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ARTICLE *in* BIOELECTROCHEMISTRY · OCTOBER 2005

Impact Factor: 4.17 · DOI: 10.1016/j.bioelechem.2005.02.004 · Source: PubMed

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Bioelectrochemistry 67 (2005) 115 – 124

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

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

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Received 27 August 2004; received in revised form 7 January 2005; accepted 16 February 2005

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.

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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doi:10.1016/j.bioelechem.2005.02.004

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

two wavelengths (228.5 and 234.5 nm) using BSA as standard for the calibration curve [33].

2.3. Electrochemical measurements

2.3.1. Electrode modification

The laccase-modified electrodes serving as working electrodes were made from spectrographic graphite (SPG) or highly ordered pyrolytic graphite (HOPG edge plane) electrodes. The surface of the SPG electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm in diameter) was prepared by first polishing with fine emery paper (Tufback Durite, P1200), then thoroughly rinsed with Millipore water and finally allowed to dry. An aliquot of 10 μl of laccase solution (10 mg/ml) was placed on the electrode surface and after 20 min the electrode was rinsed with water again. The surface of the HOPG electrode (GE Advanced Ceramics, Cleveland, OH, USA, 5.0 mm diameter) was polished on an alumina FF slurry (0.1 μm, Struers, Copenhagen, Denmark) and rinsed with Millipore water. Then, a permselective membrane electrode (PME) [34] was prepared by trapping 15 μl of laccase solution (10 mg/ml) between the HOPG electrode surface and a dialysis membrane (MWCO 6000-8000). The solution of the enzyme was first allowed to dry at the surface of the electrode in air, at room temperature, for 30 min. Then the dialysis membrane (pre-soaked in buffer) was pressed onto the electrode and fitted tight to the electrode with a rubber O-ring. The final enzyme concentration between the electrode and the membrane was approximately 30 mg/ml.

2.3.2. Cyclic voltammetry and square wave voltammetry measurements

Cyclic voltammograms (at sweep rates varying from 1 to 1000 mV s^{-1}) and square wave voltammograms of the laccase-modified electrodes were carried out using a conventional three-electrode system connected to a potentiostat (BAS CV-50W Electrochemical Analyzer with BAS CV-50W software v. 2.1, Bioanalytical Systems, West Lafayette, IN, USA) and a one-compartment electrochemical cell (volume 10 ml). In these measurements an $Ag|AgCl|KCl_{sat}$ (201 mV vs. NHE) reference electrode and a platinum counter electrode were used.

2.3.3. Determination of the steady-state potentials of the laccase-modified electrodes

For steady-state redox potential measurements a two-electrode voltammeter (Ionomer I-130.2M, APK Energoservis, St. Petersburg, Russia) was used. The reference electrode was an Ag|AgCl|3 M NaCl (210 mV vs. NHE) and the laccase-modified electrodes were used as the indicator electrodes in the potentiometric measurements. In all these experiments a one-compartment electrochemical cell (volume 10 ml) was used. The equilibrium potential values were registered under aerobic conditions.

3. Results

The key biochemical properties such as molecular mass, pH-optimum, thermal stability, copper content, pI, and specific activity have been previously reported for laccases from *T. hirsuta*, *T. ochracea*, *C. maxima*, *C. unicolor*, and *C. fulvocinerea* [21,35,36]. Some of these properties are summarised in Table 1, in which it can be seen that the enzymes have a molecular weight ranging from 64 to 70 kDa, contain 4 copper ions per molecule, and have pH-optima between 3.4 and 6.0. Like all known laccases, the enzymes are glycoproteins and contain from 4% to 32% carbohydrates per molecule of protein. The redox potentials of the T1 copper sites of *T. hirsuta*, *T. ochracea*, *C. fulvocinerea*, and *C. maxima* laccases range between 750 and 790 mV vs. NHE as specified in Table 2 [20,21,36]. From this it can be concluded that these laccases possess a rather high potential of the T1 site, which makes them highly active in homogeneous solutions. Electrochemical investigations of these laccases are of special interest for understanding and exploiting properties of these enzymes in heterogeneous systems.

