectra of Y108F SLAC in the initial ascorbate-reduced state were recorded on a Bruker Elexsys E580 X-band spectrometer equipped with a SuperHiQ resonator. An ESR 910 helium flow cryostat with ITC503 controller (Oxford Instruments) was used for temperature control in both instruments. A reference N@C60 sample ($g = 2.00204$) at ambient temperature was used to calibrate the magnetic field strength at the sample (field offsets: WT, 24.3 G; Y108F, –18.5 G). Data acquisition parameters at 40 K: modulation amplitude, 5 G (for 2000–3800 Gauss spectra) and 4 G (for 3000–3500 Gauss spectra); microwave power, 4.0 mW; microwave frequency, WT: 9.3956 GHz and Y108F: 9.3965 GHz

RESULTS AND DISCUSSION

Enzyme preparation.

SLAC is an extracellular enzyme expressed by *Streptomyces* bacteria that belong to the largest genus of *Actinobacteria*. These bacteria are abundant in soil and play a central role in carbon cycling, owing to their ability to hydrolyze a wide variety of polysaccharides.^{25–28} Interestingly, *Streptomyces* requires a large amount of copper for its aerial growth, indicating the importance of this metal for native synthesis of proteins.^{25,29} Proper incorporation of copper ions into the protein matrix of numerous laccases is essential for preserving activity and thermal stability. 2D-lacs differ from 3D-lacs in that they not only lack a third domain, but six His Ne atoms coordinate to Cu_{T3}, rather than five Ne and one N δ as in 3D-laccases.^{12,30} In 3D-lacs, this difference distinguishes the two Cu_{T3} atoms, referred to as Cu_{T3 α} , and Cu_{T3 β} .³¹ 2D-lacs lack this distinction. Moreover, in 2D-lacs, the TNC cluster bridges two polypeptide monomers, so proper folding of the quaternary

structure is essential. Structural studies have demonstrated that the small laccases (*e.g.*, Ssl1 from *Streptomyces sviveus*) have narrower Cu_{T2} access channels for incorporation of Cu ions than those in 3D-laccases.³⁰ The narrow channel can result in greater difficulty filling the Cu_{T2} site, consistent with the observation that the Ssl1 Cu_{T2} site could only be filled when cells were overexpressed in the presence of a high concentration of copper salt.³⁰ We have found similar behavior in SLAC where full Cu occupancy in the native protein required enrichment of the expression media with CuSO₄.

Hole-hopping analysis.

We hypothesized that Tyr108 prevents oxidative damage to the enzyme by providing an escape route for highly oxidizing holes generated by incomplete O₂ reduction at the TNC cluster. To provide such a protective role, the radical formed on Tyr108 would have to be rapidly transferred from the enzyme active site to the enzyme surface where it could be scavenged by cellular reductants. The radical transfer would involve hole hopping through chains of Tyr and Trp residues.¹

We used the web-based eMap application (see SI) to identify hole hopping paths through chains of Tyr/Trp residues originating from Tyr108 in SLAC. The search criteria required <10 Å distance between residues and at least one surface-exposed residue at the end of a pathway. With these constraints we identified two routes: one propagating through W132; the other through W284 (Fig. 1B).^{32,33} To explore the effect of inactivation of the two chains, we prepared wild-type (WT) SLAC and three variants: Y108F, W132F and W284F. The Y108F mutant was designed to probe the effects of inactivation of both redox chains, while W132F and W284F were chosen to explore the consequences of interrupting respective chains.

Catalytic activity.

