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

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Chapter 6

Mechanism of O_2 reduction by (T1D) SLAC

In this chapter, we propose a mechanism for the reduction of O_2 by reduced T1D SLAC based on our (rapid) freeze-quench multi-frequency EPR experiments on T1D and T1D Y108F SLAC presented in chapter 4 and previously obtained optical data for these mutants. The mechanistic differences and similarities of SLAC, a 2dMCO, with the 3dMCO's are discussed.

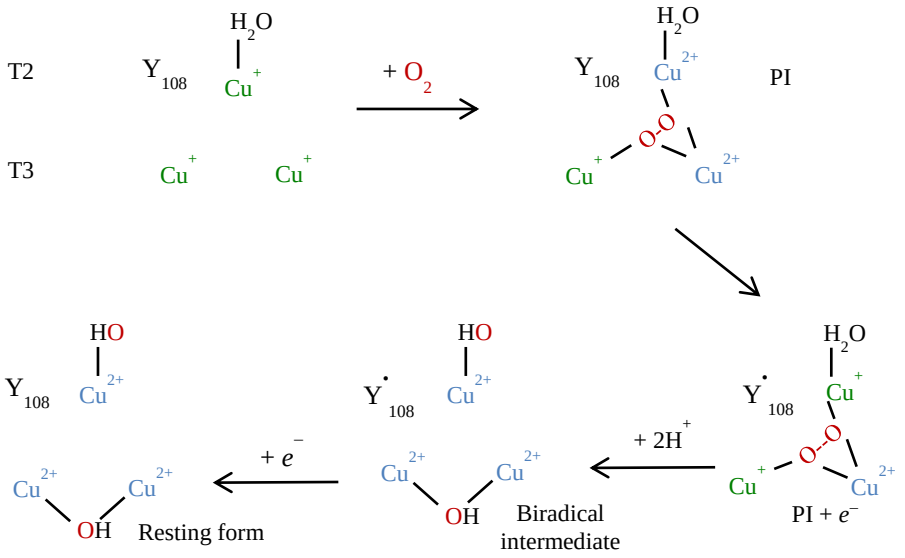
According to the commonly accepted mechanism for the reduction of oxygen by three-domain multicopper oxidases (3dMCO's), the reaction proceeds via two sequential two-electron steps[1,2,3,4,5,6]. The binding of O_2 to the reduced trinuclear cluster (TNC) is followed by a two-electron reduction forming the peroxide intermediate (PI). Since the PI is highly oxidizing, it quickly gets two more electrons and is converted to the native intermediate (NI). Therefore it is difficult to detect the PI for the native enzymes, whereas it has been detected in several MCO's lacking the T1 Cu[7,8,9,10,11,12,13]. The T1 Cu was either removed by mutating the cysteine residue at the T1 site to serine (T1D) or replaced by a redox-innocent Hg^{2+} ion (T1Hg). There exists ambiguity as regards the mode of peroxide binding and the redox state of the Cu's in the TNC of the PI. According to spectroscopic, mutagenesis and computational data on Fet3p, the peroxide bridges the three Cu's in the TNC with the T2 Cu and one T3 Cu (T3β) being oxidized[3,11,14,15]. Calculations on the electronic structure point to the role of a conserved carboxylate residue (e.g. D94 in Fet3P) located between T2 and T3β[15]. The negative charge of the carboxylate lowers the reduction potential of these Cu's, and in this way promotes the transfer of the first two electrons to O_2[11]. According to crystallographic data on a three-domain laccase from *Steccherinum ochraceum*, the peroxide binds to the two T3 Cu's[16]. The discrepancy could be due to the fact that crystallographic data were obtained at cryogenic temperature, while the other data concern room temperature. On the other hand, the mode of peroxide binding might not be the same for different MCOs.

The decay of the PI is thought to be triggered by a rapid intramolecular electron transfer from the T1 to the TNC. Computational studies on Fet3p suggest that in PI + e^- the T3α Cu and T2 Cu are reduced[15]. The cleavage of the O−O bond takes place with the concerted two-electron reduction of the PI resulting in the formation of the NI[10,17,18]. In the NI all four Cu's are oxidized. One of the

oxygen atoms from O_2 bridges the three Cu's in the TNC as an oxo ion (O^{2-}) and the other one bridges the two T3 Cu's as a hydroxide ion (OH^-)[19]. The bridging of Cu's by oxygen results in a magnetically coupled TNC for which the optical and the paramagnetic signatures differ from those of the resting form. The NI slowly decays to the resting form in which the T2 Cu is magnetically decoupled[20].

