Enzymatic reduction of oxygen by small laccase. A rapid freeze-quench EPR study

Nami, F.

Chapter 5

A Rapid Freeze-Quench EPR Study of the Enzymatic Reduction of Oxygen by Wild-type SLAC

In this chapter EPR spectroscopy is combined with the rapid freeze-quench technique to study the reoxidation of wild-type (wt) small laccase (SLAC) under similar experimental conditions as used for the study of T1 Cu depleted (T1D) SLAC described in the previous chapter. The EPR spectra of fully reduced wt SLAC mixed with oxygen on the time scale of milliseconds and longer can be described as the sum of T1 Cu and T2 Cu signals. We find no indications of the involvement of tyrosine 108, as observed with T1D SLAC, or of a native intermediate, as observed with other multicopper oxidases, in the single turnover of wt SLAC.

5.1 Introduction

Laccases are a subfamily of multicopper oxidases (MCO's), enzymes that catalyze the reduction of oxygen to water while oxidizing a wide variety of substrates. The function of these enzymes is based on the distinctive redox ability of four copper ions to carry out the four-electron reduction of molecular oxygen. The coppers are distributed in three distinct sites, categorized according to their spectroscopic features. In the oxidized state, the type 1 (T1) copper is characterized by an intense absorption band around 600 nm and an EPR signal with a small parallel copper hyperfine coupling of 120 to 300 MHz[1]. The type 2 (T2) copper shows no appreciable optical absorption in the visible range and a larger parallel copper hyperfine coupling of 480 to 600 MHz. The binuclear type 3 (T3) copper center is characterized by an absorption shoulder at about 330 nm and does not give rise to an EPR signal due to the antiferromagnetic coupling of the copper spins. The T2 Cu and the two T3 Cu's form a trinuclear cluster (TNC)[2]. The T1 Cu, at a distance of about 13 Å from the TNC, is the primary electron acceptor from the substrate and the electrons are subsequently transferred to the TNC, where O_2 binds and gets reduced. The reaction of O_2 with the fully reduced enzyme has been proposed to proceed via two sequential two-electron steps, the first generating a peroxide intermediate (PI) and the second generating the native intermediate (NI). The PI is characterized by absorption bands at 340 and 480 nm, while it lacks any EPR signal[3,4]. The PI was trapped in enzymes in which the copper in the T1 site was depleted (T1D) or replaced by mercury, and is believed to be a catalytic precursor to the NI. In the NI, all four coppers are oxidized and an oxygen bridge couples the three coppers in the TNC. The NI is characterized by absorption bands at 318, 330, 365 and 614 nm and a broad EPR signal at low temperature (<20 K) in the region $g<2$[2,5]. NI-like features have been observed in several MCO's and the

lifetime of the intermediate varies from milliseconds to minutes[5,6,7]. The NI decays to the resting form of the enzyme in which the T2 Cu is magnetically uncoupled from the Cu's in the T3 center[8].

The molecular mechanism of O_2 reduction at the TNC is being studied extensively, with the focus on the geometric and electronic structure of the intermediates[2,9,10,11]. An open issue is whether the overall description given above applies to all MCO subfamilies, which stem from different sources, function under different conditions, and are structurally different. MCO's are found in different organisms such as bacteria, fungi, plants, insects and humans[12,13]. Based on the conditions to which they are exposed, enzymes might find different pathways to efficiently reduce O_2 to H_2O without releasing reactive oxygen-species, which might harm the enzyme. As for the structure, most of the MCO's are composed of three cupredoxin-like domains (3dMCO) such as three-domain laccases (3dLACs), ascorbate oxidase, CueO and Fet3p, and there are laccases with only two domains (2dLAC's)[14]. Ceruloplasmin is another variant of the MCO's, which is composed of six domains[15]. 3dMCO's and ceruloplasmin are active in monomeric form, while 2dLAC's function as a homotrimer.