We first examined the effect of single mutations on the overall catalytic performance of SLAC using a Michaelis-Menten (MM) kinetics model. As SLAC exhibits high stability and activity towards organic substrates over broad ranges of pH (3.0 – 10.0) and temperature (–80 °C), it is attractive for industrial applications.^{5,27,34–36} We used a common laccase substrate, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS²⁻),^{35–38} to evaluate SLAC activity (pH ≈ 4.0;^{36,37} 60 °C^{35–37}) at a single O₂ (250 μM) concentration where rates were limited by the ABTS²⁻ concentration. All variants proved to be active with only modest variations in catalytic parameters: catalytic rate constants (k_{cat}) varied by less than the measurement uncertainty (~15%); Michaelis constants (K_{M}) varied by no more than factor of 2 (details in Supporting Information). A prior study of Y108F and Y108A SLAC mutants with N,N,N',N'-tetramethyl-p-phenylenediamine as substrate found that k_{cat} decreased by a factor of ~2.4, but a corresponding reduction in K_{M} resulted in relatively modest changes in activity ($k_{\text{cat}}/K_{\text{M}}$) for the two mutants (Y108F, –25%; Y108A, +19%).¹⁷

Dioxygen reactivity.

We probed the reaction of reduced SLAC with O₂ using a combination of UV-vis absorption and electron paramagnetic resonance (EPR) spectroscopic methods. Reduced SLAC was prepared by treating an anaerobic solution of the resting enzyme with excess ascorbate ($E^{\text{'}}$

= 0.25 V vs. NHE, pH 7.5)³⁹ for 25 min. Strong ascorbate UV absorption obscured SLAC spectral features below 400 nm. A 330-nm feature due to OH-Cu_{T3}²⁺ charge transfer³¹ could be detected but, as we were unable to extract reliable information about the Cu_{T3} site in this spectral region, we restricted our analysis to the 400–700 nm region. Spectra of reduced SLAC before addition of O₂ (Figure 2A) did not exhibit any features in this region attributable to the enzyme.

EPR spectra of the resting oxidized enzyme exhibited features of Cu_{T1}²⁺ and Cu_{T2}²⁺ (Figure 3). The two Cu_{T3}²⁺ centers are antiferro-magnetically coupled and are EPR-silent. Following treatment of oxidized wild-type and Y108F SLAC with excess ascorbate for 25 min, EPR spectra confirmed that the Cu_{T2} center remained at least partially oxidized (Figure 3; SI, Figure S9). This observation suggests that the reduction potential of SLAC Cu_{T2} is substantially lower than that of Cu_{T1} and ascorbate at pH 7.5. The reduced enzyme reacted with O₂ (*vide infra*), indicating that two Cu⁺ centers occupy the Cu_{T3} site. These data demonstrated that a substantial population of the ascorbate-reduced enzyme lacks the four Cu electrons required for complete conversion of O₂ to H₂O.

The X-band EPR spectrum of Cu_{T2} in ascorbate-reduced WT SLAC offered evidence for Cu_{T2}²⁺ in two distinct environments (Figure 3). Two sets of four Cu hyperfine lines centered at ca. g_{||}=2.26 and g_{||}=2.31 (total of 8 lines) were resolved in the g_{||} region, and we detected two sets of 5 superhyperfine lines (A_{N1} = 1.6 mT and A_{N2} = 1.4 mT) arising from 2N coordination to the Cu_{T2} center.^{40,41} Two types of Cu_{T2} have been observed before, albeit with slightly different g_z values (g_z=2.352 and g_z=2.246),⁴² and interpreted as heterogeneity in the orientation of the imidazole ligands to Cu_{T2}.

Exposure of ascorbate-reduced WT SLAC to 1 equivalent of O₂ led to rapid oxidation of Cu_{T1} (Figure 2B). Quantitation of the 583-nm absorption band immediately after treatment with O₂ indicated formation of 0.9 equivalents of Cu_{T1}²⁺ (based on an extinction coefficient of 4400 M⁻¹cm⁻¹ per Cu_{T1} site).²⁷ The 583-nm absorption decreased over the ensuing 10–15 min (Table 1), owing to reaction with excess ascorbate (Figure 2B). The background-subtracted UV-vis absorption spectrum recorded immediately after O₂ addition also showed an absorption maximum at 424 nm, consistent with the Tyr108[•] radical that had been identified previously by Canters and coworkers.^{17,18} The initial yield of Tyr108[•] corresponds to ≈0.1 equivalents (based on an extinction coefficient of 2300 M⁻¹cm⁻¹).^{43–45} A likely explanation for radical formation is that Tyr108 provided the fourth electron for the reduction of O₂ in those enzymes with Cu_{T2}²⁺ present after treatment with ascorbate. This explanation is consistent with Canters' suggestion that Tyr108[•] radical formation in SLAC protects the enzyme from damage by reactive oxygen species when the Cu centers cannot deliver the four electrons required for O₂ reduction. That the Tyr108[•] absorption signal disappeared much more rapidly (< 10 s, Table 1) than the Cu_{T1}²⁺ absorption demonstrates that electrons from ascorbate do not flow through Cu_{T1} and on to Tyr108[•]. The likely explanation is that Tyr108[•] is reduced directly by ascorbate through an alternate pathway (Figure 1).

