Unraveling the mechanism of multicopper oxidases : from ensemble to single molecule

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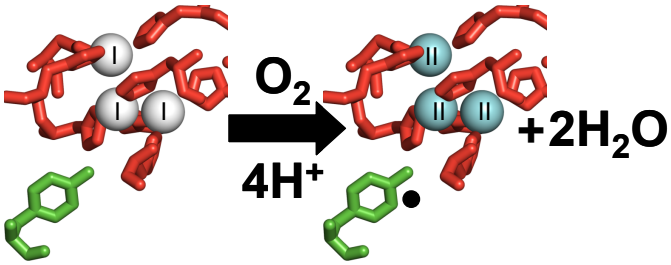
Chapter 2

Involvement of Tyr108 in the Enzyme Mechanism of the Small Laccase from *Streptomyces coelicolor**

Abstract:



The enzyme mechanism of the multicopper oxidase (MCO) SLAC from *Streptomyces coelicolor* was investigated by structural (XRD), spectroscopic (optical, EPR) and kinetics (stopped-flow) experiments on variants in which residue Tyr108 had been replaced by Phe or Ala through site-directed mutagenesis. Contrary to the more common three-domain MCOs, a tyrosine in the two-domain SLAC is found to participate in the enzyme mechanism by providing an electron during oxygen reduction, giving rise to the temporary appearance of a tyrosyl radical. The relatively low k_{cat}/K_M of SLAC and the involvement of Y108 in the enzyme mechanism may reflect an adaptation to a milieu in which there is an imbalance between the available reducing and oxidizing co-substrates. The purported evolutionary relationship between the two-domain MCO's and human ceruloplasmin appears to extend not only to the 3D structure and the mode of binding of the Cu's in the trinuclear center, as noted before, but also to the enzyme mechanism.

*Adapted from: Gupta, A.; Nederlof, I.; Sottini, S.; Tepper, A.W.J.W.;Groenen, E.J.J; Thomassen, E.A.J.; Canters, G.W. *J. Am. Chem. Soc.* **2012**, *134*, 18213.

2.1 Introduction

Multicopper oxidases (MCOs) catalyze the oxidation of a wide variety of substrates while reducing molecular oxygen to water. To achieve this, they utilize four copper atoms: a type 1 (T1) Cu which accepts reducing equivalents from the substrates and a trinuclear Cu cluster (TNC) where oxygen binds and gets converted to water. The TNC consists of a normal or type 2 (T2) Cu and a binuclear type 3 (T3) Cu pair. The mechanism of O_2 reduction at the TNC has been extensively studied by a variety of techniques.[1,2] It has been proposed that O_2 first binds at the TNC and then gets reduced in two $2e^-$ steps while others have argued for (left open) a first two–electron reduction step and subsequently two consecutive one–electron transfer steps.[2-5] The conserved residues between T1 Cu and TNC form a covalent link and promote rapid electron transfer from T1 Cu to the TNC.[6] However, views about O_2 binding and the mechanism of reduction have changed over time and still remain a topic of debate.[2,7-9] A brief account is presented in Chapter 1.

Analysis of the genome of *Streptomyces coelicolor* revealed the presence of a gene possibly encoding an MCO,[10] "small laccase" (SLAC), which owes its name to its smaller molecular weight as compared to the other well–known MCOs such as ascorbate oxidase, laccase, Fet3p and CueO. SLAC was found to be active as a homotrimer unlike most other MCOs described until now, which are monomeric proteins in solution.[11-14] It has been suggested that the three–domain ascorbate oxidase, the three–domain laccases and the six–domain ceruloplasmins have evolved via formation of a trimer of two–domain cupredoxins.[15] The recent crystal structure of SLAC clearly shows that the enzyme has such a trimeric form with a canocical TNC.[16,17] The structure together with structures of other 2–domain MCOs has been used in an attempt to fill in the gaps in the proposed evolution of MCOs.[16,18,19] Thus, it was of interest to study SLAC not only from a fundamental point of view to understand the structure–function relationship of this new enzyme, but also to seek footprints of the proposed ancestor that may have been carried over or discarded by the generations that followed after. Apart from that, SLAC holds potential for its applications in industry and its use as a cathode in biofuel cells to cater for the demands of green energy.[20,21]

SLAC was found to be excreted in the growth media of *S. coelicolor* cultures and, thus, was identified as an extracellular enzyme. The physiological roles of most extracellular enzymes, including SLAC, are unclear, researchers have mixed views,[22] but it is well known that members of the *Streptomycetes* genus produce dozens of antibiotics as secondary metabolites using such secreted enzymes.[23] For ease of expression and purification in higher yields, the gene encoding SLAC was isolated and recombinantly expressed in *E. coli*. Following the preliminary characterization, it was recently reported that reduced, type 1 depleted (T1D) SLAC, upon reaction with oxygen, forms an unusual biradical intermediate which has not been reported for the more common laccases.[24] Spectroscopic signatures, when compared with those of other enzymes and model systems, led to the hypothesis that a ferromagnetically coupled triplet state arises in SLAC due to exchange coupling of two unpaired spins, one residing on T2 Cu and the other on a tyrosyl radical ~5Å away. Similar spectroscopic features were also observed during turnover of the native enzyme, and a role of the radical in catalytic turnover was implicated (see also ref 21[25]). The present study attempts to identify the position of the radical and its role in enzyme catalysis. The preliminary results indicate that Tyr108 is the site carrying the unpaired spin. Its absence in site–directed mutants affects the enzyme kinetics. To the best of our knowledge, this is the first example where direct involvement of a tyrosyl radical in MCO catalysis has been demonstrated.

