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

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Chapter 5

General Conclusions and Outlook

This chapter provides general conclusions relating to the work presented in this thesis. Currently ongoing experiments and promising future directions are also discussed in brief. For conclusions related to a specific chapter, the reader is referred to the Ist page of either chapter which contains a graphical abstract of the study concerned.

The research presented in this thesis was aimed to investigate the catalytic mechanism of a newly discovered protein: small laccase (SLAC) from *Streptomyces coelicolor*. SLAC, like other multicopper oxidases (MCO's), catalyzes the four electron reduction of O_2 to H_2O for which the electrons are donated by a reducing co–substrate. MCO's are found in all life forms and have diverse roles. However, this category of enzymes is gaining increasing attention by the energy industry. This is because the enzyme, when immobilized on a conducting electrode, can function as a (bio)cathode in a fuel cell. To be able to exploit full potential of such biochemical electrodes, it is important to understand their mechanism of operation. In the case of studying SLAC, there is an additional interest, i.e., to understand its evolutionary relationship with other multicopper proteins as postulated by phylogenetic analysis.

Chapter 2 of this thesis focuses on understanding the mechanism of O_2 reduction at the SLAC trinuclear Cu cluster (TNC). It was demonstrated, using a variety of spectroscopic and kinetics measures, that a tyrosine residue (Y108) may have a participatory role in the reduction of O_2 at the TNC. In addition, this tyrosine residue appears to be conserved among the sequences of the homologous two–domain multicopper oxidases and also seems to have structural homology with human ceruloplasmin. It is postulated that the role of Y108 may become crucial when there is an imbalance of oxidizing and reducing equivalents in the environment surrounding the protein. Although, kinetics and spectroscopic data strongly suggest a role for Y108 during steady–state turnover, we have not yet established its presence under these conditions. Further, the characterization of the biradical species that was observed upon reaction of type 1 depleted (T1D) SLAC is still lacking. Finally, Y108A variant doesn't show formation of transient paramagnetic species but Y108F does. Is this an oxidized phenylalanine or another nearby residue (W284) that we are observing? A number of the above questions may be answered by trapping the intermediates on a short time–scale using rapid–freeze–quench and study them with multifrequency EPR. These experiments are currently being performed by Ms. Faezeh Nami, in the group of Prof. Edgar J. J. Groenen and Prof. Gerard W. Canters. Although the mutations at position Y108 had a clear effect on enzyme kinetics, the effect was small. It would be interesting to study the pH dependence of the steady–state and pre–

steady–state kinetics to establish conditions where the role of Y108 residue in the enzyme mechanism can be concluded with certainty.

Chapter 3 of this thesis was partly a follow up of the study presented in Chapter 2. The main question to be answered was: Does the enzyme (SLAC) really need a stock of 5 redox active components (4 Cu's and Y108) at any given time and if not, then which ones are actually essential? It could be concluded, from Cu depletion and kinetics experiments, that the presence of type 2 (T2) Cu is not essential for the enzyme activity. Moreover, the H102G/Y/F/Q mutations led to a more than 2 orders of magnitude drop in enzyme activity but did not lead to T2 Cu depletion. Though preliminary, the experiments in this chapter seem to suggest that SLAC is capable of turning over with as little as three Cu's in its active site, which is so far not observed for any of the MCO's studied. The question to be answered is: How is it possible and why might nature have recruited a fourth Cu site (the T2 Cu)? The latter might be reformulated and asked as: Whether, in the evolution of multicopper blue proteins, the T2 Cu site evolved first or the T3 Cu site? Future investigations may focus on understanding the steady–state kinetics of the T2 Cu depleted (T2D) SLAC to realize whether the effect on turnover rate is related to the binding of O_2 (K_M of O_2) at the TNC. Further, stopped–flow and rapid–freeze–quench EPR experiments may be utilized to identify the early intermediates in the reaction of fully reduced T2D SLAC or H102G/F variants with O_2. We are currently collaborating with Dr. Igor Nederlof to solve the crystal structure of the T2D SLAC and the H102G/Y/F/Q variants to confirm whether the removal of Cu or the mutation of a Cu coordinating residue disturbs the active site or overall fold of the enzyme which may explain the difference in activity. It will be interesting to see whether the T2D SLAC can be reconstituted back with Cu(II) and/or other transition metal ions like Zn(II), Hg(II) or Co(II) and observe the effect of such enzyme forms on their activity and spectroscopy.

Chapter 4 of this thesis focused on studying the electron transfer (ET) processes between the T1 and TNC of SLAC. A special interest was to measure the intramolecular ET during steady–state turnover and to do so we studied the enzyme kinetics at the single molecule level. We could, for the first time, measure the forward (T1→TNC) and backward (TNC→T1) electron transfer rate constants (k's) for a molecule under steady–state turnover conditions. It was demonstrated that there exists a heterogeneity/disorder across k's measured for many molecules of the population and we were able to obtain the mean and spread of this distribution. The broad and asymmetric k distribution actually corresponds to a narrow and symmetric distribution of activation energy which was elaborated further in terms of reorganization energy and driving force. A reasonable and straightforward follow–up of the study would be to measure the ET rate constants with different reducing substrates and at varying O_2 concentrations. In addition, the protein may be labeled with a different fluorophore to measure the ET kinetics. Since we are measuring an intramolecular process, any of the extrinsic changes mentioned above should yield similar results. A more stringent approach would be to utilize a second label (say tryptophan fluorescence) or another extrinsically attached fluorophore to monitor the redox changes at the TNC and then obtain a cross correlation from the two color measurements.