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Mechanistic study of direct electron transfer in bilirubin oxidase

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ABSTRACT

The mechanism of direct electron transfer in multicopper oxidases is not well understood. In this work, the mechanism of oxygen reduction in Bilirubin oxidase (BOD) is analyzed using a rotating ring-disc electrode (RRDE). The glassy carbon disc potential was swept from 0.8 V to 0 V while the platinum ring potential was held at 0.8 V, resulting currents were measured. Minimal hydrogen peroxide evolution from BOD is detected on the Pt ring, independent of rotation rate. The electron transfer rate constant, k_{et}, is calculated to be 1.14×10^{-3} cm/s. The number of electrons transferred per molecule of oxygen is calculated to be 3.92 electrons using Koutecky–Levich equation and 3.7 ± 0.2 electrons using mass/charge balance which corresponds to $7.5 \pm 5\%$ of oxygen reduction via the 2 electron pathway with a hydrogen peroxide intermediate. The 4 electron transfer mechanism is preferred because it is more efficient than the two electron mechanism.

1. Introduction

Multi-copper oxidase (MCO) is a family of enzymes, evolutionarily and structurally related, involved with pigmentation, morphogenesis, detoxification, and lignin degradation. This family of enzymes includes ascorbate oxidases, laccases, nitrite reductases, cuprous oxidases, ceruloplasmin (also known as ferroxidases), and bilirubin oxidases found in plants and fungi [1–3]. The majority of MCO's contain four copper active sites (per monomer of a protein molecule) consisting of one copper ion at the T1 or blue site and three copper ions at the trinuclear cluster or T2/T3 sites [2,4,5]. This family of enzymes reduces dioxygen to water while simultaneously oxidizing, in a 1 electron oxidation, the reducing substrate [6–9]. Much research has been devoted to unveiling the mechanism of oxygen reduction reaction by MCO's in solution [4,6–8,10–16]. It has been shown that the reduction to water takes place on the T2/T3 site, and no free hydrogen peroxide has been registered as evolving product. It has been postulated that 4 electron mechanism is thus prevalent and this understanding has been transformed to all electrochemical systems involving MCO and demonstrating direct electron transfer (DET) [16,17]. While all advances being made in incorporating of MCO into biofuel cathodes it has not explicitly confirmed whether the mechanism of oxygen reduction in MCOs such as Bilirubin oxidase (BOD) is a 4 electron transfer [18–22]:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad 1.23\,V \text{ vs. NHE} \tag{1}$$

or a 2-electron transfer with a hydrogen peroxide intermediate (Scheme 1) [23,24]:

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad 0.68\,V \text{ vs. NHE} \tag{2}$$

$$H_2O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad 1.77\,V \text{ vs. NHE} \tag{3}$$

The formation of H_2O_2 decreases the number of electrons transferred per molecule of O_2 reacted making the 4 electron, direct transfer mechanism more desirable. This research aims to clarify the mechanism of oxygen reduction in the MCO enzyme BOD.

It is a task of this study to establish the number of electrons exchanged in BOD-catalyzed oxygen reduction reaction (ORR) by a standard electrochemical technique and to confirm the absence of the hydrogen peroxide as a free intermediate corresponding to a 4 electron mechanism. The mechanism for oxygen reduction was studied using electrochemical measurements carried out on a rotating ring disc electrode (RRDE) with an inert (glassy carbon) disc electrode. The disc was modified by a thin film containing the enzyme catalysts and used for measuring the oxygen reduction reaction. The catalytic Pt ring electrode simultaneously measures a second reaction, the decomposition of hydrogen peroxide at constant potential [25]. In this work, the catalyst, BOD was deployed on the disk in a form of an ink (suspension) of multi-walled carbon nanotubes (MWNT), with the enzyme immobilized onto their surface and dispersed in alcohol/water suspension of tetrabutylammonium bromide (TBAB) modified Nafion^{TM}, Fig. 1.

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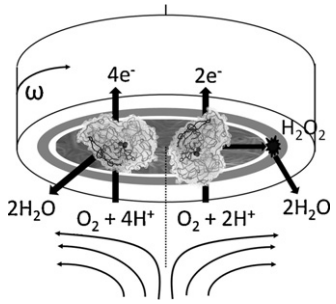

Scheme 1. 4 and 2 electron transfer on the carbon nanotube coated rotating ring-disc electrode.

2. Experimental

2.1. Glassy carbon electrode disk preparation

A RRDE with a glassy carbon disc and a platinum ring (Pine Instruments) was cleaned with alumina, 1 μm, 0.3 μm, followed by 0.05 μm grit with a washing of water in between each grit. A suspension of 5% multi-walled carbon nanotube (MWNT obtained from cheaptubes.com) in 4:1 water:ethanol suspension (reagent grade, Sigma, St. Louis, MO) was bath sonicated for 30 min. The MWNT suspension was then reacted with 10 mM 1-pyrenebutanoic acid, succinimidyl ester (PBSE), 10 mg/ml BOD. Then one of two polymers was then added to the MWNT suspension:

- Chitosan polymer, 0.5% in 0.3 M acetic acid, or
- TBAB-Nafion polymer, 0.1% in absolute ethanol, a gift from the laboratory of Prof. Shelley Minteer, St. Louis University, MO [26,27].