2.2 Results and Discussion

The crystal structure of wild–type (*wt)* SLAC (PDB: 3CG8)[16] shows the presence of a tyrosine residue (Y108) ~5Å away from the T2 Cu, as predicted from electron paramagnetic resonance (EPR) results.[24] This residue is located at the interface of two subunits in the trimeric form of the enzyme. Site–directed mutagenesis was carried out to prepare SLAC variants in which the tyrosine is replaced by phenylalanine (Y108F) or alanine (Y108A) in both the *wt* and the T1D (C288S) sequences.[26] All variants containing mutations in the wt sequence at position 108 were crystallized and analyzed by X–ray diffraction to a resolution of 2.7–2.8 Å.[26] The diffraction data confirm single amino acid

replacements at the desired position as well as intact active sites. No significant changes in the overall fold of the enzyme or near the active sites were observed (Figure 1). This facilitates a direct comparison of the enzyme kinetics and spectroscopic features of the mutants to those of the *wt* SLAC.[26]

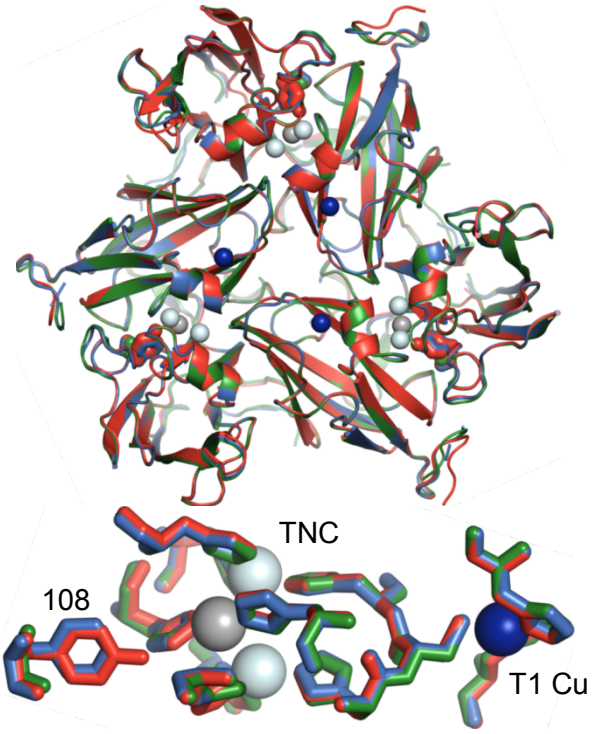


Figure 1: Ribbon representations of *wt* SLAC (red, PDB: 3CG8) overlaid with those of the mutants Y108F (blue, PDB: 4GXF) and Y108A (green, PDB: 4GY4). The bottom shows an expanded view of residues near the T1 Cu and TNC. The T3 Cu's are shown in light blue and the T2 Cu is colored in gray. Clearly the overall fold and active sites are intact in the mutants.

Steady–state kinetics experiments were performed using *N,N,N'N'*–tetramethyl–*p*–phenylenediamine (TMPD) as a substrate.[26] The enzymatic rate of reaction was monitored by following the formation of the oxidation product of TMPD at 610 nm at a given concentration of O_2. Alternatively, the rates of O_2 consumption during the reaction were measured using a Clark–type O_2 electrode.[26] TMPD is a one electron reductant, and the ratio of reaction rates monitored by optical spectroscopy versus those obtained by monitoring O_2

consumption was ~4 for any given concentration of substrates for the mutants studied. This implies complete reduction of O_2 to H_2O and shows that no H_2O_2 is released to solution. Addition of catalase to the reaction chamber, did not affect apparent O_2 uptake kinetics, nor did it show any increase in O_2 concentration, which strengthens this conclusion. The kinetic parameters obtained from the fits to the data are shown in Table I. The ratio of ${}^{app}k_{cat}$ obtained by the two methods is not exactly 4 owing to the fact that the solubility of O_2 is limited in buffer, and therefore rates at enzyme–saturating O_2 concentrations could not be measured.

Table 1: Turnover Number (k_{cat}) and Second–Order rate constant k_{cat}/K_M of *wt* and Mutant SLAC at 295 K in Phosphate Buffer at pH 6.[a]

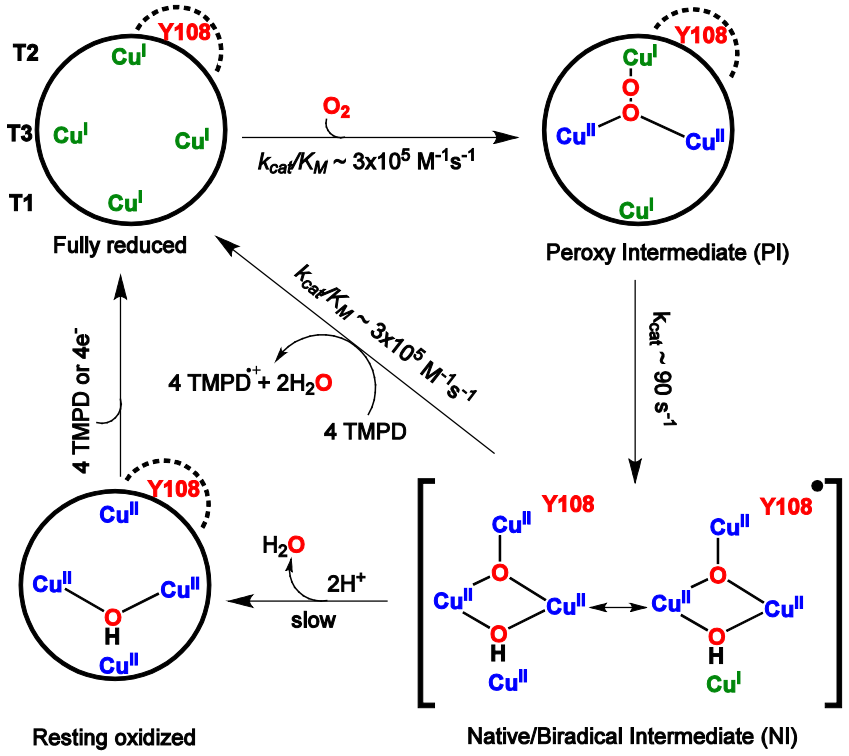
	${}^{app}k_{cat}$ (s^{-1})		${}^{app}k_{cat}/K_M$ ($M^{-1}s^{-1}$) (x10^{-5})	
	O_2[b]	TMPD[c]	O_2[b]	TMPD[c]
wt–SLAC	90 ± 3	315 ± 9	2.8 ± 0.2	3.2 ± 0.4
Y108A	34 ± 1	128 ± 3	2.7 ± 0.2	3.8 ± 0.4
Y108F	35 ± 1	132 ± 4	2.6 ± 0.2	2.4 ± 0.3

