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Direct electron transfer reactions of laccases from different origins on carbon electrodes

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Abstract

Electrochemical studies of laccases from basidiomycetes, i.e., *Trametes hirsuta*, *Trametes ochracea*, *Coriolopsis fulvocinerea*, *Cerrena maxima*, and *Cerrena unicolor*, have been performed. Direct (mediatorless) electrochemistry of laccases on graphite electrodes has been investigated with cyclic voltammetry, square wave voltammetry as well as potentiometry. For all mentioned high potential laccases direct electron transfer (DET) has been registered at spectrographic graphite and highly ordered pyrolytic graphite electrodes. The characteristics of DET reactions of the enzymes were analysed under aerobic and anaerobic conditions. It is shown that the T1 site of the laccase is the primary electron acceptor, both in solution (homogenous case) and at surface of the graphite electrode (heterogeneous case). A mechanism of ET for the process of the electroreduction of oxygen at the laccase-modified graphite electrodes is proposed and the similarity of this heterogeneous process to the laccase catalysed oxygen reduction homogeneous reaction is concluded.
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Keywords: Laccase; Redox potential; T1 site; Carbon electrode

1. Introduction

Studies of direct electron transfer (DET) reactions between proteins and electrodes yield important information on the thermodynamics and kinetics of biological redox processes. The understanding of the heterogeneous reactions facilitates practical applications of biomolecules in biosensors, biofuel cells, bioelectroorganic synthesis, etc. DET reactions with electrodes have been shown for many proteins [1–8] including large redox enzymes, e.g., laccases from different sources [9–12].

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyse the oxidation of *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions coupled to the reduction of molecular oxygen to water [13,14]. They are widely distributed in plants and fungi and have been identified in bacteria [15] and insects [16]. The enzyme is a copper protein and contains four metal ions classified into three types, denoted T1, T2, and T3 [13,14]. The T2 and T3 coppers form the T2/T3 cluster, where molecular oxygen is reduced to water.

The key characteristic of laccase is the standard redox potentials of the T1 site. The values of this redox potential

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid); DET, Direct electron transfer; ET, Electron transfer; HOPG, Highly ordered pyrolytic graphite; IET, Intraprotein electron transfer; MRT, Mediated redox titration; PME, Permselective membrane electrode; SPG, Spectrographic graphite.

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in different laccases have been found to be between 430 and 790 mV vs. the normal hydrogen electrode (NHE) as determined using potentiometric titrations with redox mediators [17–21]. It has been shown that the T1 site is the primary centre, at which electrons from reducing substrates are accepted [13,14,22]. Moreover, the catalytic efficiency (k_{cat}/K_M) for the majority of the aromatic reducing substrates depends on the redox potential of the T1 copper [22,23], which makes laccases with a high redox potential of the T1 site of special interest in biotechnology, e.g., for bleaching [24,25] and bioremediation processes [26,27].

The first publication on DET for a large redox protein with enzymatic activity concerned a high redox potential laccase from the basidiomycete *Polyporus versicolor* [9]. In that work, a mediatorless electroreduction of oxygen was shown, catalysed by *P. versicolor* laccase adsorbed at a carbon electrode. As a proof a reduction current proportional to the oxygen concentration was recorded at the laccase-modified carbon electrode caused by a DET reaction between the electrode and the adsorbed laccase. This process depended on the electrode material, the method of its preparation and the partial pressure of oxygen in the system [9]. A second paper about the electroreduction of oxygen at laccase-modified carbon electrodes was published in 1984 [28]. In this paper the authors observed the electroreduction of oxygen using highly ordered pyrolytic graphite electrode coated with laccase from *P. versicolor.* The potential at which the laccase started to catalyse the electroreduction of oxygen (about 735 mV vs. NHE) was in the vicinity of the redox potential of the T1 site of this enzyme (780 mV vs. NHE). Under anaerobic conditions the laccase-modified graphite electrode, however, exhibited cyclic voltammograms that were indistinguishable from the control voltammograms in the absence of the enzyme. In the presence of an electrochemically inactive promoter, 2,9-dimethylphenanthroline, cyclic voltammograms of laccase modified HOPG electrodes exhibited a wave with a formal potential of 645 mV vs. NHE. Additionally, some kinetic parameters of the electroreduction for molecular oxygen at carbon electrodes modified with adsorbed laccase have been reported [28–30]. In 1998 a DET process was reported as small cyclic voltammetric peaks of the high potential laccase from *P. versicolor* under anaerobic conditions at pyrolytic graphite electrodes [31]. The process was quasi-reversible with a midpoint redox potential (E_m) close to the redox potential of the T1 site of this laccase (ca. 780 mV vs. NHE). Under aerobic conditions a clear catalytic wave was shown appearing close to the potential of the T1 site.

Despite these data the molecular mechanism behind the DET reaction between laccases and electrodes under both anaerobic and aerobic conditions remains largely unknown. Specifically, many heterogeneous DET processes recorded with cyclic voltammetry were not correlated with the formal potential values of the T1 copper site. Moreover, a well-pronounced electrochemical response of the enzyme on carbon electrodes under anaerobic conditions has been shown only recently [11,12], however, without answers to many question, e.g., which is the first process in the heterogeneous catalytic cycle of the enzyme (oxygen binding to the T2/T3 site or the electrochemical reduction of the copper sites in the enzyme).

The objectives of this work were (i) to investigate the possibility of achieving DET for laccases from different origins under aerobic and anaerobic conditions on carbon electrodes, (ii) to compare the mechanism of electron transfer in homogeneous and heterogeneous catalysis, and (iii) to propose a mechanism of oxygen reduction catalysed by the laccases adsorbed on the graphite surface. It is believed that these studies of laccases from different organisms should be beneficial in clarifying the true mechanism of the function of laccase at different conditions as well as opening up new opportunities for their use in various areas in biotechnology.

2. Experimental

2.1. Chemicals

Buffer chemicals, H_2SO_4, Na_2HPO_4, KH_2PO_4, NaOH, KCl, and NaCl were obtained from Merck (Darmstadt, Germany); catechol and $K_4Fe(CN)_6$ were from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade. The buffers were prepared using water (18 MΩ) purified with a Milli-Q system (Millipore, Milford, CT, USA).

2.2. Enzymes

Two strains of the basidiomycetes *Trametes hirsuta* and *Trametes ochracea* were obtained from the laboratory collection of the State Research Institute of Protein Biosynthesis (Moscow, Russia). *Cerrena unicolor* was obtained from the culture collection of the Biochemistry Department, Maria Curie Sklodowska University, Lublin, Poland. Extracellular laccases from *T. hirsuta*, *T. ochracea*, and *C. unicolor* were isolated from the culture media of the individual strains and purified to homogeneity according to published procedures [21,32]. Partially purified preparations of extracellular laccases from *Cerrena maxima* and *Coriolopsis fulvocinerea* have been kindly provided by Drs. O.V. Koroleva and V.P. Gavrilova in the frames of joint research performed with the support of the INCO Copernicus grant No. ICA2-CT-2000-10050. They were used without any additional purification.

The preparations of the laccases (10 mg/ml) were stored in 0.1 M phosphate buffer, pH 6.5, at −18 °C. The laccase concentrations were measured spectrophotometrically at

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