Cyclic voltammograms of bare and *T. ochracea*, *C. fulvocinerea*, *C. maxima*, *C. unicolor* laccases-modified SPG electrodes in oxygen-saturated solutions at pH 3.0 are shown in Fig. 1. When adsorbed on SPG electrodes all laccases largely decreased the overvoltage needed for the electroreduction of molecular oxygen. As can be seen from the CVs (Fig. 1) the electrocatalytic current at the electrodes modified with laccases starts at potentials ranging from +900 mV to +840 vs. NHE with a half wave potential of oxygen electroreduction between +800 mV and +740 mV vs. NHE (Table 2). The half wave potential for the electroreduction of oxygen changed with 5–30 mV/pH in the pH region between 3.0 and 5.0 (Table 2). A similar electrochemical behaviour was also observed for another carbon electrode material, namely HOPG. As an example, CVs of bare and *T. hirsuta* laccase-modified HOPG electrode in air-saturated solutions at pH 3.0 are shown in Fig. 2A.

CVs of SPG electrodes with adsorbed laccases were recorded to investigate whether proofs of direct heterogeneous ET of the laccase could be seen also under anaerobic conditions. Unfortunately, the CVs recorded between pH 3 and 7 did not reveal any redox transformation of any of these

Table 1
Some biochemical properties of the investigated laccases

Laccase	MW (kDa)	pI	pH-optimum	Carbohydrate content (%)	Copper content
Trametes ochracea	64±2	4.7±0.1	3.7–4.9	10±1	4
Trametes hirsuta	70±2	4.2±0.1	3.5–4.5	12±1	4
Cerrena maxima	67±2	3.5±0.1	3.5–4.5	13±1	4
Cerrena unicolor	64±4	4.5±0.2	4.0–6.0	N.d.	4
Coriolopsis fulvocinerea	65±2	3.5±0.1	3.9–5.2	32±1	4

"N.d."—not determined. If uncertainty values are not indicated, the standard deviations have been calculated from results of three independent experiments, and were found to not exceed 10%.

Table 2
Some electrochemical properties of the investigated high potential fungal laccases as well as of *Rhus vernicifera* and *Polyporus versicolor* laccase such as the redox potential of the T1 site ($E^{0'}$) the half wave potential (E 0_2, CV, mV) and the half wave potential of oxygen electroreduction by the laccase-modified SPG electrodes (E 0_2, CV, mV/pH), the redox potentials of the peak on the SWVs (Peak E, SWV), and the steady-state potentials of the laccase-modified SPG electrodes (Steady-state E, mV)

Laccase	$E^{0'}$ T1 site, MRT, mV (pH 6.5)	E 0_2, CV, mV (pH 3.0)	E 0_2, CV, mV/pH	Peak E, SWV, mV (pH 6.5)	Steady-state E, mV (pH 6.0)
Trametes ochracea	790±10	750	25	800	790±15
Trametes hirsuta	780±10	780	15	790	780±15
Coriolopsis fulvocinerea	780±20	740	5	–	790±15
Cerrena maxima	750±5	790	20	780	780±15
Cerrena unicolor	N.d.	800	30	N.d.	790±15
*Polyporus versicolor**	780*	750*	N.d.	N.d.	820*
*Rhus vernicifera**	430*	450*	N.d.	N.d.	N.d.

"*"—literature values; "–"—absence of the electrochemical activity; "N.d."—not determined; if the error range does not indicate, the standard deviations have been calculated from five independent experiments and were found do not exceed 10%. For *Polyporus versicolor* laccase the different steady-state potentials established on a pyrolytic graphite electrode at pH 5.0 [9] and electroreduction of oxygen was measured at pH 3.8 [31]. For *Rhus vernicifera* laccase the potential of electroreduction of oxygen was measured at pH 5.0 [10]. The standard redox potentials of the T1 sites of *Rhus vernicifera* and *Polyporus versicolor* laccases were published in [17]. All potentials are given vs. NHE.

