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Chapter 5

The structural effect of mutating Tyr108 on the Active Site conformation of the Small Laccase from *Streptomyces coelicolor*5

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Abstract

Close to the active site Cu's of the Small Laccase protein, a Tyr is found, which is likely involved in the enzyme mechanism. The Tyr was mutated (to Phe and to Ala) and the effect of these mutations on the conformation of the active site was shown by X-ray crystallography to be negligible. Comparison of the enzymatic activities of these mutants suggests that, contrary to the more common three-domain MCOs, the tyrosine participated 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

5 In this chapter I present my contribution to the following paper:

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


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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. The enzyme mechanism of the multicopper oxidase (MCO) SLAC from Streptomyces coelicolor was investigated by structural (XRD) and compared to spectroscopic (optical, EPR), and kinetics (stopped-flow) experiments on variants in which residue Tyr108 had been replaced by Phe or Ala through site-directed mutagenesis.

5.1 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). 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 canonical 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, possibly Tyr108. 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).

In order to examine the role of Tyr 108 by site directed mutagenesis, we had to confirm that mutating this residue did not affect the active site other than removing a potential location for the radical that forms during the reaction. For this purpose, we crystallized the SLAC mutants Y108F and Y108A, and determined their structure.
5.2 Materials and methods

5.2.1 Site-directed Mutagenesis
Site-directed mutagenesis was carried out to prepare SLAC variants in which the tyrosine is replaced by phenylalanine (Y108F) or alanine (Y108A), and 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 **GCC** GTC CAG GCC GTG C–3′

Desired mutations were confirmed by DNA sequencing (BaseClear).

The purification of the wildtype (wt) SLAC and the mutants was carried out as reported previously (6). The proteins were aliquoted and stored at -80 °C till further use. The absorption spectra of the wt SLAC, Y108A and Y108F mutants are shown in figure 1.
5.2.2 Crystallization and Data Collection

Crystallization experiments were carried out using the sitting drop vapor diffusion technique in SD-2 plates (Innovadyne). Rockmaker software (Formulatrix) was used for designing the experiments and tracking crystal growth. A Genesis (Tecan) was used for dispensing the screening solutions in the reservoirs. 200 nl of reservoir solution and 200 nl protein solution in sitting drop wells were dispensed by an ORYX 6 (Douglas Instruments) crystallization robot. Crystallization experiments with SLAC were setup around conditions previously published (12). Plates were stored at 20 °C and imaged using the automated imaging system Rock Imager (Formulatrix). Chemicals used for experiments were purchased from Sigma. Crystals of the SLAC mutants 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 beam line ID 14-4 (Grenoble) using an ADSC Q315 X-ray detector at a temperature of 100 K.
Figure 5.2 Crystals of SLAC mutant Y108A.

<table>
<thead>
<tr>
<th></th>
<th>SLAC Y108F</th>
<th>SLAC Y108A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P4$_3$2$_1$2</td>
<td>P4$_3$2$_1$2</td>
</tr>
<tr>
<td>Unit cell parameters</td>
<td>$a = b = 176.66 , c = 176.85$</td>
<td>$a = b = 178.44 , c = 177.77$</td>
</tr>
<tr>
<td>Resolution range Å</td>
<td>53.27 – 2.73 (2.88 – 2.73)</td>
<td>79.80 – 2.67 (2.81 – 2.67)</td>
</tr>
<tr>
<td>No. of observations</td>
<td>370849 (46537)</td>
<td>1047174 (131331)</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>59361 (8756)</td>
<td>82018 (11803)</td>
</tr>
<tr>
<td>Data completeness</td>
<td>80.5 (82.3)</td>
<td>100.0 (100.0)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>6.2 (5.3)</td>
<td>12.8 (11.1)</td>
</tr>
<tr>
<td>Mosaicity (°)</td>
<td>0.45</td>
<td>0.65</td>
</tr>
<tr>
<td>Mean I/σ (I)</td>
<td>12.9 (3.5)</td>
<td>5.7 (0.9)</td>
</tr>
<tr>
<td>R$_{pim}$</td>
<td>0.043 (0.202)</td>
<td>0.099 (0.768)</td>
</tr>
</tbody>
</table>

Table 5.1 Data collection and processing statistics parameters for the SLAC variants studied (values in parenthesis correspond from highest resolution shell).
5.2.3 Data Analysis