We also used EPR spectroscopy to probe the reaction of ascorbate-reduced WT SLAC with O₂. After the reduced enzyme was mixed with ≈1 equivalent of O₂, it was frozen in

liquid nitrogen. As the shortest freezing time was approximately 2 min, we were not able to observe a signal for Tyr108[•] ferromagnetically coupled to Cu_{T2}²⁺.^{17,18} Interestingly, double integration of the Cu_{T2}²⁺ EPR spectra indicated that the signal amplitude (determined by double integration) increased about 40% during the first 6 min after exposure to O₂. This is a surprising observation, given that the reaction with O₂ is complete in less than 1 s and UV-vis spectra indicated that Cu_{T1}²⁺ reduction occurred on this timescale.

Predictably, reaction of ascorbate-reduced Y108F SLAC with 1 equivalent of O₂ proceeded differently. The initial yield of Cu_{T1}²⁺ was just 60% of that expected, and the 583-nm signal decayed twice as fast as that of the wild type with a roughly comparable ascorbate concentration (Table 1, Supporting Information). An absorption maximum at 408 nm appeared after exposure to O₂ but, unlike WT, the signal persisted for over 30 min, remaining even after Cu_{T1}²⁺ was fully reduced. Although a band maximum at 408-nm is consistent with a Tyr[•] radical, the persistence of the absorption in the presence of a high concentration of ascorbate suggests a different origin, likely an irreversible oxidation product.

The Cu_{T2}²⁺ EPR spectrum of ascorbate-reduced Y108F SLAC is similar to that of the WT enzyme, although the distribution between the two coordination environments is somewhat altered (Figure 3). The first spectrum recorded after exposure to O₂ showed no signs of an organic (e.g., Tyr[•]) radical. Double integration of the Cu_{T2}²⁺ signal in subsequent spectra revealed that the amplitude increases by a factor of five during the first 6 min. This result may indicate Cu_{T2} is reduced to a greater extent in Y108F than in WT SLAC, possibly owing to a somewhat higher reduction potential.

Two other mutant enzymes (W284F and W132F) displayed disparate reactivity with O₂. In the W284F enzyme, the initial yield of Cu_{T1}²⁺ was about 60%, but increased to 90% in the first 100 s after exposure of the ascorbate-reduced enzyme to O₂. During this time period a small 424-nm signal corresponding to 10% yield of a Tyr[•] radical decayed to baseline. This behavior is consistent with electron transfer from Cu_{T1}⁺ to Tyr108[•]. In contrast, a 90% yield of Cu_{T1}²⁺ appeared initially in the W132F mutant and the signal decayed with a 153 s time constant. A small 408-nm signal appeared with the W132F mutant but, as with Y108F, the signal did not decay and no radical signal was detected in the EPR spectrum of a Cu_{T1}-depleted W132F mutant (Supporting Information).

Mechanistic implications.