Small laccase (SLAC), the focus of this study, is a 2dLAC, which was identified some years ago when searching the genome of *Streptomyces coelicolor* for the presence of copper proteins[16]. Characterization of the protein revealed that besides structural differences, SLAC represents some other features distinct from those of 3dMCO's. Surprisingly, the T1D SLAC is able to complete the reduction of oxygen to water in a single turover[17], while for 3dMCO's the reduction is not completed in the absence of the T1 Cu. Reaction of the reduced T1D SLAC with oxygen results in the formation of a biradical intermediate detected by electron paramagnetic resonance (EPR) spectroscopy

on the time scale of minutes[17]. Such an intermediate has not been reported for the 3dMCO's. Primary characterization of this intermediate led to the hypothesis that the biradical signal originates from two exchange-coupled spins, one localized on the T2 Cu and the other one on an amino-acid-derived radical, most probably a tyrosyl radical. The proposal was later supported by the crystal structure of SLAC[18], which revealed that the tyrosine residue Y108 is about 5 Å away from the T2 Cu. In chapter 4, we have characterized the biradical intermediate using rapid freeze-quench (RFQ) multi-frequency EPR on the time scale of milliseconds. From multi-frequency EPR spectra of the intermediate, an exchange interaction of about 12 GHz was derived between the T2 Cu and the tyrosyl radical. When Tyr 108 was replaced with phenylalanine (Y108F) by site-directed mutagenesis, no biradical signal was observed on the time scale of milliseconds and longer. As the O_2 reduction to H_2O requires four electrons and each copper center provides one electron, in the absence of T1 an insufficient number of electrons is available to complete the reaction. For T1D SLAC, Tyr 108 is proposed to provide the fourth electron required to complete the reduction, in order to prevent the generation of reactive oxygen species (ROS), which might damage the enzyme.

Besides the occurrence of the biradical intermediate, the lack of an optical PI signal during the reoxidation of T1D SLAC is another striking difference between this enzyme and 3dMCO's. However, PI-like features in the optical absorption were observed for Y108F/Y108A T1D SLAC, i.e., when neither the T1 Cu nor the tyrosine is present, while for 3dMCOs the PI was observed during reoxidation of the T1D enzymes. In addition, no NI-like species was observed, neither for T1D SLAC nor for Y108F T1D SLAC. For Y108F T1D SLAC, a clear signal from T2 Cu is observed for the freeze-quench samples at

both 9 and 94 GHz, which is not compatible with the NI, as for NI the T2 Cu is coupled to the T3 Cu's.

The remarkable observation of a biradical intermediate in the reoxidation of T1D SLAC and the absence of signals characteristic of a PI and an NI, calls for a search for such intermediates in the case of wild-type (wt) SLAC. Here we report on the EPR study of the reoxidation of wt SLAC under similar experimental conditions as used for the study of T1D SLAC described in the previous chapter. The EPR spectra of fully reduced wt SLAC mixed with oxygen on the time scale of milliseconds and longer can be described as the sum of T1 Cu and T2 Cu signals. We find no indications for a biradical or an NI in the single turnover of wild-type SLAC.

5.2 Materials and methods

5.2.1 Protein isolation. The wt SLAC was isolated from *E. coli* cultures harboring the pET20b overexpression plasmid as previously described[16]. The purity of the preparations was checked by SDS PAGE. The protein was stored in 10mM phosphate buffer pH 7.2 at −80° C until required for use. Protein concentration was determined by absorption spectroscopy using an extinction coefficient of 38 mM^{-1} cm^{-1} at 280 nm.

5.2.2 Chemicals. All chemicals were of analytical grade, purchased from Sigma-Aldrich and used without further purification.

5.2.3 EPR measurements

The 9.5 GHz spectra were recorded on an ELEXSYS E680 spectrometer (Bruker BioSpin GmbH) equipped with a He-flow ESR900 Cryostat (Oxford Instruments) and a standard TE102 cavity.