As compared to the mechanism for 3dMCO's, a proposal for the mechanism of the reaction of O_2 with the reduced T1D SLAC, a 2dMCO[21], has to account for the following distinct observations: (1) on the millisecond time scale not the PI but a biradical intermediate involving a tyrosyl radical has been observed, and (2) there is no indication of the NI as precursor of the resting form.

On the basis of our rapid freeze-quench multi-frequency EPR experiments on T1D and T1D Y108F SLAC presented in chapter 4 and previously obtained optical data for these mutants[22,13], we propose the mechanism presented in scheme 1 for the reduction of O_2 by reduced T1D SLAC. Oxygen binds to the fully reduced TNC, which results in the formation of PI. In PI two Cu's are oxidized and one T3 Cu remains reduced. In the next step, Y108 gets oxidized and reduces the T2 Cu. Then oxygen accepts two more electrons with two protons and the biradical intermediate is formed. In the biradical intermediate the TNC is fully oxidized and the T2 Cu is magnetically separated from the T3 Cu's. The biradical intermediate gets an electron from an unknown source to reduce the Y108 and forms the resting form.



Scheme 1. A possible mechanism for the reduction of O_2 by reduced T1D SLAC

Upon reaction of O_2 with reduced T1D SLAC, optical and EPR signals appear that point to a tyrosyl radical. The PI is not observed under circumstances for which it is commonly observed for 3dMCOs. For T1D and T1Hg 3dMCO's, the idea is that in the absence of the T1 Cu an insufficient number of electrons is available to complete the reaction. Consequently, the PI is stabilized and becomes optically detectable. Apparently, for T1D SLAC the fourth electron is provided by tyrosine Y108, which results in a fast decay of the PI. In agreement with this interpretation, the PI is only detected when both the T1 Cu and Y108 are absent, i.e., for T1D Y108F/A SLAC[13].

As yet, studies of the mode of peroxide binding have not been performed for the PI of SLAC. The comparison of the 3D structure of SLAC (pdb code: 3CG8) with that of Fet3P (1ZPU), a 3dMCO, reveals that the catalytically relevant

carboxylate residue located between T2 and T3β (D94 in Fet3P) is also conserved in SLAC (D259). As shown for SLAC in figure 6.1, this carboxylate residue is part of a network of hydrogen bonds, which involves two histidines, coordinated to T2 and T3β respectively, the water ligand of T2, and a bulk water molecule. Electronic structure calculations suggested[15] that negative charge of this carboxylate decreases the redox potential of T2 and T3β Cu's, and consequently promotes the transfer of the first two electrons to O_2. Most likely, the same hypothesis also applies to SLAC. Therefore we represent in scheme 1 the PI with the T2 Cu and one T3 Cu oxidized, although we do not exclude other binding modes. For SLAC both T3 Cu's are coordinated by the three ε2N-His[21], while for 3dMCOs there is a distinct asymmetry between T3α Cu (two ε2N-His and one δ2N-His) and T3β Cu (three ε2N-His)[14]. For the moment, we also refer to the Cu coordinated to His 104, which is close to D259, as T3β Cu and the other as T3α Cu.

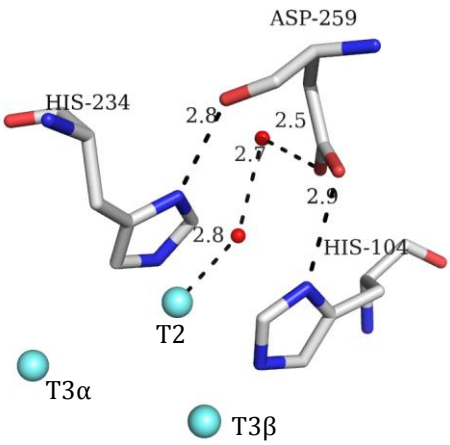


Figure 6.1. The hydrogen-bond network of D259 with the His234 and His104 coordinated to T2 and T3β respectively, and also to the water ligand of T2 through a bulk water molecule. Color code: Cu, cyan; C, gray; N, blue; O, red.