Our EPR measurements demonstrate that ascorbate does not fully reduce Cu_{T2}²⁺ at pH 7.5. Farver *et al.* noted earlier, without supporting data, that the failure of ascorbate to reduce Cu_{T2}²⁺ indicates that its formal potential must be relatively low.¹⁵ It is possible that the Cu_{T2}²⁺ formal potential in the “resting-oxidized” enzyme is lower than that of the “native intermediate” that is populated during enzyme turnover.^{8,9} Indeed, Farver *et al.* reported that Cu_{T1}⁺ to Cu_{T3} electron transfer is faster in enzymes that had been “cycled” (reduced then reoxidized with O₂).¹⁵ Our EPR measurements, however, revealed that Cu_{T2}²⁺ resists reduction with ascorbate even after reduced enzyme has been oxidized with O₂. Moreover, the enzyme substrates tetramethylphenylenediamine (TMPD, $E^\circ = 0.26$ V vs. NHE)^{17,46} and ABTS²⁻ ($E^\circ = 0.72$ V vs. NHE)⁴⁷ likely will not reduce Cu_{T2}²⁺.

The clear implication is that Cu_{T2} is not an obligatory participant in SLAC catalysis. The observation of Tyr108[•] formation following exposure of the reduced enzyme to O₂ indicates that Tyr108 can supply the fourth electron required for O₂ reduction when Cu_{T2} remains oxidized. In this way, Tyr108 acts as an extended catalytic site reminiscent to the role of Tyr244 in CcO. It is surprising, then, that the enzymatic activity toward ABTS²⁻ is about the same for WT and Y108F mutant SLACs. Three-electron reduction of O₂ would produce a hydroxyl radical that might attack the protein in the vicinity of the TNC.⁴⁸ It is possible that the persistent 408 nm absorption feature observed in the reaction of Y108F SLAC with O₂ results from OH[•] attack on nearby aromatic residues in the protein. Various isomers of dihydroxyphenylalanine (DOPA) are produced in the reaction of OH[•] with Tyr and Phe.^{48–50} In addition, quinone methides, which have absorption maxima near 400 nm,⁵¹ have been invoked as intermediates and products in Tyr and DOPA oxidations.^{52–54}

CONCLUSIONS

In the accepted mechanism for MCO catalysis, the four electrons required for O₂ reduction are supplied by the four Cu centers in each enzymatic unit. *S. coelicolor* SLAC is an extracellular enzyme¹⁷ and as such will be exposed to a wide range of substrates with diverse formal potentials and concentrations. The apparently low formal potential of Cu_{T2}²⁺ in SLAC means that, depending on substrate conditions, it will not always be able to donate an electron to O₂ during catalysis. Tyr108 can replace Cu_{T2} as an electron donor in WT SLAC and the resulting Tyr108[•] radical is reduced by substrate through a Cu_{T1}-independent path. That catalysis continues unabated when this residue is removed suggests that other Tyr or Trp residues near the TNC might also serve a similar function. Ultimately, irreversible oxidation of aromatic amino acids could limit enzyme survival when Tyr108 is unavailable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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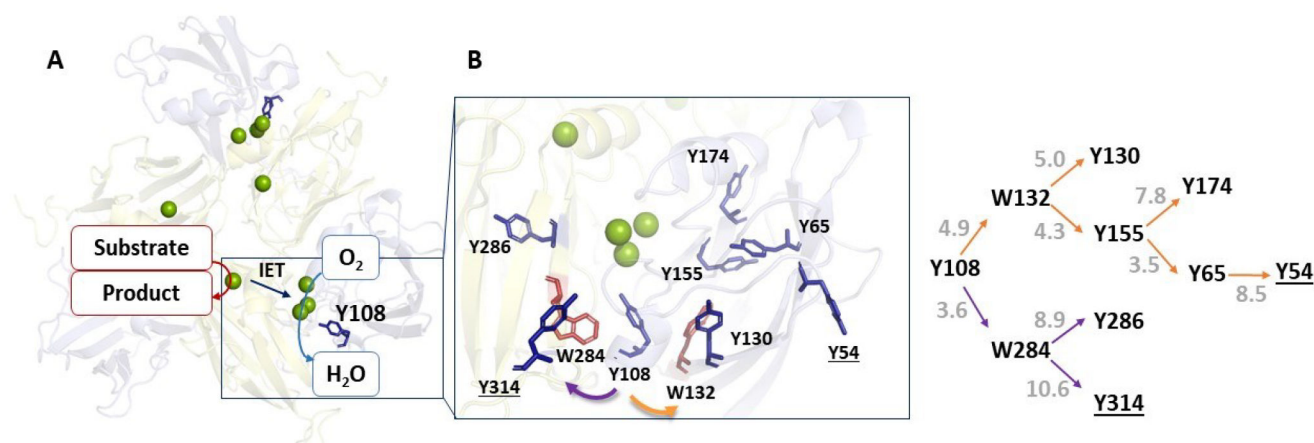