5.2.4 RFQ sample preparation

The RFQ apparatus used for the sample preparation is described in chapter 4. To prepare the RFQ samples, the enzyme was anaerobically reduced by addition of a slight excess of anaerobically prepared sodium dithionite (DT) in a glovebox. The reduction was monitored on a mini UV-Vis spectrometer (Ocean Optics) as a decrease of the 330 and 590 nm bands, which completely disappear for a fully reduced wt SLAC. The excess of DT was removed in the glovebox using a 10K Amicon centrifugal filter from Millipore. Before loading the reduced enzyme into the RFQ syringe, the UV-Vis spectrum was recorded to ensure that no DT is remained in the solution. The syringe assemblies for the enzyme were made rigorously anaerobic in the glovebox one day prior to the experiment. The samples were prepared by 1:1 mixing of the reduced enzyme with oxygen-saturated sodium phosphate buffer (100mM, pH 6.8). The mixing was conducted at room temperature and the reaction mixture was sprayed into cold isopentane. Frozen particles were then packed into a 3mm (i.d.) EPR tube using the sucking method[19], which is described in chapter 2, and a steel rod.

The sample with a reaction time of two seconds was frozen in a different way. The reactants were mixed by the RFQ setup and the reaction mixture was injected into an EPR tube and immediately frozen on dry ice.

5.3 Results

As stated above, the experiments were designed to investigate the possible occurrence of a tyrosyl radical and/or the native intermediate during the reoxidation of fully reduced wt SLAC with O_2. The X-band EPR spectra at 40 K of RFQ samples of reduced wt SLAC reacted with oxygen for different times are presented in Figure 5.1. The overall shape of the spectra hardly varies with

the reaction time between 8 ms and 2 seconds, which means that the ratio of the different components that contribute to the spectra does not significantly change during this time. The 270 mT signal and the high-field signal (337-347 mT) refer to oxidized T2 Cu. The 290 mT signal and the blue color of the RFQ samples refer to oxidized T1 Cu. The spectra obtained for the RFQ samples are virtually identical to the spectrum of the resting form of wt SLAC, also shown in Figure 5.1. The spectrum of the resting form is the superposition of T1 and T2 Cu signals and this seems to apply to the spectra of the RFQ samples as well.

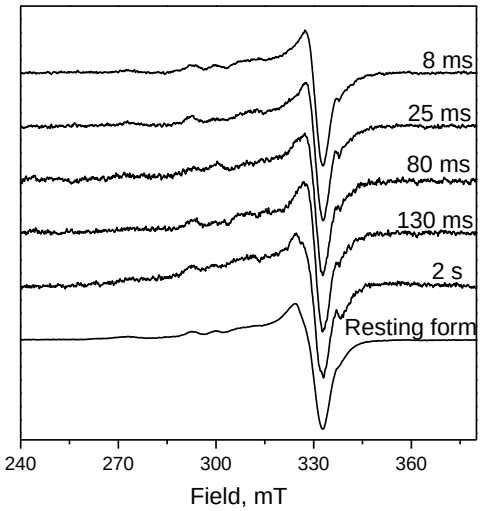

Figure 5.1. The cw X-band EPR spectra of RFQ samples of reduced wt SLAC reacted with oxygen for different times. Enzyme and oxygen concentration are 320 μM (360 μM for the 8 ms-sample) and 650 μM, respectively, both in 100 mM sodium phosphate buffer, pH 6.8. EPR measurements: microwave frequency 9.5 GHz, T = 40 K, microwave power 0.16 mW and modulation amplitude 0.5 mT.

In order to quantify the contribution of each component, we first determined the T1 Cu spectrum. The T1 Cu spectrum was obtained by subtracting the spectrum

of the resting form of T1D SLAC (only T2 signal) after normalizing at 272 mT from that of wt SLAC (figure 5.2a).

