JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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

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Supporting Information

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(XRD), spectroscopic (optical, EPR), and kinetics (stopped-flow) experiments on variants in which residue Tyr108 had been replaced by Phe or Ala through site-directed mutagenesis.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 MCOs 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.

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] It has been proposed that O_2 first binds at the TNC and then gets reduced in two $2e^-$ steps.[2] The conserved residues between T1 Cu and TNC form a covalent link and promote rapid electron transfer from T1 Cu to the TNC.[3] However, views about O_2 binding and the mechanism of reduction have changed over time and still remain a topic of debate.[4,5]

Analysis of the genome of *Streptomyces coelicolor* revealed the presence of a gene possibly encoding an MCO,[6] "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.[7−10] 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.[11] The recent crystal structure of SLAC clearly shows that the enzyme has such a trimeric form with a canocical TNC.[12,13] This structure, together with structures of other two-domain MCOs, has been used in an attempt to fill in the gaps in the proposed evolution of MCOs.[12,14,15] 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.[16,17]

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, and researchers have mixed views,[18] but it is well known that members of the *Streptomycetes* genus produce dozens of antibiotics as secondary metabolites using such secreted enzymes.[19] For ease of expression and purification in higher yields, the gene encoding SLAC was isolated and recombinantly expressed in *E. coli*.[6] 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.[20] 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 ∼5 Å 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). 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.

The crystal structure of wild-type (wt) SLAC (PDB: 3CG8)[12] shows the presence of a tyrosine residue (Y108) ∼5 Å away from the T2 Cu, as predicted from electron paramagnetic resonance (EPR) results.[20] This residue is located at the interface of two subunits in the trimeric form of the

Received: September 6, 2012
Published: October 24, 2012

dx.doi.org/10.1021/ja3088604 | J. Am. Chem. Soc. 2012, 134, 18213−18216

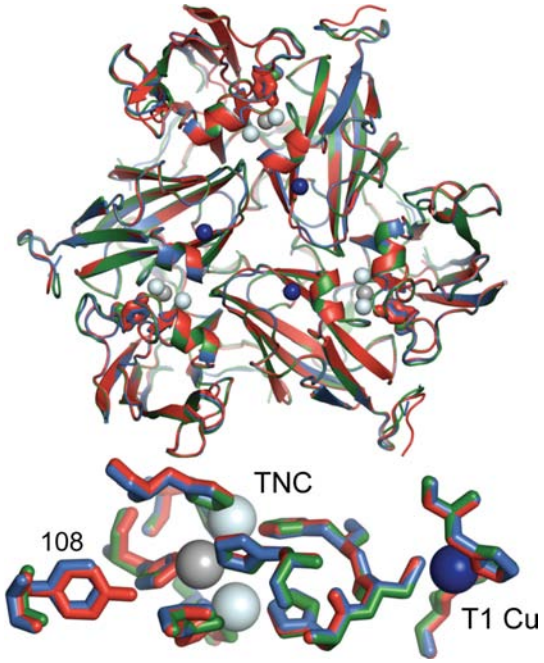


Figure 1. Ribbon representations of wt SLAC (red, PDB: 3CG8) overlaid with those of the mutants Y108F (blue, PDB: 4GXF) and Y108A (green, PDB: 4GY4). The bottom shows an expanded view of residues near the T1 Cu and TNC. The T3 Cu's are shown in light blue and the T2 Cu in gray. Clearly the overall fold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

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.[22] 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.[22] 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.[22]

Steady-state kinetics experiments were performed using *N,N,N′N′*-tetramethyl-*p*-phenylenediamine (TMPD) as a substrate.[22] 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.[22] 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 formed. 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 1. 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 Wild-Type and Mutant SLAC at 295 K in Phosphate Buffer at pH 6[a]

	$^{app}k_{cat}$ (s^{-1})		$^{app}k_{cat}/K_M$ ($\times 10^5$ M^{-1} s^{-1})	
	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