[a]For Michaelis–Menten plots see SI.[26] Parameters based on concentration of single subunit of SLAC as determined from the 280nm absorption (*i.e.*, equal to three times the SLAC concentration). [b]monitoring decrease in O_2 concentration *vs* time. [c]monitoring increase in absorption as a result of TMPD oxidation *vs* time.

It is evident from the data in Table 1 that the mutation affects only the turnover number (k_{cat}) of the enzyme and not the second–order rate constants (k_{cat}/K_M). While one would expect such a result for TMPD, as the mutations are far away from the T1 Cu reaction site for the TMPD (Figure 1), it is not immediately obvious why the second–order rate constant for O_2 remains unaffected. After all, the mutations are close to the O_2 reaction site (*i.e.*, the TNC). For any given enzyme, k_{cat}/K_M encompasses the steps from substrate binding up to and including the first irreversible step, whereas k_{cat} signifies the steps related to turnover of the ES complex and/or product release.[27] From single–turnover experiments on SLAC, it is found that O_2 binding to the TNC is practically irreversible.[26] This is in agreement with the enzymatic mechanism proposed for laccases, where the binding of O_2 followed by its reduction to the peroxide intermediate (PI) at the TNC was found to be irreversible.[28] We conclude that

the rate-limiting step(s) must occur after the binding of O_2 and reduction to PI and may involve the decay of PI to the native intermediate (NI).[29] TMPD or another co-substrate may then reduce the NI, thereby completing the reaction cycle and regenerating fully reduced SLAC ready to bind and reduce oxygen (Scheme I). Since k_{cat} is affected by the mutations at position 108, the rate-limiting step(s) must involve Y108 in the case of *wt* SLAC.

Scheme 1: Proposed Pathway in the Reaction Mechanism of SLAC with a role for Y108 as postulated initially.[30] The scheme may need modification in the light of more recently obtained evidence [33].[a]



[a]Reduced copper sites are depicted in light green and oxidized ones in blue. Tyrosine is shown in red. The rate constants are those obtained for SLAC in this study. Work is going on to characterize the Native/Biradical Intermediate (NI) in more detail.

The results from transient absorption spectroscopy and EPR spectroscopy support the original hypothesis about the localization of the unpaired spins on Y108 and T2 Cu in the T1D SLAC.[24] Experiments analogous to those

performed earlier[24,26] with the T1D SLAC were now carried out with the double mutants, T1D–Y108F and T1D–Y108A, i.e., where both C288S and Y108A or Y108F mutations are present. Pre–steady–state kinetics experiments reveal that the absorption feature around 410 nm which was earlier attributed to the formation of a tyrosyl radical, is not observed in these variants. Instead, an intermediate resembling the PI[31] (absorption maxima around 340, 470 and 710 nm) is observed (Figure 2a). In conjunction with the results above, no biradical signal is observed in the EPR spectrum of T1D–Y108A mutant (Figure 2b). It is interesting to note that a new radical signal is observed in the T1D–Y108F mutant, which is distinct from the signal reported for T1D itself (not shown). However, the intensity of the new radical signal compared to that of the total spin is very low (<10%). While similar turnover numbers are observed for Y108A and Y108F variants, we conclude that this signal cannot be catalytically relevant and is formed only in the absence of Y108. High–field magnetic resonance spectroscopy studies are underway to determine if this signal may correspond to a phenylalanine or possibly a tryptophan (W284, see SI) radical.

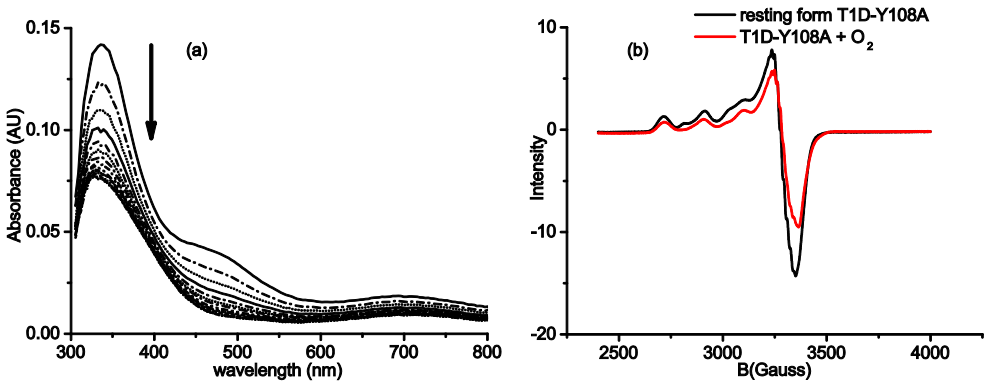


Figure 2: (a) Decay of the peroxide intermediate (PI) monitored by absorption spectroscopy following rapid mixing of reduced T1D–Y108A mutant (100 µM) with air–saturated phosphate buffer (pH 6.8) at 295 K. The PI is formed within 200 ms of mixing and then decays slowly with a half–life of 2.5 s. (b) X–band EPR spectrum of the resting form of T1D–Y108A mutant (black) overlaid with the spectrum of the same enzyme after it had been reduced, reoxidized, and frozen immediately (red). The spectra were recorded at 40 K (see also SI).