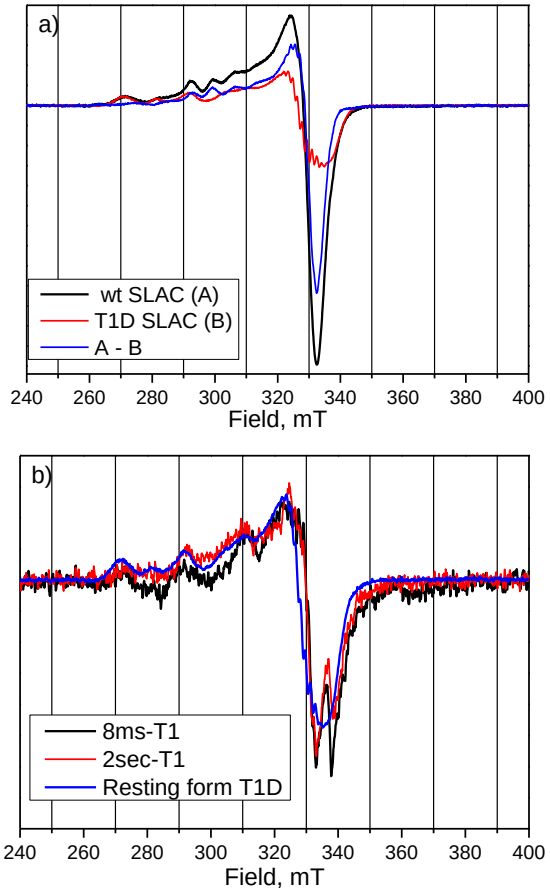

Figure 5.2. a) The X-band EPR spectra of the resting forms of wt and T1D SLAC and the difference of the two spectra, which represents the spectrum of T1 Cu. b) The T1-subtracted X-band EPR spectrum of the RFQ sample of reduced wt SLAC reacted with oxygen for 8ms and 2 seconds overlaid with the spectrum of the resting form of T1D SLAC. To subtract the T1 spectrum, the spectra were normalized at the low-field signal of T1 (~292 mT). EPR measurements: microwave frequency 9.5 GHz, T = 40 K, microwave power 0.16 mW and modulation amplitude 0.5 mT.

We assumed that the T1 Cu spectrum for the resting form is the same as that for the RFQ samples. The T1 Cu signal was subtracted from the spectra of the RFQ samples after normalizing at the low-field signal of T1 (~292 mT). The remaining spectra resemble the spectrum of the T2 Cu, as shown by the comparison with the spectrum of the reoxidized form of T1D in which we have only the T2 Cu (Figure 5.2b). A small signal around g = 2, which is a sign of an organic radical, can be seen on top of the T2 Cu signal. We neglect this small signal, which contributes less than 1% to the whole spectrum. Spin quantification reveals that the ratio of T1 to T2 Cu in the RFQ samples of wt SLAC is approximately 1:1. The spectra show no indication of a tyrosyl radical, neither as an isolated radical nor as a radical in interaction with a copper.

In order to investigate the possible presence of NI, the X-band EPR spectrum of the RFQ sample of reduced wt SLAC reacted with oxygen for 8ms was obtained at 8 K, high microwave power of 100 mW and high modulation amplitude of 1.6 mT. The spectrum is presented in Figure 5.3. The low-field features of T1 Cu and T2 Cu between 270 to 310 mT are not resolved at such high power due to saturation. We do not observe any indication of a broad band above 350 mT ($g \approx 1.85$), which would be a sign of an NI intermediate.

