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

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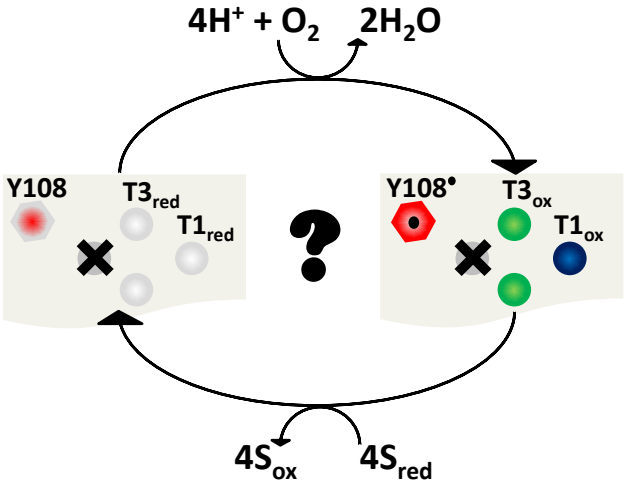
Chapter 3

Small laccase from *S. coelicolor* can turnover without Type 2 Cu but not without its Coordinating Histidine*

Abstract:



The type 2 Cu of the Small Laccase (SLAC) from *Streptomyces coelicolor* was removed from the protein under reducing conditions utilizing a Cu(I) chelator at neutral pH. The enzyme form thus produced still possesses about 33% activity relative to the wt protein. However, mutation of a coordinating (to type 2 Cu) His residue (H102) to a glycine, tyrosine, phenylalanine or glutamine causes enzyme activity to diminish by more than two orders of magnitude but doesn't lead to Cu depletion. Atomic absorption and EPR spectroscopy together with kinetics and inhibition studies suggest that T2 Cu is not involved in binding O_2 at the trinuclear Cu site and the enzyme is capable to function without this Cu. We hypothesize that during the evolution of Cu proteins, the type 3 Cu sites may have been recruited prior to the type 2 Cu sites in multicopper proteins. However, the question remains: how the protein containing only three Cu's (i.e. the T1 Cu and the T3 Cu pair) is capable of reducing O_2 to H_2O.

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3.1 Introduction

Biochemical conversion of O_2 to H_2O is an essential process from where respiratory beings derive the energy required for their vital processes. The reaction, requiring formally four electrons and four protons, is typically mediated by heme and/or Cu proteins. Multicopper oxidases (MCO's) constitute a family of enzymes containing four copper ions arranged within the protein matrix to catalyze this process. The reducing equivalents, from substrates, are accepted at the type 1 (T1) Cu site which transmits them through a conserved HisCysHis pathway to the trinuclear Cu cluster (TNC) where O_2 binds and gets reduced.[1] The TNC is composed of a normal or type 2 (T2) Cu and a binuclear type 3 (T3) Cu pair. It has been demonstrated from site–directed mutagenesis, spectroscopy, kinetics and theoretical approaches that the MCO's cannot catalyze the reduction of O_2 if the T2 Cu site is absent.[2] We demonstrate first clear evidence that small laccase (SLAC), from *S. coelicolor*, still exhibits catalytic activity after the T2 Cu site has been chemically removed. Further, the substitution of a T2 Cu coordinating His102 residue causes the activity to diminish by more than two orders of magnitude but doesn't lead to depletion of the T2 Cu.

The quest to identify polyphenol oxidase from lacquer trees as being a copper containing metallo–oxidase is more than a century old.[3] It is interesting to note that a clear demonstration of the Cu containing nature of these proteins comes from reconstitution experiments whereby completely inactive apo–protein was reconstituted with various metal ions and only cupric ion seemed to restore the activity of the protein.[4,5] Later on, MCO's were isolated from other sources including plants and fungi. Whereas the role of Cu in oxidase activity was established, the challenge remained to identify the contribution of individual Cu's to the spectroscopy and kinetics of these proteins. While the electron paramagnetic resonance (EPR) spectroscopic features and the mechanistic role of the blue or T1 Cu were well understood in terms of an electron transfer cofactor, the role of another paramagnetic Cu, the T2 Cu, was puzzling. It was shown that inorganic anions, which have strong Cu binding properties, inhibited the enzyme activity and also displayed spectroscopic evidence of binding to T2 Cu.[6] Further,