It is of interest here to contrast the sequence of O_2 reduction events as observed for SLAC (Scheme 1) with the sequence observed for the three–domain laccases. In *Rhus vernicifera* laccase (Lac), for instance, in which the T1Cu site has been inactivated by replacement of the T1Cu with a redox–innocent mercuric ion (T1Hg), complete reduction of the enzyme followed by reaction with O_2 results in the PI.[28] Similar behavior and rate constants are observed for another member of the three–domain MCOs, Fet3p, where the T1 site is inactivated by site–directed mutagenesis to prepare a T1D form of Fet3p.[32] Since in both cases there are only three electrons available in the reduced enzyme (i.e., at the T2 and the T3 sites), reduction of O_2 stops at the PI. In SLAC, on the other hand, under similar circumstances no PI is observed, but the optical and paramagnetic signatures of a Tyr radical appear instead. This is because the fourth electron, which is lacking in the T1D or T1Hg Lac, is now provided, apparently, by the Tyr moiety in the T1D SLAC, leading to the complete reduction of O_2 to H_2O. Consistent with this interpretation, we observe that only when the T1Cu site ***and*** Y108 have been deleted in SLAC does the spectroscopic evidence of PI be observed. We have not characterized the mode of peroxide binding at the TNC and other binding modes are possible than the one presented in Scheme 1. In addition, we can't exclude the possibility that under turnover conditions, two– or three–electron reduced SLAC can already start reacting with O_2.[33]

Multiple sequence alignment of SLAC with other multicopper proteins reveals that Y108 is conserved among the homologous two–domain MCOs[26] and also in human ceruloplasmin (hCp), for which a crystal structure has been published.[34] The appearance of a 410 nm intermediate (presumably oxidized tyrosine) during oxidation of fully reduced hCp was observed several years ago[35,36] but was not investigated further owing to the challenging mutagenesis of the recombinant protein and its purification in soluble form.[37] By analogy with SLAC, we now can assign, tentatively, this intermediate to a tyrosine radical. It is noteworthy that, among all known MCOs, only the six–domain ceruloplasmins and SLAC bind to the Cu sites with eight 2Nε–His coordination whereas the three–domain laccases and ascorbate oxidase contain seven 2Nε–His and one 1Nδ–His as a ligand, leading to a distinct asymmetry between the T3α (two 2Nε–His and one Nδ1–His) and T3β (three 2Nε–His) Cu's.[38] It has been suggested that this

structural difference between the T3α and T3β Cu's has important mechanistic consequences for O_2 binding and reduction in the three–domain laccases.[38] It will be of interest to see if the more symmetric coordination of the T3 site in SLAC (as in hCp) leads to different reaction kinetics than in the 3–domain laccases. Thus, SLAC and hCp share not only the conserved Y108 (Y107 in hCp) and the above same features of unique copper binding motifs but possibly also a similar enzyme mechanism.[26] We therefore propose SLAC to be suited as a model system to study the structure–function relationship of the more complicated hCp. We conclude that, not only from a structural viewpoint but also from a mechanistic point of view, our experiments appear to support the earlier postulated evolution of copper proteins, where the two–domain MCOs are proposed to be ancestors to the six–domain hCp.[15,39,40]

As for the evolutionary history of the three–domain laccases, it may be that the evolution of ascorbate oxidase and other three–domain MCOs such as the laccases took separate divergent paths or that, in the evolutionary process, subtle changes were incorporated in the primary coordination sphere of the TNC and also in the nearby protein environment by reorganizing the residues and the overall arrangement of the cupredoxin domains to provide a more efficient oxidase activity in laccases as compared to SLAC and hCp. In this connection, it must be noted that SLAC is secreted outside the cell and is supposed to perform its function there at relatively higher levels of oxygen (~5–8–fold) than inside the cell.[41] Thus, the 7–fold difference observed between the k_{cat}/K_M (and K_M) of O_2 between three–domain laccases and SLAC might reflect an evolutionary adaptation to the O_2 rich environment SLAC operates in. The lower k_{cat}/K_M of SLAC might be brought about by a reduced accessibility of the TNC connected with the access channel to the TNC in the trimeric form of SLAC.[42] Moreover, *S. coelicolor* is a soil and aqueous dwelling aerobe. It is conceivable that the concentration of reducing co–substrate is substantially less than that of oxygen. This may result in a slow loading of the enzyme with reducing equivalents, entailing the risk of producing long–lived forms of a three–electron loaded (PI + $1e^-$) intermediate which could lead to the generation of reactive oxygen species and consequent damage to the enzyme or the organism. In this case Y108 would be able to provide the fourth electron, thereby reducing the lifetime of a

deleterious three–electron reduced oxygen intermediate. This way, Y108 would act as a kinetic buffer of redox equivalents, thus preventing the generation of reactive oxygen species that might harm the enzyme and possibly also the bacterium. The danger posed at the same time by the presence of a reactive tyrosyl radical might be mitigated by the surrounding protein shell.