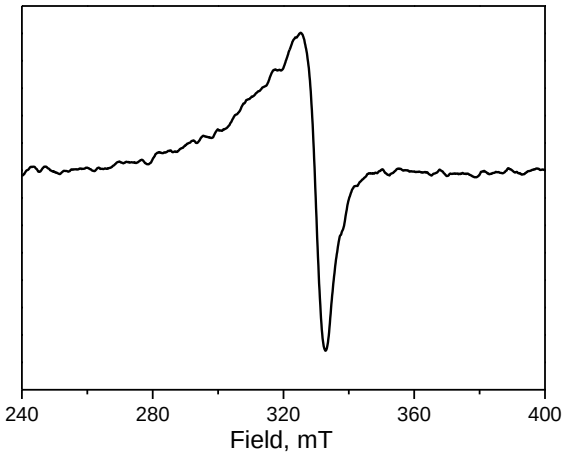

Figure 5.3. The X-band EPR spectrum at high microwave power and modulation amplitude of the RFQ sample of reduced wt SLAC reacted with oxygen for 8ms. Enzyme and oxygen concentration are 360 μM and 650 μM, respectively, both in 100 mM sodium phosphate buffer, pH 6.8. EPR measurement: microwave frequency 9.5 GHz, T = 8 K, microwave power 100 mW and modulation amplitude 1.6 mT.

5.4 Discussion

In this study, we have investigated the reoxidation of fully reduced wt SLAC with molecular oxygen at pH 6.8 using RFQ EPR on the time scale of milliseconds. The EPR spectra at 40 K for the RFQ samples with a reaction time between 8 ms and 2 seconds are found to be the superposition of T1 Cu and T2 Cu in approximately 1:1 ratio. The shape of the EPR spectrum hardly changes after 8 ms, which means that the ratio of T1 to T2 Cu remains constant. Quantitative EPR experiments are necessary to find out whether the overall signal increases with time. If not, both T1 and T2 Cu are completely reoxidized within 8ms.

In the reoxidation of T1D SLAC, clear evidence for the participation of a tyrosine radical in the catalytic cycle of the enzyme was obtained by EPR spectroscopy, optical spectroscopy and mutagenesis studies[17,20]. We have not obtained any indication from EPR of the presence of a tyrosyl radical during the reoxidation of wt SLAC, neither a signal of an isolated radical nor of a radical in interaction with a copper. In agreement with this results, no 420 nm absorption band of tyrosine was detected during the reoxidation of wt SLAC by transient absorption spectroscopy under similar experimental conditions of pH (6.8) and concentration of oxygen (610 μM) [A. Tepper and G. W. Canters, unpublished data]. This seems to imply that, in contrast to T1D SLAC, the tyrosine residue Y108 plays no role for wt SLAC. However, the 420 nm absorption band was previously observed during the reoxidation of wt SLAC in the presence of a lower amount of oxygen (130 μM)[17]. Moreover, studies by optical spectroscopy showed that the replacement of Y108 with alanine or phenylalanine in wt SLAC affects the turnover number of the enzyme in the steady state[20]. For the moment the observations as regards the participation of tyrosine Y108 in the oxygen reduction to water by wt SLAC seem contradictory, and more investigations under a variety of reaction conditions are necessary before definite conclusions can be drawn.

Our RFQ EPR experiments did not provide any indication of the presence of the native intermediate in the reoxidation of wt SLAC. The EPR spectra recorded at 40 K clearly show the signal from T2 Cu, which is not expected for NI because of the coupling with the T3 Cu's. As observed with other MCO's, the coupled TNC shows a broad signal with $g < 2$ at low temperature (<20 K)[2]. The EPR spectrum of the RFQ sample of reduced wt SLAC reacted with oxygen for 8 ms recorded at 8 K, high microwave power and high modulation amplitude did not show a broad band above 350 mT ($g \approx 1.85$). Such an intermediate has also not

been observed for T1D and T1D Y108F SLAC under similar experimental conditions. The lack of detection of NI during the single turnover of wt SLAC and different mutants means that either NI is not formed or its lifetime is shorter than 8 ms. The lifetime of the NI varies between milliseconds and minutes for different MCOs for which such an intermediate has been detected. For instance in the absence of reducing substrate, the NI in wt *Rhus Vernicifera* laccase is formed within 1 ms and is stable for minutes[5] while in the case of wt human ceruloplasmin the half-lifetime of NI was estimated to be 200-500 ms[6]. On the other hand no indication of NI-like features was observed for the wt Laccase from *Trametes versicolor* (a 3dMCO) even on the time scale of microseconds[21]. This suggests that the differences in the quaternary structure, the second-sphere residues around the coppers and the source of the enzyme may lead to differences in the pathway of O_2 reduction by MCO's. As yet, we have no evidence of a role of NI for SLAC, but more experiments under different reaction conditions (pH and concentration of O_2) are required to exclude that NI is part of the reaction cycle for SLAC.