Figure 1.

(A) Structure of the small laccase (SLAC: PDB ID 3CG8) with copper ions and Tyr108 marked in green and blue, respectively. Substrate oxidation coupled to oxygen reduction is shown schematically. (B) Tyr/Trp redox pathways: (I) $Y108 \rightarrow W284 \rightarrow Y286/Y314$, (II) $Y108 \rightarrow W132 \rightarrow Y130/Y155 \rightarrow Y65 \rightarrow Y174/Y54$, indicated by the purple and orange arrows, respectively. Numbers refer to shortest atom-atom distances in Å.

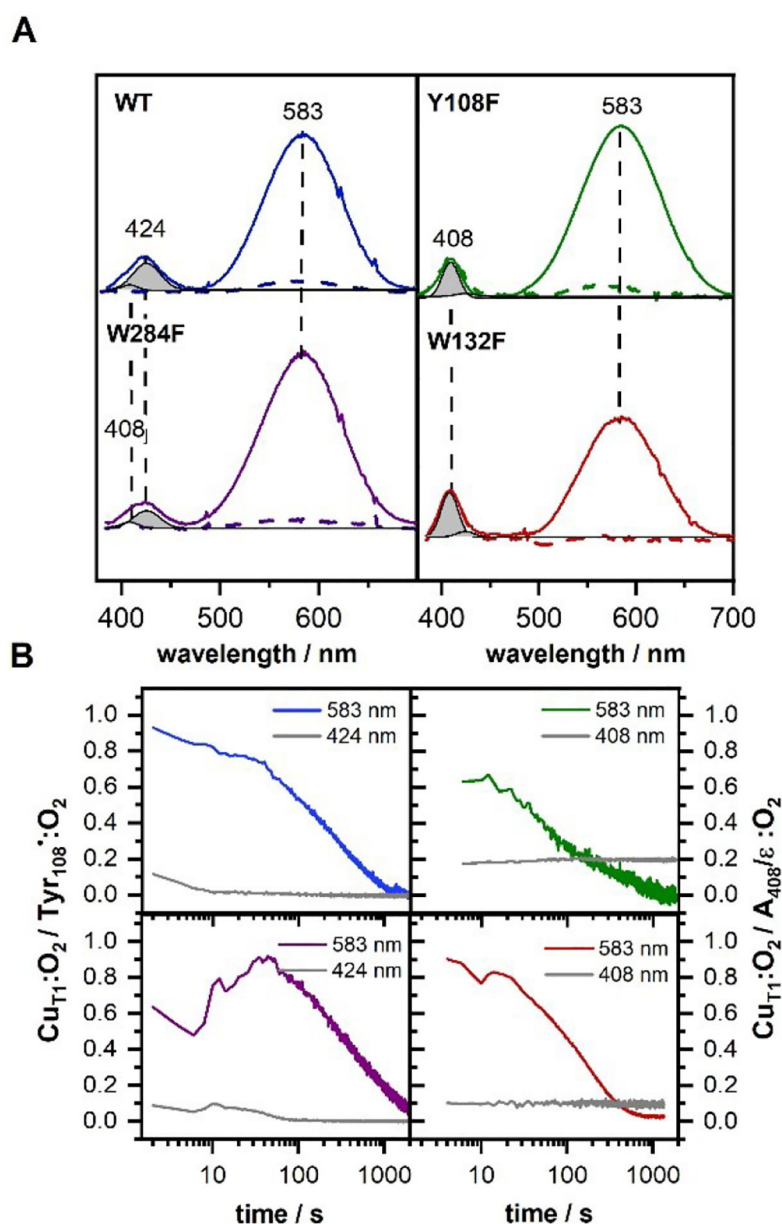
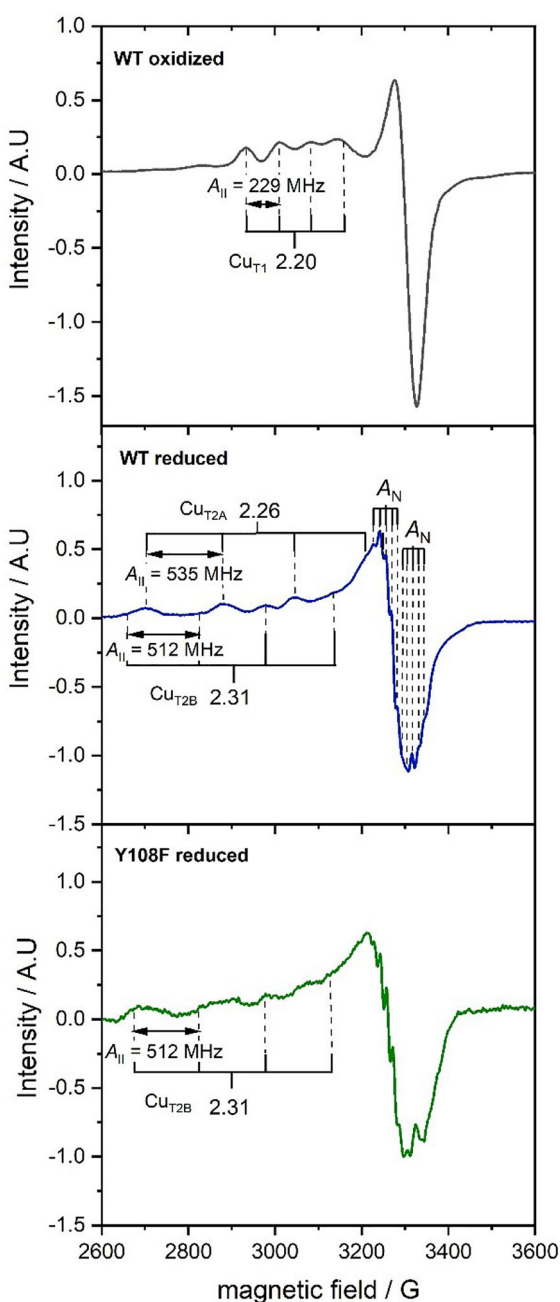