While the physiological significance of SLAC in the morphological development or metabolic system of *S. coelicolor* is not clear, along with the question of its natural substrates, it is evident that residue Y108 does form an integral part of the active site and is involved in the oxidase activity of this enzyme. If the natural substrate of SLAC has much higher turnover number for the *wt* SLAC, as known for the ferroxidase in yeast (Fet3p) and hCp,[43] the effect of mutation may be much more pronounced across the mutants. While we have noticed that SLAC catalyzes oxidative coupling of *o*–phenylenediamines and *o*–aminophenols to phenazines and phenoxazines, respectively, which are speculated to be extracellular secondary metabolites for signaling or self–defense of *Pseudomonas aeruginosa* and *S. antibioticus*,[44,45] their turnover numbers are too low to identify them as the natural substrates. The ability of SLAC to catalyze such reactions, along with a recombinant expression system, indeed holds new promises for the use of SLAC in industry for large–scale production of antibiotics for further studies or for human welfare. We are currently investigating other possible substrates and also attempting to identify the substrate binding pocket using crystallography, which may shed more light on the enzyme function and its similarities and dissimilarities relative to the other members of the family of MCOs.

2.3 Supplementary Information

Site–directed mutagenesis: Site–directed mutagenesis was carried out using the Quick Change site–directed mutagenesis kit (Stratagene). The primers used for respective mutations are given below where mutations are in bold and underlined.

Y108F

Forward primer: 5'–G CAC GGC CTG GAC **TTC** GAG ATC TCC AGC G–3'

Reverse primer: 5'–C GCT GGA GAT CTC **GAA** GTC CAG GCC GTG C–3'

Y108A

Forward primer: 5'–G CAC GGC CTG GAC **GCC** GAG ATC TCC AGC G–3'

Reverse primer: 5'–C GCT GGA GAT CTC **GGC** GTC CAG GCC GTG C–3'

Same set of primers were used to make mutations in the wt–SLAC or T1D–SLAC where the C288S mutant was used as a template. Desired mutations were confirmed by DNA sequencing (BaseClear).

The purification of the wt–SLAC and the mutants was carried out as reported previously.[10] The proteins were aliquoted and stored at –80 ^{0}C till further use. The absorption spectra of the *wt*–SLAC, Y108A and Y108F mutants are shown in Figure S1.

Crystallization and data collection: Crystallization experiments with SLAC were setup around conditions previously published.[16] SLAC crystals grew after 3 days in 35–45 % polyethylene glycol–550–monomethyl ether, 0.15–0.5 M NaCl and 0.1 M TRIS pH 8.5. Crystals were transferred to cryo–protectant containing 80 % mother liquor and 20 % glycerol, and frozen in liquid nitrogen. Single–crystal diffraction data were collected at the ESRF on beamline ID 14–4 (Grenoble) using an ADSC Q315 x–ray detector at a temperature of 100 K.

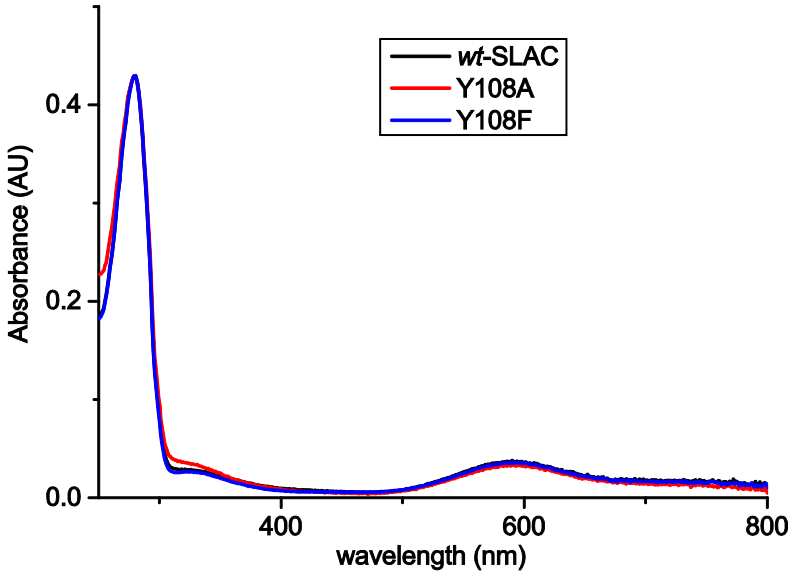


Figure S1: Absorption spectrum of the *wt*–SLAC overlaid with that of Y108A and Y108F. The spectra have been normalized at the 280nm absorption.

Diffraction data were indexed and integrated using Mosflm and imported into the CCP4 software package. SCALA was used for scaling the data. Crystals of SLAC variants belonged to the P43212 spacegroup and the cell dimensions are mentioned in table S1. Molrep was run for molecular replacement using 3CG8 as a homologous structure. Refmac was used to refine the structure. Terminal residues and mutations were built using Coot. The final structure parameters are summarized in Table S2.

Table S1. Data collection and processing statistics parameters for the SLAC variants studied (values in parenthesis correspond from highest resolution shell).

	Slac Y108F	Slac Y108A
Space Group	$P4_32_12$	$P4_32_12$
Unit Cell Parameters Å	$a = b = 176.66$ $c = 176.85$	$a = b = 178.44$ $c = 177.77$
Resolution Range Å	53.27 – 2.73 (2.88 – 2.73)	79.80 – 2.67 (2.81 – 2.67)
No of observations	370849 (46537)	1047174 (131331)
No of unique reflections	59361 (8756)	82018 (11803)
Data Completeness	80.5 (82.3)	100.0 (100.0)
Redundancy	6.2 (5.3)	12.8 (11.1)

Mosaicity (°)	0.45	0.65
Mean I/σ (I)	12.9 (3.5)	5.7 (0.9)
R_{pim}	0.043 (0.202)	0.099 (0.768)

Table S2. Structure parameters of SLAC variants.