T2 Cu could be selectively removed from fungal laccase where the decrease of enzyme activity paralleled the extent of removal of T2 Cu.[7] These studies established clear evidence for a direct role of the T2 Cu in the fungal laccase enzyme mechanism. The ability to selectively remove the T2 Cu and to study the spectroscopy and kinetics of other MCO's triggered intense investigations and discussions. The method was further improved and was shown to work for ascorbate oxidase and other laccases.[8-15]

Apart from the T1 and T2 Cu's, there are two additional Cu ions in the MCO's which do not give rise to an EPR signal. This is because they are bridged by an oxygen atom which renders the Cu–pair diamagnetic properties by antiferromagnetic coupling. It provides a spectroscopic feature in absorption (λ_{max} ~ 330 nm) arising from a $O^{2-} \rightarrow$ Cu(II) charge transfer transition. The overall organization of Cu's in the protein became clearer when the crystal structure of ascorbate oxidase was solved.[16,17] The structure showed that the TNC is located ~13 Å away from the T1 Cu and that the organization of the two sites was in agreement with what was already anticipated from the spectroscopic studies. Despite the structural similarities of MCO's from various sources, there has been a strong debate about the mechanistic aspects of O_2 binding and reduction at the TNC.[18,19] X–ray diffraction experiments on ascorbate oxidase crystals soaked with H_2O_2 have demonstrated that the peroxide becomes terminally coordinated to one of the T3 Cu(II) ions.[20] A recent study on crystals of a blue laccase, from *Steccherinum ochraceum*, shows X–ray dose dependent reduction of the active site of the laccase as well as evidence of a peroxy group bound symmetrically between the two T3 Cu ions.[21] Yet another set of detailed spectroscopic and theoretical investigations on ferrous oxidase (Fet3p), from *Saccharomyces cerevisiae*, demonstrate that the O_2 binds between the T2 Cu and one of T3 Cu's called T3Cu–β.[2] Thus, the question about catalytically relevant intermediates present during the steady–state turnover of respective MCO's remains open. It won't be surprising to observe that different MCO's from different sources have unique catalytic mechanisms.

The advent of entire genome sequencing made it possible to identify gene fragments characteristic of MCO's in all domains of life and allowed researchers

to further investigate the similarities and differences among the members of this family of proteins.[22-24] Recently, a number of MCO's exhibiting structures different than those of the known MCO's, were identified, isolated and crystallized, seemingly filling in the voids in existing evolutionary theories of Cu proteins.[24-27] SLAC is one such protein which was identified as a homo–trimer of monomers, each monomer consisting of two cupredoxin domains unlike the three–domain monomeric laccases or ascorbate oxidase.[22,26] Careful mechanistic and spectroscopic studies enabled identification of the involvement of Tyr108 in the enzymatic reduction of O_2 at the TNC (see Chapter 2), a feature so far not seen for other laccases.[28] SLAC might be unique in this respect but this tyrosine appears to be conserved among all known sequences of the homologous putative two–domain multicopper proteins and also in human ceruloplasmin. Here we report another astonishing observation, viz. that SLAC doesn't lose its activity when the T2 Cu is chemically removed. This demands serious attention as it not only demonstrates the singular behavior of SLAC and possibly other members of the family, but also calls for a revised mechanism of O_2 binding and reduction to H_2O at the TNC.

3.2 Results and Discussion

SLAC was recombinantly expressed in *E. coli* and purified as reported earlier.[22] To selectively remove the T2 Cu from MCO's, majority of the methods reported in literature make use of prolonged dialysis of the protein in the presence of strong Cu(I)/Cu(II) chelators like bathocuprone disulfonate, dimethyl glyoxime and *N,N*–diethyldithiocarbamate.[7-10] Since the T2 Cu is coordinated by only two histidine residues, it is relatively labile and gets removed preferentially while the other Cu's remain bound to the protein. Several of these approaches were attempted to selectively remove the T2 Cu from SLAC without much success. In most of these experiments, the protein lost multiple Cu's and eventually got denatured. It became apparent that the Cu's in SLAC are much more weakly bound as compared to those of laccases and therefore somewhat mild conditions must be utilized to selectively remove the T2 Cu. The weak binding might arise from the fact that the TNC in SLAC is located at the interface of two SLAC monomers unlike the laccases where the TNC sits

