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

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Summary

Since the discovery of enzymes as biological catalysts, the mechanism of enzymatic reactions has been a key question to enzymologists. Elucidating the reaction kinetics and the nature of enzymatic intermediates are necessary to understand such reactions. An important challenge in these studies is the limited lifetime of such intermediates, usually on the time scale of milliseconds to seconds. Therefore, a suited trapping method is required. Rapid freeze-quench (RFQ) is a proven technique to trap the intermediates on the time scale of milliseconds. The RFQ technique has been mostly combined with electron paramagnetic resonance (EPR) spectroscopy to reveal the nature of the paramagnetic intermediates involved in enzymatic reactions. In this thesis, we improve and extend the combination of conventional RFQ technique with EPR spectroscopy up to a microwave frequency of 275 GHz to trap and characterize the intermediates involved in the enzymatic reduction of O_2 by small laccase (SLAC).

The SLAC enzyme belongs to the group of multicopper oxidases (MCO's) enzymes. It was discovered in 2004 when searching the genome of *Streptomyces coelicolor* for the presence of copper proteins. Characterization of the protein revealed that SLAC represents structural features distinct from those of other laccases. However, the active-site morphology of SLAC, which involves a type 1 (T1) Cu and a trinuclear cluster (TNC), is the same as for other MCO's. The SLAC consists of only two cupredoxin-like domains (2dMCO) and it is active as a homotrimer, contrary to the more common three-domain MCO's (3dMCO's), which are active in monomeric form. Considering these structural differences, it is of great interest to see the possible mechanistic differences and similarities of SLAC, a 2dMCO with the 3dMCO's. To answer

this question, the reaction of the reduced T1 depleted (T1D) SLAC with oxygen was initially followed by EPR on the time scale of minutes. The results showed the presence of a biradical intermediate, which had not been reported for the 3dMCO's. Preliminary studies led to the hypothesis that the biradical signal originates from two exchange-coupled spins, one localized on the type 2 (T2) Cu and the other one on an amino-acid-derived radical, most probably a tyrosyl radical. Characterization of this intermediate and any other possible paramagnetic intermediates formed during the reoxidation of wild type and mutants of SLAC on the timescale of milliseconds is the subject of this thesis.

In chapter 2, we present a method to improve the collection of freeze-quench particles from isopentane and their packing in an EPR tube for X-band spectroscopy. The method is based on sucking the particle suspension into an EPR tube with a filter at the end. As compared to the conventional approach, the sucking method results in a significant reduction of the required volume of reactants, which allows the economical use of valuable reactants such as proteins. The method provides for a reproducible, efficient and fast collection of the freeze-quench particles.

In Chapter 3, we report on the extension of the conventional RFQ/EPR methodology to higher microwave frequencies based on the sucking method described in chapter 2. In this way, the particles are efficiently packed into the capillaries with inner diameters down to 150 μm that fit into the single-mode cavities for high-frequency EPR. We demonstrate that one RFQ sample for each point in time suffices for EPR experiments at multiple microwave frequencies. We validate the application of the method to biological samples using the reaction of binding azide to myoglobin, combining RFQ with EPR at 9, 94 and 275 GHz.

In chapter 4, we make use of RFQ multi-frequency EPR to trap and characterize paramagnetic intermediates formed during the reaction of fully reduced T1D SLAC and of T1D Y108F SLAC with oxygen on the time scale of milliseconds. The X-band EPR spectra of the T1D SLAC intermediate with the reaction time of milliseconds are essentially identical to those previously derived from the difference of the spectra corresponding to different reaction time during the decay of the intermediate on the time scale of minutes. The combined analysis of EPR data at 9, 94 and 275 GHz of RFQ samples of T1D SLAC unambiguously indicates that the intermediate consists of the T2 Cu and the tyrosyl 108 radical. For T1D Y108F SLAC, we observed no sign of a biradical intermediate during the reoxidation process on the time scale of milliseconds to seconds. This reinforces our conclusion as regards the crucial role of tyrosine 108 in the reaction of T1D SLAC with molecular oxygen. For T1D Y108F SLAC, we observed an intermediate with a small contribution from a radical, which is tentatively assigned to a tryptophanyl radical.

In chapter 5, we investigate the reoxidation of wild-type SLAC by RFQ/EPR under similar experimental conditions as used for the study of T1D SLAC described in chapter 4. We search for the involvement of the tyrosyl radical and the so-called native intermediate (NI) during the reoxidation of the wild-type enzyme. The EPR spectra of fully reduced wild-type SLAC mixed with oxygen on the time scale of milliseconds and longer can be described as the sum of T1 Cu and T2 Cu signals. We find no indications of a tyrosyl radical nor of an NI in the single turnover of wild-type SLAC. The lack of detection of these intermediates during the single turnover of wild-type SLAC means that either these are not formed or their lifetime is shorter than 8 ms.

In chapter 6, 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 SLAC 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. For wild-type SLAC, our observations seem contradictory, and for the moment a conclusion as regards the role of Y108 cannot be drawn. Additional experiments under different reaction conditions are necessary to investigate whether a mechanism like that described for T1D SLAC applies to wild-type SLAC as well.