	SLAC Y108F	SLAC Y108A
R_{work}	0.18204	0.21452
R_{free}	0.19858	0.22710
R_{all}	0.18238	0.21515
Mean *B*-factor (Å^2)	36.507	49.31
RMSD bond length from ideal (Å)	0.010	0.020
RMSD bond angles from ideal (°)	1.590	1.925
No of non hydrogen atoms	6888	6694
No of monomers in asymmetric units	3	3
No of water molecules	280	232
Other localized molecules	12 x Cu, 3 O , 8 PEGs	12 x Cu, 3 O , 8 PEGs
Solvent content (%)	84	84
Matthews Coefficient (Å^{3}DA^{-1})	7.52	7.78

EPR spectroscopy: Samples for EPR measurements were prepared as reported earlier.[24] Typically the enzyme concentration was ~1mM in 100mM phosphate buffer (pH 6.8). Spectra were recorded for the enzyme in the resting form. Alternatively, the enzyme was titrated with sodium dithionite to afford complete reduction of the copper centers and then reoxidized and immediately frozen in liquid N_2. The cw–EPR measurements were performed at X– band on a Bruker Elexsys E680 spectrometer at a temperature of 40K. All the spectra were acquired with a microwave power of 2 mW, a field modulation of 100 kHz, and a modulation amplitude of 0.5 mT. Spin quantification was performed against azurin or $CuSO_4$ as standard which reveals the content of paramagnetic Cu(II) in the resting form of the variants studied. The EPR spectrum of the resting form of *wt*–SLAC overlaid with those of Y108A and Y108F is shown in Figure S2.

Steady–state kinetics: Steady state kinetics measurements were performed in sodium phosphate buffer (200mM) at pH 6. The buffer concentration had no effect on the enzyme kinetics. A high buffer concentration was used owing to the

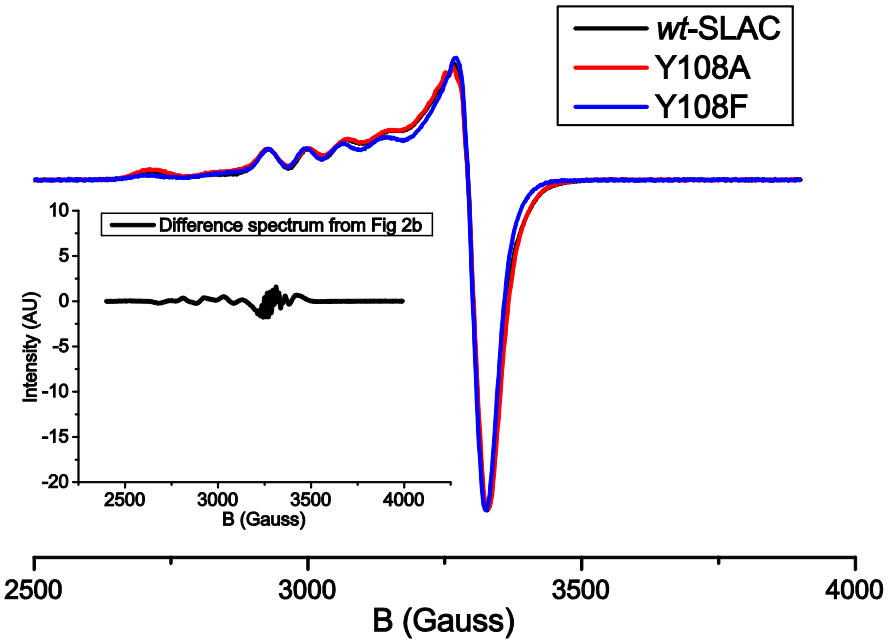


Figure S2: Normalized EPR spectra of the resting forms of *wt*–SLAC (black), Y108A (red) and Y108F (blue) overlayed on each other. The corresponding double integration reveals 2.0 ± 0.2 equivalents of Cu(II) per mol of monomer for *wt*–SLAC and Y108A and 1.7 ± 0.2 equivalents for Y108F mutant. The type–3 copper atoms are anti–ferromagnetically coupled and invisible, therefore, in the EPR spectrum. The inset shows the difference between the spectra reproduced in Figure 2b after normalization, which corresponds to less that 5% of total spin (~1.1 ± 0.2 equivalents of Cu(II) since T1 site is vacant).

higher concentration of the hydrochloride salts of the substrates used for the assays. The temperature was kept constant at 22 ^{0}C using a circulating water bath (Neslab). The measurements were made in two different ways: (1) by monitoring the absorption spectrum of the oxidized substrate with a UV–vis spectrometer (Varian) and (2) by monitoring O_2 consumption using a Clark–type O_2 electrode (Hansatech). Several organic and inorganic substrates were tested for activity and TMPD (N,N,N',N' tetramethyl–*p*–phenylenediamine) was chosen for measurements because of its high turnover and linear response with time. For solubility reasons, the dihydrochloride salt of this substrate was used.

TMPD oxidation was monitored at 610 nM (ϵ_{610} = 12,200 $M^{-1}cm^{-1}$). The stock solution of substrate was prepared fresh by dissolving the required amount in distilled water (MilliPore) up to a concentration of 250mM. The stock was diluted in desired buffer immediately prior to the measurement and the reaction was initiated by enzyme injection. Absorbance increase at 610 nM or O_2 consumption was monitored against time and the method of initial rates was used to analyze the data. In a typical measurement, the initial concentration of one of the substrates (O_2 or TMPD) was kept fixed while that of the other was varied to obtain data points at low rates and up to saturation (where possible). For O_2, the concentration is limited by its solubility (~1.3mM at O_2 saturation at room temperature) and so the data for higher concentrations could not be obtained. The rates for background oxidation of substrates or O_2 electrode drifts were subtracted before analyzing the data. The rates were fitted to a Michaelis–Menten type equation to obtain the kinetic parameters. As an example the plots are shown in Figure S3.