5.5 Conclusion

We have investigated the reoxidation of wild-type SLAC by RFQ/EPR under similar experimental conditions as used for the study of T1D SLAC described in chapter 4 to search for the involvement of the tyrosyl radical and NI during the reoxidation of the wt enzyme. The EPR spectra of fully reduced wt SLAC mixed with oxygen on the time scale of milliseconds and longer can be described as the sum of T1 Cu and T2 Cu signals. We find no indications of a tyrosyl radical nor of an NI in the single turnover of wt SLAC. The lack of detection of these intermediate during the single turnover of wt SLAC means that either these are not formed or their lifetime is shorter than 8 ms. More

experiments under a variety of reaction conditions are necessary to exclude the involvement of these intermediates in the reaction cycle of wt SLAC.

References

(1) Solomon, E. I.; Hadt, R. G. Recent Advances in Understanding Blue Copper Proteins. *Coord. Chem. Rev.* **2011**, *255* (7–8), 774–789.

(2) Jones, S. M.; Solomon, E. I. Electron Transfer and Reaction Mechanism of Laccases. *Cell. Mol. Life Sci.* **2015**, *72* (5), 869–883.

(3) Cole, J.; Ballou, D.; Solomon, E. I.; Solomon, E. Spectroscopic Characterization of the Peroxide Intermediate in the Reduction of Dioxygen Catalyzed by the Multicopper Oxidases. *J. Am. Chem. Soc.* **1991**, *113* (22), 8544–8546.

(4) Palmer, A. E.; Sang Kyu Lee; Solomon, E. I. Decay of the Peroxide Intermediate in Laccase: Reductive Cleavage of the O-O Bond. *J. Am. Chem. Soc.* **2001**, *123* (27), 6591–6599.

(5) Lee, S. K.; George, S. D.; Antholine, W. E.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. Nature of the Intermediate Formed in the Reduction of O_2 to H_2O at the Trinuclear Copper Cluster Active Site in Native Laccase. *J. Am. Chem. Soc.* **2002**, *124* (21), 6180–6193.

(6) Machonkin, T. E.; Solomon, E. I. The Thermodynamics, Kinetics, and Molecular Mechanism of Intramolecular Electron Transfer in Human Ceruloplasmin. *J. Am. Chem. Soc.* **2000**, *122* (50), 12547–12560.

(7) Morishita, H.; Kurita, D.; Kataoka, K.; Sakurai, T. Study on Dioxygen Reduction by Mutational Modifications of the Hydrogen Bond Network Leading from Bulk Water to the Trinuclear Copper Center in Bilirubin Oxidase. *Biochem. Biophys. Res. Commun.* **2014**, *450* (1), 767–772.

(8) Yoon, J.; Liboiron, B. D.; Sarangi, R.; Hodgson, K. O.; Hedman, B.; Solomon, E. I. The Two Oxidized Forms of the Trinuclear Cu Cluster in the Multicopper Oxidases and Mechanism for the Decay of the Native Intermediate. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (34), 13609–13614.

(9) Ferraroni, M.; Matera, I.; Chernykh, A.; Kolomytseva, M.; Golovleva, L. A.; Scozzafava, A.; Briganti, F. Reaction Intermediates and Redox State Changes in a Blue Laccase from Steccherinum Ochraceum Observed by Crystallographic High/low X-Ray Dose Experiments. *J. Inorg. Biochem.* **2012**, *111*, 203–209.