Diffraction data were indexed and integrated using MOSFLM (22) and imported into the CCP4 software package (23). SCALA (24) was used for scaling the data. Crystals of SLAC variants belonged to the P4$_3$2$_1$2 space group and the cell dimensions are mentioned in table 1. Molrep (25) was run for molecular replacement using 3CG8 (12) as a homologous structure. 10 rounds of REFMAC (26) were run to refine the structure. Terminal residues, mutations and water molecules were built using Coot (27) and another 10 refinement runs were done using REFMAC. The final structure parameters are summarized in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>SLAC Y108F</th>
<th>SLAC Y108A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{work}$</td>
<td>0.18204</td>
<td>0.21452</td>
</tr>
<tr>
<td>$R_{free}$</td>
<td>0.19858</td>
<td>0.22710</td>
</tr>
<tr>
<td>$R_{all}$</td>
<td>0.18238</td>
<td>0.21515</td>
</tr>
<tr>
<td>Mean B-factor ($\text{Å}^2$)</td>
<td>36.507</td>
<td>49.31</td>
</tr>
<tr>
<td>RMSD bond lengths from ideal ($\text{Å}$)</td>
<td>0.010</td>
<td>0.020</td>
</tr>
<tr>
<td>RMSD bond angles from ideal ($^\circ$)</td>
<td>1.590</td>
<td>1.925</td>
</tr>
<tr>
<td>No. of non hydrogen atoms</td>
<td>6888</td>
<td>6694</td>
</tr>
<tr>
<td>No. of monomers in asymmetric units</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>280</td>
<td>232</td>
</tr>
<tr>
<td>Other localized molecules</td>
<td>12 x Cu, 3 O, 8 PEGs</td>
<td>12 x Cu, 3 O, 8 PEGs</td>
</tr>
<tr>
<td>Solvent content (%)</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>Matthews coefficient ($\text{Å}^3\text{Da}^{-1}$)</td>
<td>7.52</td>
<td>7.78</td>
</tr>
</tbody>
</table>

Table 5.2 SLAC variants structure parameters.
5.2 Results

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 theT1D (C288S) sequences. 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. 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 3). This facilitates a direct comparison of the enzyme kinetics and spectroscopic features of the mutants to those of the wt SLAC.
Figure 5.3 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.

**Discussion and Conclusion**

Mutation of Tyr 108 in SLAC to either Phe or Ala does not affect the other residues of the active site. This allows interpreting activity assays and spectroscopic analysis of these mutants in mechanistic terms. Below the implications of these additional experiments (described in
Gupta et al., 2012 (42) are summarized.

**Kinetics**

<table>
<thead>
<tr>
<th></th>
<th>( \text{app} k_{\text{cat}} ) (s(^{-1}))</th>
<th>( \text{app} k_{\text{cat}}/K_M ) (x10(^2) M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( O_2^b )</td>
<td>( \text{TMPD}^c )</td>
</tr>
<tr>
<td><strong>wt SLAC</strong></td>
<td>90 ± 3</td>
<td>315 ± 9</td>
</tr>
<tr>
<td></td>
<td>2.8 ± 0.2</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td><strong>Y108A</strong></td>
<td>34 ± 1</td>
<td>128 ± 3</td>
</tr>
<tr>
<td></td>
<td>2.7 ± 0.2</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td><strong>Y108F</strong></td>
<td>35 ± 1</td>
<td>132 ± 4</td>
</tr>
<tr>
<td></td>
<td>2.6 ± 0.2</td>
<td>2.4 ± 0.3</td>
</tr>
</tbody>
</table>

**Table 5.3** Turnover Number (\( k_{\text{cat}} \)) and Second-Order Rate Constant (\( k_{\text{cat}}/K_M \)) of Wild Type and Mutant SLAC at 295 K in Phosphate Buffer at pH6.

It is evident from the data in Table 3 that the mutation affects only the turnover number (\( k_{\text{cat}} \)) of the enzyme and not the second-order rate constants (\( k_{\text{cat}}/K_M \)). 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 3), 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, \( k_{\text{cat}}/K_M \) encompasses the steps from substrate binding up to and including the first irreversible step, whereas \( k_{\text{cat}} \) signifies the steps related to turnover of the ES complex and/or product release (28). From single-turnover experiments on SLAC, it is found that \( O_2 \) binding to the TNC is practically irreversible. 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 (29). 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) (30). 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 \( k_{\text{cat}} \) is affected by the mutations at position 108, the rate-limiting step(s) must involve Y108 in the case of wt SLAC. Structural studies proved that no other alterations were present to the proteins active site or overall 3 tertiary structure.

\[
\text{Scheme 5.1 Proposed Pathway in the Reaction Mechanism of SLAC with a Role for Y108. Reduced copper sites are depicted in light green and oxidized ones in blue. Tyrosine is shown in red. The rate constants are those obtained for SLAC in this study. Work is going on to characterize the native/ biradical intermediate (NI) in more detail.}
\]

**Spectroscopic Experiments**

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) 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 4b). In conjunction with the results above, no biradical signal is observed in the EPR spectrum of T1D-Y108A mutant (Figure 4b). 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 radical.

![Absorbance and Intensity](image)

**Figure 5.4** (a) Decay of the peroxide intermediate (PI) monitored by absorption spectroscopy following rapid mixing of reduced T1DY108A 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.
It is important to note that Y108 is conserved among the homologous two-domain MCOs and also in human ceruloplasmin (hCp), for which a crystal structure has been published (32). The appearance of a 410 nm intermediate (presumably oxidized tyrosine) during oxidation of fully reduced hCp was observed several years ago (33,34) but was not investigated further owing to the challenging mutagenesis of the recombinant protein and its purification in soluble form (35). 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 1N6-His as a ligand, leading to a distinct asymmetry between the T3α (two 2Nε-His and one N61-His) and T3β (three 2Nε-His) Cu’s (36). It has been suggested that this structural difference between the T3α and T3β Cu’s has important mechanistic consequences for O2 binding and reduction in the three-domain laccases (36). 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. 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 sixdomain hCp (11,37,38).

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 (39), 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 (40,41),
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.

References