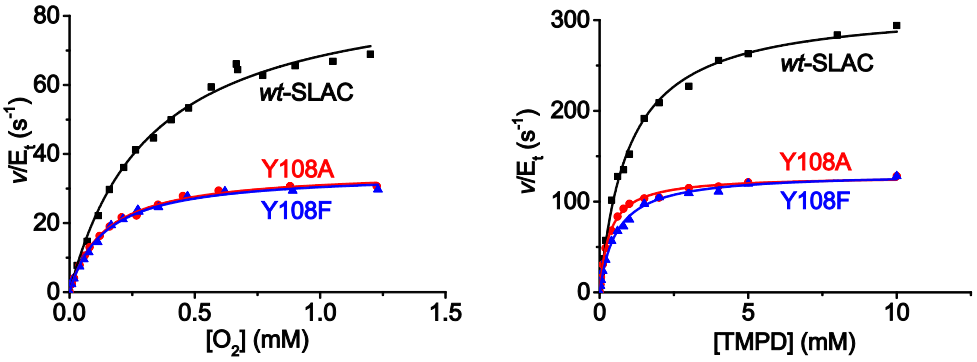


Figure S3: (a) Rate of O_2 consumption plotted against the respective O_2 concentration while keeping the initial TMPD concentration fixed at 10mM for each measurement. (b) Rate of TMPD oxidation plotted against the respective TMPD concentration while keeping the O_2 concentration fixed at maximum O_2 solubility (~ 1.3mM). Typical enzyme concentrations used in the assays were around 15–50 nM. Measurements were made at 295K in 200mM phosphate buffer (pH 6). The data were fitted to Michelis–Menten equation shown in bold lines. *wt*–SLAC shown in black, Y108A mutant in red and Y108F mutant in blue.

Transient kinetics: Transient kinetics was monitored using a stopped flow instrument (SX.18MV– Applied Photophysics). The instrument was used in a single mixing mode. It was made rigorously anaerobic by purging argon through all tubing and, where necessary, the argon flow was maintained throughout the measurement. One of the syringes was filled with buffer of known O_2 concentration while the other was filled with anaerobic buffer containing reduced enzyme. The enzyme concentration was typically 100 µM. Extinction coefficient of the ~340 nm band of the PI (Figure 2a) was estimated to be ~3000 $M^{-1}cm^{-1}$. The instrument dead time is ~2.5ms and 400 data points were recorded for each experiment. The spectra were recorded using a photo–diode array detector (300–1200nM). Data analysis was performed by global fitting of time traces using the software Pro–K. Reoxidation of completely reduced *wt*–SLAC or T1D SLAC was found to be irreversible under single turnover conditions as the y–axis intercept in Figure S4 is virtually zero ($k_{off} \approx 0$, see caption to Figure S4). Second order rate constants (k_{on}) similar to those presented in Table I were obtained.

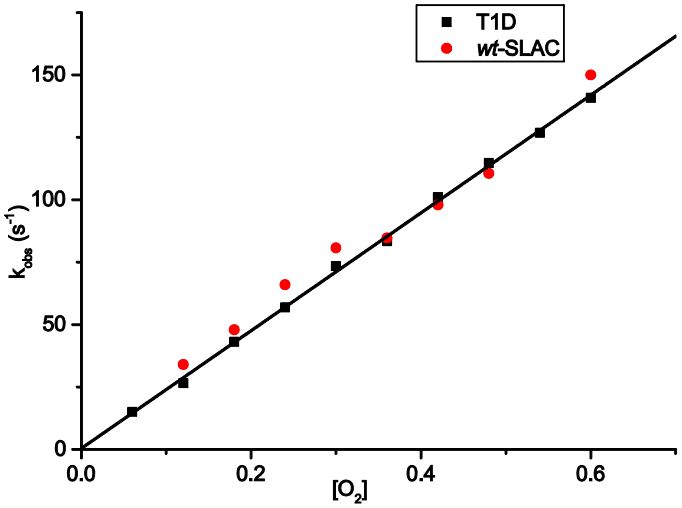


Figure S4: Rate of reoxidation of completely reduced *wt*–SLAC (red circles) or T1D (black squares) plotted against the respective O_2 concentration. The measurements were made following the fluorescence of tryptophan at 338 nm (excitation at 280nm) using the FRET principle as reported earlier.[42] The linear fit ($k_{obs} = k_{on}[O_2] + k_{off}$) yields a second order rate constant (k_{on}) of 2.3×10^5 $M^{-1}s^{-1}$ and a dissociation rate constant (k_{off}) of $<1s^{-1}$.

Relationship to Ceruloplasmin: Figure S5 represents the structural alignment of SLAC (3CG8) with human ceruloplasmin (2J5W). Cp is rendered in yellow, while the three domains of SLAC are represented in red, blue and green. The leftmost panel is a view perpendicular to the threefold symmetry axis of SLAC, the middle panel represents a view along this axis. The rightmost picture shows a close–up of the T1 site (dark blue for hCp ***and*** SLAC) and the TNC (light blue for hCp ***and*** SLAC). The hCp structure is shown again in yellow, while for SLAC the blue domain has been depicted. Moreover Tyr108 (SLAC) has been depicted in green and Tyr107 (hCp) in yellow. For completeness a conserved Trp has been depicted as well for SLAC (W284, blue) and hCp (W1019, yellow). The possible relevance of this residue for the function of the enzyme is under investigation. The alignment was performed using the Protein structure comparison service Fold at the European Bioinformatics Institute (http://www.ebi.ac.uk/msd–srv/ssm). The structural alignment of the quaternary structures of both proteins shows conserved domains and secondary structures including the highly conserved residues around the active site. It should be noted that hCp is a monomer and the TNC is located between the N and C terminus of the protein. SLAC is a trimer and the TNC are located at the N and C terminus of adjacent subunits.