between the N– and C–terminus of the same peptide chain.[26] The successful strategy of Cu removal was derived from a modification of the method by Malkin *et al.*[7] Briefly, 10mM of sodium bicinchoninate (BCA) was added to ~100uM SLAC in 200mM sodium phosphate buffer at pH 7.2. Thereafter, sodium ascorbate (Asc) was added to a final concentration of 100mM. Immediately upon addition of ascorbate the solution turned purple owing to the formation of $Cu(I)(BCA)_2$ complex (Figure 1a). The absorption of the complex was monitored at 560 nm (ϵ_{560} = 8000 $M^{-1}cm^{-1}$) for 30–60 mins till it reached a plateau. From the absorption, with the knowledge of protein concentration and extinction coefficient of the $Cu(I)(BCA)_2$ conplex, it was clear that one equivalent of Cu had formed a complex with BCA. The solution was then passed through a desalting column or dialysed against 100mM NaPi buffer at pH 7.2 to remove the excess ascorbate and the $Cu(I)(BCA)_2$ complex. The protein thus prepared (T2D SLAC) was checked for its Cu content by atomic absorption spectroscopy (AAS) and EPR. AAS revealed presence of only 3.0 Cu's per SLAC monomer, which was as expected (Table 1). The EPR spectrum showed no signs of the broad hyperfine lines in the low field region due to T2 Cu indicating that this Cu has been selectively removed from the protein (Figure 1b). The protein was checked for its activity as reported earlier and still possessed almost one–third of the wt–SLAC activity (Table 1).

A number of features are worth pointing out. First, the Cu was easily removed at neutral pH unlike previous experiments where Cu depletion was observed at pH <4.5 under mild denaturing conditions.[7] Second, the protein solution after desalting was blue in color and possessed almost identical absorption in the 600 nm region as before T2 Cu depletion and slightly diminished absorption around 330 nm (Figure 1c). There has been an intense debate about the redox state and spectroscopic properties of the T3 Cu site following T2 Cu removal from *Rhus* laccase which was largely dependent on the method employed for Cu depletion.[11-13,15,29-31] It has been suggested that there may exist multiple isotypes of T2D laccase, depending on the method of preparation, which might be responsible for the differences in observations.[32] Third, all the T2 Cu depleted MCO preparations in the past have yielded inactive protein indicating a direct role of the T2 Cu in O_2 reduction.[7-10] However, SLAC only partially loses

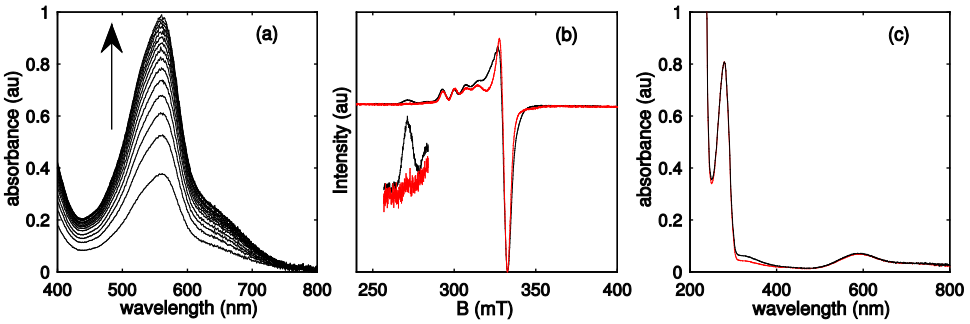

Figure 1: (a) Absorption of the $Cu(I)(BCA)_2$ complex increases and reaches saturation when wt SLAC (100 µM) is treated with BCA (10 mM) and Asc (100 mM) in sodium phosphate buffer (200mM) at pH 7.2. Normalized (b) EPR and (c) absorption spectrum of the wt SLAC (black) overlaid with the spectrum of the protein from where T2 Cu had been chemically removed (red) by the method used in (a). The inset in (b) shows the zoomed in portion of the spectra where the absence of the T2 Cu signal in the T2 Cu depleted SLAC spectrum is clearly visible.

Table 1: Catalytic rate of enzyme turnover (v/E_T) and copper quantification (ratio of Cu concentration vs the protein concentration) using atomic absorption spectroscopy. The turnover rate was measured at 5 mM DMPD in 200 mM air saturated (~270 µM O_2) sodium phosphate buffer (pH 6) at 295 K.

	v/E_T (s^{-1})*	[Cu]/[protein]
wt SLAC	135 ± 5	3.9
T2D SLAC	45 ± 2	3.0
H102G	<1	3.8
H102Y	<1	3.6
H102F	<1	4.1
H102Q	<1	3.7
T2D Y108A	30 ± 2	2.9

* v denotes the catalytic rate and E_T is the total enzyme concentration. Thus, v/E_T denotes the normalized unimolecular rate constant.