(10) Wherland, S.; Farver, O.; Pecht, I. Multicopper Oxidases: Intramolecular Electron Transfer and O_2 Reduction. *J. Biol. Inorg. Chem.* **2014**, *19*, 541–554.

(11) Komori, H.; Sugiyama, R.; Kataoka, K.; Miyazaki, K.; Higuchi, Y.; Sakurai, T. New Insights into the Catalytic Active-Site Structure of Multicopper Oxidases.

Acta Crystallogr. Sect. D Biol. Crystallogr. **2014**, *70* (3), 772–779.

(12) Giardina, P.; Faraco, V.; Pezzella, C.; Piscitelli, A.; Vanhulle, S.; Sannia, G. Laccases: A Never-Ending Story. *Cell. Mol. Life Sci.* **2010**, *67* (3), 369–385.

(13) Sharma, P.; Goel, R.; Capalash, N. Bacterial Laccases. *World J. Microbiol. Biotechnol.* **2007**, *23* (6), 823–832.

(14) Komori, H.; Higuchi, Y.; Ions, F. C. Structural Insights into the O_2 Reduction Mechanism of Multicopper Oxidase. *J. Biochem.* **2015**, *158* (4), 293–298.

(15) Bento, I.; Peixoto, C.; Zaitsev, V. N.; Lindley, P. F. Ceruloplasmin Revisited: Structural and Functional Roles of Various Metal Cation-Binding Sites. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2007**, *63* (2), 240–248.

(16) Machczynski, M. C.; Vijgenboom, E.; Samyn, B.; Canters, G. W. Characterization of SLAC: A Small Laccase from Streptomyces Coelicolor with Unprecedented Activity. *Protein Sci.* **2004**, *13* (9), 2388–2397.

(17) Tepper, A. W. J. W.; Milikisyants, S.; Sottini, S.; Vijgenboom, E.; Groenen, E. J. J.; Canters, G. W. Identification of a Radical Intermediate in the Enzymatic Reduction of Oxygen by a Small Laccase. *J. Am. Chem. Soc.* **2009**, *131* (33), 11680–11682.

(18) Skálová, T.; Dohnálek, J.; Østergaard, L. H.; Østergaard, P. R.; Kolenko, P.; Dušková, J.; Štěpánková, A.; Hašek, J. The Structure of the Small Laccase from Streptomyces Coelicolor Reveals a Link between Laccases and Nitrite Reductases. *J. Mol. Biol.* **2009**, *385* (4), 1165–1178.

(19) Nami, F.; Gast, P.; Groenen, E. J. J. Rapid Freeze-Quench EPR Spectroscopy : Improved Collection of Frozen Particles. *Appl. Magn. Reson.* **2016**, *47*, 643–653.

(20) Gupta, A.; Nederlof, I.; Sottini, S.; Tepper, A. W. J. W.; Groenen, E. J. J.; Thomassen, E. A. J.; Canters, G. W. Involvement of Tyr108 in the Enzyme Mechanism of the Small Laccase from Streptomyces Coelicolor. *J. Am. Chem. Soc.* **2012**, *134* (44), 18213–18216.

(21) Matijošyte, I.; Arends, I. W. C. E.; Sheldon, R. A.; De Vries, S. Pre-Steady State Kinetic Studies on the Microsecond Time Scale of the Laccase from Trametes Versicolor. *Inorganica Chim. Acta* **2008**, *361* (4), 1202–1206.

(22) Augustine, A. J.; Quintanar, L.; Stoj, C. S.; Kosman, D. J.; Solomon, E. I. Spectroscopic and Kinetic Studies of Perturbed Trinuclear Copper Clusters: The Role of Protons in Reductive Cleavage of the O-O Bond in the Multicopper Oxidase Fet3p. *J. Am. Chem. Soc.* **2007**, *129* (43), 13118–13126.