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

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Summary

Multicopper oxidases (MCO's) are present in all life forms including plant, fungi and humans. The most well studied members of the MCO family are the laccases (Lc's) and ascorbate oxidase (AO). Although the biological role of most of these proteins is unknown, they invariably catalyze oxidation of substrate molecules ranging from phenols to aromatic amines to low–valent metal ions. The electrons gathered from the substrates are transferred to oxygen which is converted to water. Each enzymatic turnover requires four reducing equivalents in the form of four $1e^-$ donors, one molecule of O_2 and, of course, four protons.

In this thesis, we have studied the structure–function relationship of a newly discovered member of the family of MCO's: small laccase (SLAC) derived from *Streptomyces coelicolor*. The journey starting from the discovery of the unique characteristics of this protein and the new results obtained in this thesis are summarized in the following paragraphs.

In 2004, Michael Machczynski discovered that the growth media of *S. coelicolor* exhibited phenoloxidase activity (Protein Sci. 2004, 13, 2388). Disruption of the gene encoding SLAC in the bacterium abolished this activity. To study this protein in more detail, the corresponding gene was cloned into a pET vector suited for high level overexpression in *E. coli*. Recombinant SLAC, thus produced, contained a full load of four Cu ions and possessed laccase activity but contained only 343 amino acids contrary to the larger molecular weights of known Lc's and AO. Later, the crystal structure of SLAC revealed that this protein exists as a homotrimer, where each monomer consists of only two cupredoxin domains (unlike three domains for Lc's and AO). This new discovery was quickly adapted into the existing phylogenetic analysis which had predicted the presence of such multimeric Cu proteins. The evolutionary theory had suggested that the Lc's and AO might have evolved from trimeric two–domain MCO's. The obvious and immediate question that followed was: The structural similarities of these different MCO species are obvious but do they also follow the same enzyme mechanisms?

The first investigation into this question was made by Armand Tepper. He showed that the reaction of fully reduced type 1–depleted SLAC with O_2 resulted in the formation of a biradical intermediate which has not been observed for other Lc's (J. Am. Chem. Soc. 2009, 131, 11680). Instead, a similar reaction of type 1–depleted laccases, results in the formation of a long lived peroxide intermediate. Based on transient kinetics and spectroscopic measurements, it was proposed that the two spins in the biradical intermediate reside on two cofactors in the vicinity of each other: the type 2 (T2) Cu and a tyrosine, possibly Y108. While, it was clear that the reactivity of SLAC with O_2 is different from other Lc's, this observation demanded further investigation of the enzyme mechanism. The challenge was to identify the position of the organic radical, i.e. if the unpaired spin is localized at the Y108 or not, and to understand its involvement in enzyme catalysis, if any.

Chapter 2 details the efforts that I have undertaken to identify the position of the organic radical within SLAC. Mutants were prepared where the tyrosine residue at position 108 was replaced with an alanine (Y108A) or a phenylalanine (Y108F) residue in both the wild–type and the type 1–deplted SLAC. To support the comparisons in the enzyme activity or spectroscopy between the native enzyme and the variants, the crystal structures of the new variants were uncovered. These revealed that the active site and overall fold of the variants was essentially identical to that of the native enzyme. The steady–state kinetics measurements revealed that both Y108A and Y108F variants were less active than the wild–type SLAC, a finding that is compatible with a possible role of Y108 residue in the enzyme mechanism. Further, reaction of fully reduced T1D–Y108A SLAC with O_2 did not result in the formation of any biradical intermediate. Instead, a new kind of intermediate was observed resembling the peroxide intermediate, where O_2 has been reduced at the TNC by two electron equivalents. Thus, it was confirmed that Y108 gets oxidized and forms a radical. Y108 was also found to be conserved across all known homologous MCO's and also with human ceruloplasmin. We propose that Y108 gets oxidized to prevent formation of reactive oxygen species under the circumstances when there is an imbalance of reducing and oxidizing equivalents in the milieu. However, this raised an immediate question: Does SLAC really need a stock of five redox active

components (four Cu's and Y108) at any given point of time to carry out four electron reduction of O_2 to H_2O? If not, then which ones are actually essential?

Chapter 3 contains preliminary data that partly answers this question. The type 2 (T2) Cu of the SLAC was selectively removed. The removal of T2 Cu was confirmed by Electron Paramagnetic Resonance (EPR) and Atomic Absorption Spectroscopy (AAS). T2 Cu depleted (T2D) ascorbate oxidase and laccases have been found to be completely devoid of catalytic activity. However, T2D SLAC still possessed almost 1/3rd of the activity of the native enzyme. One hypothesis could be that Y108 replaces the role of T2 Cu when this Cu has been removed. To confirm this hypothesis, we took another route to prepare T2D SLAC. We made mutations to replace one of the T2 Cu coordinating histidine residues (H102) with a glycine (H102G), tyrosine (H102Y), phenylalanine (H102F) or glutamine (H102Q). To our surprise, EPR and AAS revealed that these variants may still possess the T2 Cu. Moreover, the activity of the variants was more than 2 orders of magnitude lower than the activity of the native enzyme. Thus, we could conclude that H102 is crucial for enzyme activity. Further experiments are required to understand how the H102 residue modulates enzyme activity and to answer the question: Can a redox active amino acid really replace the role of a Cu site?

Chapter 4 of this thesis takes a slightly different stand from the previous two chapters. In this chapter, instead of looking at the reactivity of the protein with O_2, we attempted to understand the communication between the T1 Cu and the trinuclear Cu cluster (TNC) sites of SLAC. The main goal was to study the electron transfer (ET) kinetics between the two Cu centers during steady–state turnover. We made use of a single molecule approach to monitor the ET kinetics. A method recently introduced by Sofya Kuznetsova (Anal. Biochem. 2006, 350, 52) was used here which allows fluorescence readout of the redox state of the protein cofactor, T1 Cu in the present case. Thus, by monitoring the fluctuations in the fluorescence count rate from a single molecule that is turning over, the lifetime or rate constant of the decay of the redox state of the T1 Cu (corresponding to the fluorescence intensity) could be obtained. SLAC variants were prepared to allow site specific labeling and immobilization of protein on

transparent glass surface. The fluorescence–count–rate fluctuations of single turning–over SLAC molecules were recorded on a home–built confocal microscope. From these data, we extracted ET rate constants from T1 to TNC (and back) and binned them in a histogram. The forward and backward ET rates across many molecules follow a log–normal distribution with means of 460 and 85 s^{-1}, respectively, corresponding to activation energies of 347 and 390 meV for the forward and backward ET rates. In the context of Marcus theory, the driving force and reorganization energy were calculated from above data and amount to 0.043 eV and 1.5 eV, respectively. The distribution of electron transfer rates shows more than one order of magnitude spread of these rate constants across many molecules which corresponds to a small 30 meV spread in activation energy or 0.1 eV in reorganization energy. Thus, the single–molecule strategy used here not only allows monitoring the internal dynamics of enzymes under steady–state, it is much more informative than the ensemble measurements where the heterogeneity in the sample (extrinsic or intrinsic) can be observed.

Chapter 5 provides general conclusions to the work presented in this thesis. In addition, it also provides promising directions for future research to gain more insight into the SLAC mechanism and advance our knowledge of the mechanism of MCO's in general.