The next step is the decay of the PI. For T1D SLAC, we propose that the PI accepts an electron from Y108 to form PI + e^-, in which the T2 Cu and one of the T3α Cu's are reduced. The oxidation of tyrosine is assumed to be a proton-coupled electron transfer process[23,24]. The crystal structure of SLAC shows that the oxygen of Y108 is part of a network of hydrogen bonds with the surrounding residues and water molecules close to the TNC (Figure 6.2). The carboxylate residue of D113 seems to have a rather flexible conformation, which could also be involved in the hydrogen-band network with Y108 (Private communication, Dr. Navraj Pannu, Leiden Institute of Chemistry). The proton of the tyrosyl radical might well be involved in the O_2 reduction, and more experiments are required to investigate the role of this proton.

The cleavage of the O−O bond takes place with the concerted two-electron reduction of the PI resulting in the formation of the biradical intermediate, instead of the NI in 3dMCO's. Our RFQ multi-frequency EPR investigations of the biradical intermediate indicate that the intermediate is a tyrosyl radical in exchange interaction of 12 GHz with the T2 Cu. The interspin distance was estimated to be 5.7 Å, compatible with the 3D structure of SLAC, which shows a distance of 5.6 Å between the T2 Cu and the carbon of the aromatic ring that is bonded to the oxygen of Y108. The distance to carbon is taken because for tyrosine about 75 % of the spin density is distributed over the aromatic ring[25]. Finally, the biradical intermediate slowly decays to the resting form of the enzyme.

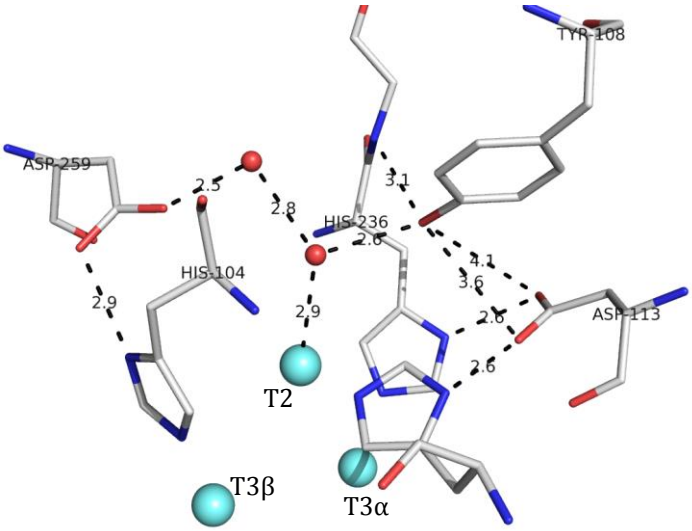


Figure 6.2. The network of hydrogen bonds of Y108 with the surrounding residues and water molecules. Color code: Cu, cyan; C, gray; N, blue; O, red.

For wt SLAC, as for T1D SLAC, no indication of the NI has been obtained, which suggests that the tyrosyl radical observed for T1D SLAC might be involved for wt SLAC as well. A tyrosyl-like absorption band was observed around 420 nm in the UV-Vis spectrum during the reoxidation of wt SLAC in the presence of 130 μM O_2[22]. Moreover, mutation of Y108 in wt SLAC to phenylalanine or alanine was found to reduce the turnover rate by a factor of three[13]. These observations indeed point to the participation of Y108 in the rate-limiting step. On the other hand, no EPR-signature of the biradical intermediate or optical signal of a tyrosyl radical was detected during the reoxidation of wt SLAC in the presence of 600 μM O_2, i.e., at the oxygen concentration for which the biradical clearly showed up in the EPR spectrum for T1D SLAC. The observations for wt SLAC seem contradictory, and for the moment a conclusion as regards the role of Y108 for wt SLAC cannot be drawn. Additional

experiments under different reaction conditions are necessary to investigate whether a mechanism like that described for T1D SLAC in scheme 1 applies to wt SLAC as well.

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