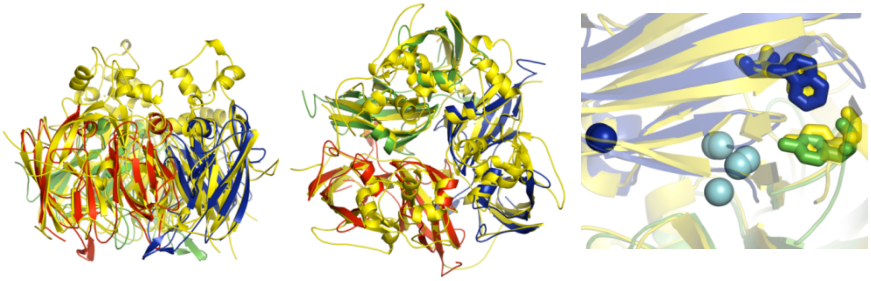


Figure S5: Overlay of human Ceruloplasmin (yellow) with SLAC (three chains shown in red, green and blue). Left: view perpendicular to the threefold symmetry axis of SLAC; middle: view along the same axis; right: close–up of the T1 site (dark blue for hCp ***and*** SLAC) and the TNC (light blue for hCp ***and*** SLAC) with hCp shown in yellow and the SLAC domain in blue. Tyr108 (SLAC) has been depicted in green and Tyr107 (hCp) in yellow. A conserved Trp (W284 (SLAC), blue; W1019 (hCp), yellow) has been depicted also.

Multiple sequence alignment of SLAC with all known 2–domain type–B MCOs: Sequence alignment of SLAC with the homologous type–B Multicopper proteins (following Nakamura's terminology[15]) is shown in Chart S1. The accession numbers are shown in the beginning and all of them are derived from various members of *Streptomycetes* genus. SLAC (gi 21225006) is highlighted on top. The residues coordinating to the Cu centers are highlighted in grey. The conserved tyrosines (corresponding to Y108 in SLAC) have been underlined. A conserved tryptophan which is shown in Figure S3 is also underlined. The sequence alignments were obtained using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Chart S1: Multiple sequence alignment of SLAC with other putative Multicopper proteins. Some of these have been recombinantly expressed and purified like EpoA (gi 15425710) from *Streptomyces griseus*. (see text above for further details)

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254393501   --------------------------------MDR----RSFGRRVLAGSAVAAG-GAA 22
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302555481   ----------------M-----------------DR----RSFNRRVLLGGAAVA------ 18
297203782   -------------MGAL-----------------DR----RGFNRRVLLGGAAVA------ 21
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297203782   --T----------------------SLSLA--------------PEARSDAGPAQ------ 38
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257056126    NPGSSFGFQVIAGEGVGPGAWMYHCHVQFHSDDGMAGIFLVRNEDGSLPPGAEEALERYR 331
257056125    NPGSSFGFQVIAGKGVGPGAWMYHCHMQVHSDDGMTGLFLVRNEDGSLPPGAEEALRRYH 314

21225006     GATAK--------SG-ESGEPTGGAA---------------AHEHEH------------- 343
294816028    GH-----------------------------------------AAR--------------- 355
291435857    HGAAS--------GKPEKAEKPAGSEKPAGS-----EKTEESEEHAH------------- 349
254393501    GH-----------------------------------------AAR--------------- 335
111559381    ---AH--------GG---GEPTADAP---------------AHQH--------------- 335
302557153    HGAAA-----------------------------------KEGQEHAH------------- 328
302555481    ----------------------HGEQ----------PP---AGGHEH------------- 325
297203782    ----------------------HSGQ--------------RAEHHH------------- 325
290955899    TAADP---GTAGTADGTTAGTTDGAG----------PT---GTSHHH------------- 355
239992058    AGATEKKAGEKAGEKTGGKAEKKSAEKTAAK-----DTGADAGEHQH------------- 353
318060531    KSG--------ATGKGTASGK---SA----------AG--EHAGHGG------------- 349
302522913    KSG--------ATGKGTASGK---SA----------AG--EHAGHGG------------- 349
326775330    AGGAEKKAGAKGAGANADKDAKGAA-----------------EHQH------------- 348
182434812    AGGTEKKAGAKGAGANADKAAKGAA-----------------EHQH------------- 348
15425710     AGGTEKKAGAKGAGANADKAAKGAA-----------------EHQH------------- 348
291455313    AP---KKSSKEAAEEKKAAEKKGEAQ--SGK-----SSQAESGDHAHH------------ 347
302540243    AG----------------------------------------RGS--------------- 344
295835579    KSGDSGTGGGAATGKGAAADKGVASG----------AG--AHAGHGG------------- 331
320007304    AG--------------------AA----------------KEHAH------------- 329
297190627    SE----------------------------------------EGHDH------------- 338
345011266    AH----------------------------------------HHG--------------- 334
345003352    AG--------------------AA----------------GEHAH------------- 333
334337260    DHQHGGHANH------------------------------------------------- 340
354612709    GHAGG------------------------------------------------------ 342
354577634    GHQGG----H------------------------------------------------- 338
257056126    NHG--------------------------------------------------------- 334
257056125    AHFS-------------------------------------------------------- 318
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[33] In recent studies presented in Chapters 3, we have obtained further evidence that T2 Cu is likely not involved in the peroxide binding a the TNC as shown in Scheme 1. Also, in Chapter 4, we fail to observe a long-lived on-state which suggests that fully reduced form of SLAC may not be part of the enzyme mechanism under turnover conditions. Thus, Scheme 1, as proposed earlier, may require further revisions in light of the new experiments.

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