activity after the removal of the T2 Cu, clearly showing that the enzyme is capable of functioning without this Cu. Moreover, it was found that azide or fluoride, which strongly inhibits activity of other MCO's by coordinating to T2

Cu,[6] did not inhibit SLAC activity, nor did the azide cause any change in the EPR spectrum of SLAC (Figure S2). On the contrary, azide boosted the SLAC activity by about 25%. Cyanide, another laccase inhibitor, showed strong inhibition of SLAC activity but it is suggested to bind to the T3 Cu site instead of the T2 Cu.[33] Taking all the above observations into account, we conclude that the O_2 binding, at least in SLAC, occurs at the T3 Cu site and the T2 Cu may only be partly involved either in binding or reducing O_2 to H_2O.

We further performed stopped–flow experiments as reported earlier (see Chapter 2) where fully reduced protein was reacted against air/O_2 saturated buffer (Figure 2a).[28] Again, these experiments indicate that fully reduced T2D SLAC is capable of reacting with O_2 unlike the T2D MCO's studied previously. We could also observe the formation of the peroxide intermediate (PI) which is formed by two electron reduction of O_2 bound to the TNC. This intermediate decayed with concomitant increase of the absorption at 590 nm, characteristic of the oxidized T1 Cu, and 410 nm, characteristic of Y108$^{\bullet}$, consistent with the mechanism reported earlier.[28] However, it is worth mentioning that the yield of the tyrosyl radical as judged from the absorption of the 410 nm ($\epsilon_{410} \approx 3250\ M^{-1}cm^{-1}$) band is very small which is puzzling. It appears that the protein abstracts the fourth electron from another site which doesn't show up in the absorption spectrum. Such an observation is consistent with the previous observations where the type–1 depleted Y108A mutant showed formation of PI followed by its rapid decay (see Chapter 2).[28] This is a subject of further study.

To further strengthen these findings, SLAC variants were prepared where one of the two coordinating histidine residues of the T2 Cu, H102, was replaced by a glycine (H102G), glutamine (H102Q), tyrosine (H102Y), or phenylalanine (H102F).[34] All these variants showed activity diminished by more than two orders of magnitude. AAS revealed presence of ~3.6–4.1 Cu's per SLAC monomer (Table 1) and the EPR spectrum showed the presence of features characteristic of T2 Cu in all these mutants (Figure 2b). This was surprising as such mutations in other MCO's in the past have been shown to yield protein containing only three Cu's per molecule as there is only one histidine residue available to bind to the T2 Cu.[2,35] Addition of EDTA didn't help remove this Cu

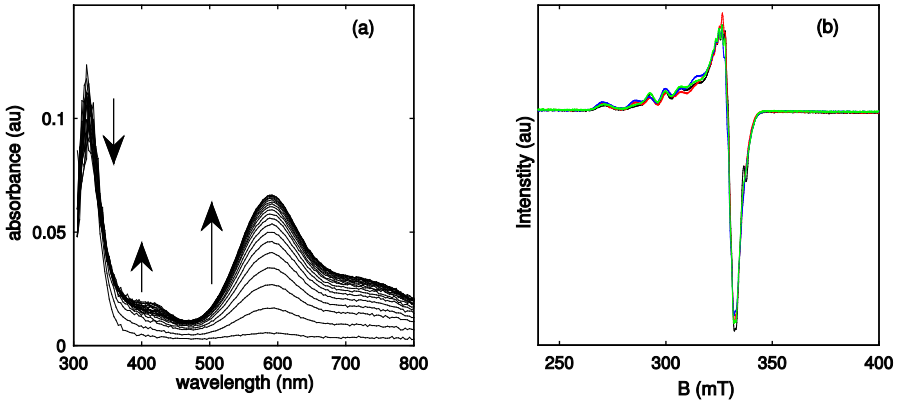

Figure 2: (a) Reaction of fully reduced T2D SLAC with air saturated buffer monitored using stopped–flow. The absorption around 320 nm (PI absorption) changes only slightly while the absorption around 600 nm (T1 Cu absorption) and 410 nm (tyrosyl radical absorption) starts to appear. The measurement was made in 100 mM sodium phosphate buffer (pH 6.8) at ~22 °C. (b) X–band cw–EPR spectra of H102G (black), H102Y (red), H102F (blue) and H102Q (green) variants overlaid on top of each other. The spectra of the variants are nearly identical to each other and to that of wt SLAC (Figure S2). The spectra were recorded in 100 mM sodium phosphate buffer (pH 6.8) at 40 K.

which suggests that this is not adventitiously bound Cu. Further, all attempts to remove the T2 Cu from these mutants by the method reported above failed as it always led to removal of all the Cu's and protein denaturation. It appears that neither the H102 residue nor the T2 Cu individually affect the enzyme stability but together provide strong foundation to the tertiary structure of the enzyme.