The MWNT suspension, containing BOD attached to MWNT through a PBSE linker, requires a binder to attach to the glassy carbon electrode. The drop-casting method relies on evaporation of the MWNT suspension to coat the electrode. Chitosan and TBAB-Nafion polymers were found to produce successful MWNT suspension attachment to the electrode and be enzyme compatible [28,29]. Chitosan, a linear polysaccharide formed from deacylated chitin obtained from crustaceans, is well known for being biocompatible, biodegradable, chemically inert, non-toxic, and having high mechanical strength [30–33]. TBAB-Nafion, a derivative of Nafion made by exchanging a proton in the sulfonic acid group for a TBAB salt, exhibits increased pore size, increased hydrophobicity, and decreased acidity [26]. The larger pore size increases mass transport and enzyme encapsulation in an enzyme compatible environment [26,34–36].

The MWNT suspension (regardless of the polymer uses) was incubated at 4 °C for 50 min. Then 10 μL of the suspension was dropcast on the glassy carbon disc and dried under nitrogen gas. Under this procedure the loading of the "catalysts", BOD-modified MWNT, was 0.40 mg/cm^2.

2.2. Electrochemical measurements of ORR activity

Electrochemical measurements were performed in a 125 mL glass electrochemical cell using a WEB30 Pine bi-potentiostat and a Pine Instruments Rotator (Pine Instruments, Raleigh, NC). Using a three-electrode setup, the reference electrode was Ag/AgCl and the counter electrode was a platinum wire. The disc potential was swept from +0.8 V to 0.0 V (in cathodic direction) at a scan rate of 10 mV/s (optimized to yield the highest ORR currents) while the potential at the ring was held constant at +0.8 V. Using the oxygen reduction reaction theory, the potential of 0.8 V was chosen because it is just below the potential where water begins to split, which was determined experimentally for the buffer used [37,38]. The optimum scan rate for the RRDE measurements was determined by varying the scan rate and measuring the corresponding observed ORR currents, Fig. 2(left). The largest ORR currents are observed for scan rates of 10 mV/s. The supporting electrolyte was 100 mM potassium phosphate at pH 7.5. Electrochemical measurements are taken under oxygen or nitrogen saturated conditions with the gas was bubbling through the cell at room temperature.

BOD is covalently linked to PBSE, which is non-covalently tethered to MWNT [39]. An ink of such BOD-modified MWNT is made by suspending them in an aqueous polymer solution containing tetrabutyl-ammonium bromide (TBAB) modified Nafion™. This ink is then drop cast on the glassy carbon disc of the rotating electrode.

2.3. Calculating the oxygen reduction reaction current

Enzyme activity is quantified through the observed ORR current. The electrochemical current (Δi) is taken as the difference in current at the onset of oxygen reduction and the reductive peak current (taken at ~300 mV).

2.4. Calculating the electrochemical accessible surface area (ECSA)

The extent of electrochemical accessibility determines the practical utilization of the high surface area MWNT modified glassy

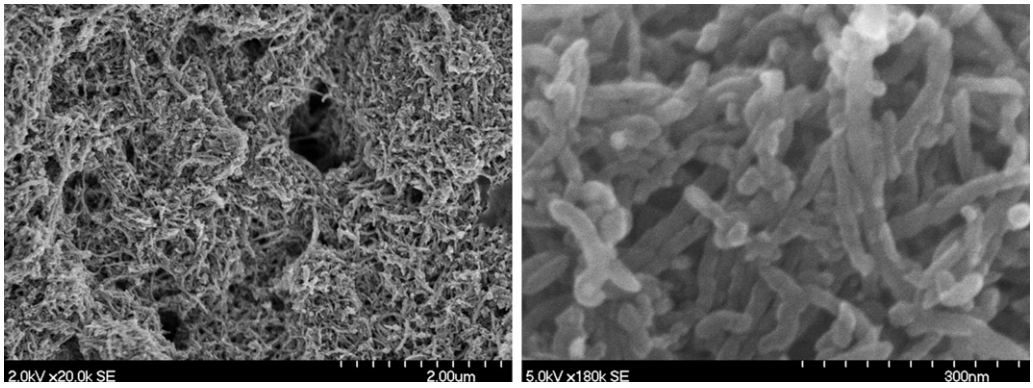

Fig. 1. SEM of dropcast ink containing single walled carbon nanotube, tetrabutylammonium bromide (TBAB) modified Nafion™, 1-pyrenebutanoic acid, succinimidyl ester (PBSE), and billirubin oxidase on glassy carbon. Scale bar 2 μm (left) and 300 nm (right).