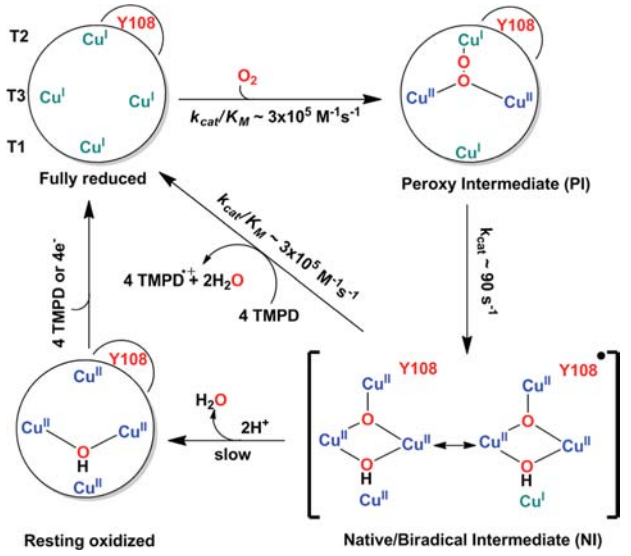
[a]For Michaelis−Menten plots see SI.[22] Parameters based on concentration of single subunit of SLAC as determined from the 280 nm absorption (i.e., equal to 3 times the SLAC concentration). [b]Monitoring decrease in O_2 concentration vs time. [c]Monitoring increase in absorption as a result of TMPD 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.[23] From single-turnover experiments on SLAC, it is found that O_2 binding to the TNC is practically irreversible.[22] 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.[24] 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).[25] 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 1). 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.

The results from transient absorption spectroscopy and EPR spectroscopy support the original hypothesis[20] about the localization of the unpaired spins on Y108 and T2 Cu in the T1D SLAC. Experiments analogous to those performed earlier with the T1D SLAC[20,22] 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[26] (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.

dx.doi.org/10.1021/ja3088604 | J. Am. Chem. Soc. 2012, 134, 18213−18216

Scheme 1. Proposed Pathway in the Reaction Mechanism of SLAC with a Role for Y108[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.

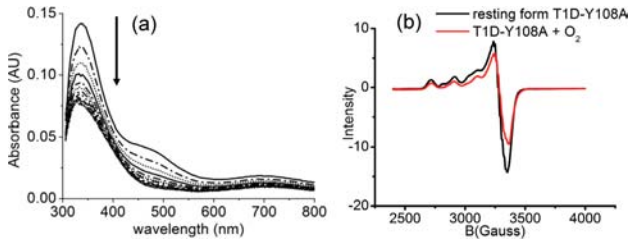


Figure 2. (a) Decay of the peroxide intermediate (PI) monitored by absorption spectroscopy following rapid mixing of reduced T1D-Y108A mutant with air-saturated phosphate buffer (pH 6.8) at 295 K. The PI is formed within 200 ms of mixing and then decays slowly with a half-life of 2.5 s. (b) X-band EPR 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.[24] 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.[27] 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 PI appear.

It is important to note that Y108 is conserved among the homologous two-domain MCOs[22] and also in human ceruloplasmin (hCp), for which a crystal structure has been published.[28] The appearance of a 410 nm intermediate (presumably oxidized tyrosine) during oxidation of fully reduced hCp was observed several years ago[29,30] but was not investigated further owing to the challenging mutagenesis of the recombinant protein and its purification in soluble form.[31] 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.[32] 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.[32] 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 three-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.[22] 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.[11,33,34]

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.[35] 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 in which SLAC operates. 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.[36] 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

dx.doi.org/10.1021/ja3088604 | J. Am. Chem. Soc. 2012, 134, 18213–18216

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,[37] 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*,[38,39] 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.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Navraj Pannu for collecting the X-ray diffraction data and for assistance during data analysis, and Faezeh Nami for assistance with the EPR experiments. We acknowledge the staff at the European Synchroton Radiation Facility for assistance during data collection. This work was supported by the Netherlands Organization for Scientific Research through the Foundation for the Chemical Sciences (NWO-CW), in part by a VENI grant (to A.W.J.W.T.) and in part by a Top-Grant (to G.W.C.).

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