The above experiments immediately trigger an interesting thought: Though the chemical depletion of the T2 Cu had only a moderate effect on SLAC activity, the His102 mutation abolished the enzyme activity even when the T2 Cu was present in these mutants. This clearly suggests a role of His102 in the O_2 reduction by SLAC. It might be possible that His102 mediates proton transfer to the O_2 bound at the T3 Cu site, a role which is thought to be played by aspartate and glutamate residues in the three domain MCO's.[18,36] We observed that the presence of imidazole in the activity assay of H102G variant caused a significant burst in the activity (~10 fold) which lasted for only few seconds. Further

experiments are underway to precisely define a role for His102 in the SLAC mechanism.

In a previous study (Chapter 2), the involvement of a tyrosine residue (Y108) in the enzyme mechanism of SLAC was discovered, where it acts as a purported redox buffer and provides an electron during O_2 reduction. Thus, one explanation for the above observations could be that after removal of the T2 Cu, the enzyme is utilizing Y108 to fulfil its role and thus, is still able to turnover. If this was true then we should have observed, using optical and EPR spectroscopy, the quantitative formation of a tyrosyl radical in the single turnover measurements as described above. Moreover, if this hypothesis is correct then the removal of T2 Cu from the Y108A mutant of SLAC, where tyrosine at position 108 is replaced by alanine, should yield completely inactive protein. Indeed, this preparation was significantly less active as compared to wt–SLAC but still possessed significant activity (Table 1) which demands further investigation. Taken together, the experiments now seem to suggest that although Y108 residue is the site of radical on the longer time scale, it is not the immediate electron donor to the O_2 and there must be another redox active residue which is crucial for enzyme activity. Regardless, this is the first example of an MCO which is capable of turnover with only three Cu's per monomer.

SLAC is active as a homotrimer in solution and thus consists of three T1 Cu sites and three TNC's. The T1 Cu sites, as revealed from the crystal structure are only ~18 Å apart. It is conceivable that these T1 Cu sites communicate with each other by exchanging electrons when required. Such a mechanism has been proposed for ceruloplasmin which contains three T1 Cu sites but only one TNC.[37] We're planning to investigate whether this is true or not from dual color single molecule experiments. Though less likely, another possibility to explain the above results would be direct reduction of partially reduced oxygen species by reducing substrate. Although it is clear that the first and the second electron reduction of the TNC in MCO's take place via T1 Cu, there are no clear experiments which prove that subsequent reductions can't take place directly by the substrate as the redox potential of the T3 Cu's and the T1 Cu are more or less identical. Once the TNC is in a more reduced state, smaller reorganization

energy would be required for further electron transfer steps which – accompanied by the strong oxidizing capability of partially reduced O_2 species – may make the direct reduction by substrate favorable.[38] A last possibility will be that upon binding of O_2 to the two electrons reduced T3 Cu site, the two electrons are transferred to the O_2 forming the PI, which remains bound to the T3 Cu's and awaits entry of two more electrons through the T1 Cu before getting fully reduced to H_2O. If this pathway is being followed, we must be able to observe formation of PI upon treatment of the SLAC with H_2O_2. No such evidence was obtained from the experiments and thus this mechanism is unlikely to be the right one. Further experiments are required to single out which one of the above mentioned pathways are likely to occur in the reduction of O_2 by SLAC containing only three Cu's per monomer.

These new findings also seem to have implications for the proposed evolution of Cu proteins. It seems that incorporation of the T2 Cu site in the MCO's must have occurred after the incorporation of the T3 Cu sites, which are sufficient to catalyze the reduction of O_2 to H_2O. Thus, the scheme as drawn by Nakamura and Go about the evolutionary relationships of multicopper proteins may require revision or further experimental data to include/exclude the new findings reported in this work.[25] Indeed the warning put forward by Keilin and Mann in their paper in 1939 seems to fit here: "…These results also serve as a warning against generalizations… and against the tendency of ascribing to one enzyme the properties of another enzyme in the same class, contrary to all the available evidence."[4] With more and more genes being identified from different organisms, it remains to be seen how the evolutionary theory and the previously proposed mechanisms will fit into one consensus.