Figure 2.

(A) Baseline-corrected UV-vis spectra (A) of SLAC variants (left: WT in navy blue, W284F in purple; right: Y108F in green, W132F in red) in deoxygenated 10 mM phosphate buffer pH 7.5 with excess of ascorbic acid, just after addition (1–2 s) of oxygenated buffer (solid line) and after reduction (1500 s) by ascorbate (dotted line). (B) Time traces of concentrations of SLAC-variant intermediates from single-turnover experiments. Ordinates indicate absorption spectra intensities at 583 (WT: blue, W284F: purple, Y108F: green and W132F: red) and 424 or 408 nm grey), converted to concentration and divided by the initial O_2 concentration.

**Figure 3.**

X-band EPR spectra: WT SLAC in its oxidized state (top); WT in the ascorbate-reduced state (middle); and Y108F in the ascorbate-reduced state (bottom). Spectra were recorded at 40 K. Spectrum of Y108F was normalized to the intensity of reduced WT.

Table 1.

Time constants ($\tau_{\text{exp}} = 1/k_{\text{exp}}$) derived from exponential fits to UV-vis kinetics traces (Fig. S3)

	τ_{exp} (583 nm) /s	τ_{exp} (424 nm) /s
WT	317	8.7
W284F	538	43
Y108F	671	-
W132F	153	-