3.3 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. Desired mutations were confirmed by DNA sequencing (BaseClear).

H102G

Forward primer: 5'– GTG CGG GCC AGC CTG **GGC** GTG CAC GGC CTG GAC –3'

Reverse primer: 5'– GTC CAG GCC GTG CAC **GCC** CAG GCT GGC CCG CAC –3'

H102Y

Forward primer: 5'– GTG CGG GCC AGC CTG **TAC** GTG CAC GGC CTG GAC –3'

Reverse primer: 5'– GTC CAG GCC GTG CAC **GTA** CAG GCT GGC CCG CAC –3'

H102F

Forward primer: 5'– GTG CGG GCC AGC CTG **TTC** GTG CAC GGC CTG GAC –3'

Reverse primer: 5'– GTC CAG GCC GTG CAC **GAA** CAG GCT GGC CCG CAC –3'

H102Q

Forward primer: 5'– GTG CGG GCC AGC CTG **CAA** GTG CAC GGC CTG GAC –3'

Reverse primer: 5'– GTC CAG GCC GTG CAC **TTG** CAG GCT GGC CCG CAC –3'

The purification of the wt–SLAC and the mutants was carried out as reported previously.[22] The proteins were aliquoted and stored at –80 ^{0}C till further use. The absorption spectra of the H102G, H102Y, H102F and H102Q variants are shown in Figure S1.

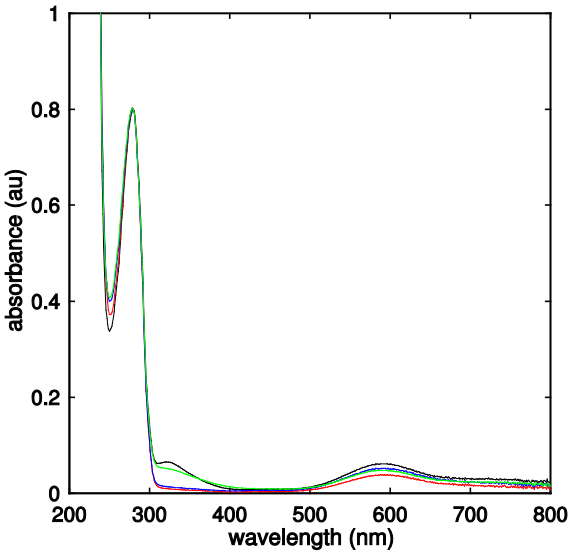

Figure S1: Absorption spectra of the H102G (black), H102Y (red), H102F (blue) and H102Q (green). The spectra have been normalized at the 280nm absorption.

EPR spectroscopy: Samples for EPR measurements were prepared as reported earlier (see Chapter 2). The EPR spectrum of the wt SLAC (~500 µM) in the presence of 10 equivalents of sodium azide (~5 mM) is shown in Figure S2.

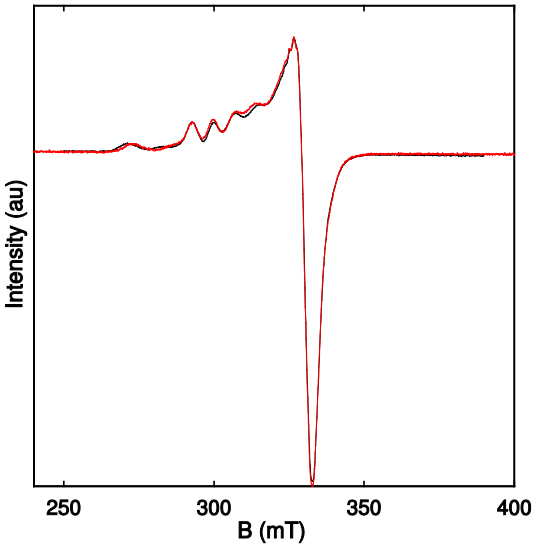

Figure S2: Normalized EPR spectrum of the wt SLAC (black) overlaid with that of the protein incubated with 10 equivalents of sodium azide (red). Clearly, the two spectra are superimposable and thus, rule out azide binding to the T2 Cu.

Steady–state kinetics: Steady state kinetics measurements were performed as reported earlier (see Chapter 2). However, in the current experiments, the rates were measured at a single concentration of TMPD, i.e. at 5 mM under air saturation.

Transient kinetics: Transient kinetics was monitored using a stopped flow instrument (SX.18MV– Applied Photophysics) as reported earlier (see Chapter 2).

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