high redox potential laccases in the potential range between +1100 and −100 mV vs. NHE. It was possible, however, to observe DET for some laccases under anaerobic conditions using the HOPG electrode. As an example, Osteryoung

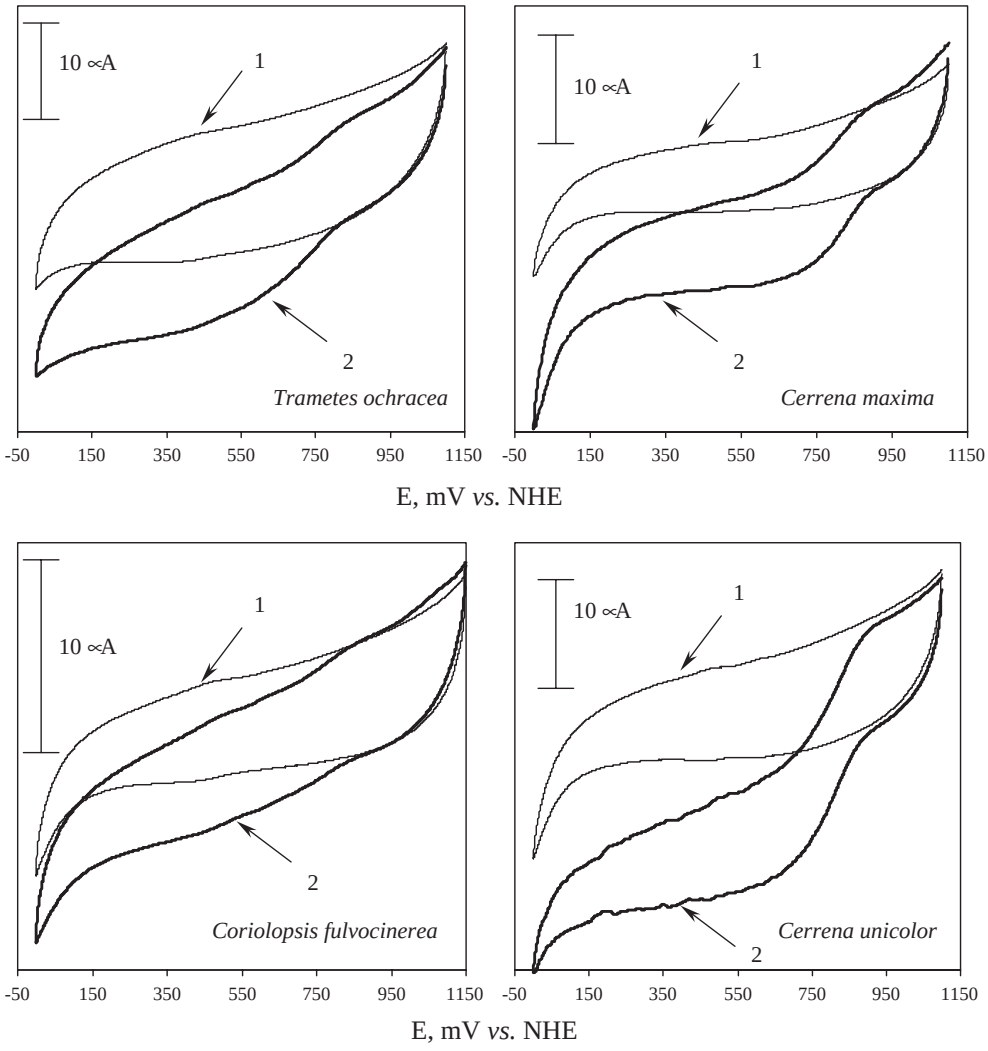

Fig. 1. Electroreduction of molecular oxygen on: (1) naked SPG electrodes; (2) SPG electrodes modified with adsorbed laccases from different basidiomycetes. Citrate-phosphate buffer, 50 mM, saturated oxygen pH 3.0; ionic strength—100 mM $NaClO_4$; scan rate—10 mVs^{-1}; second scan.

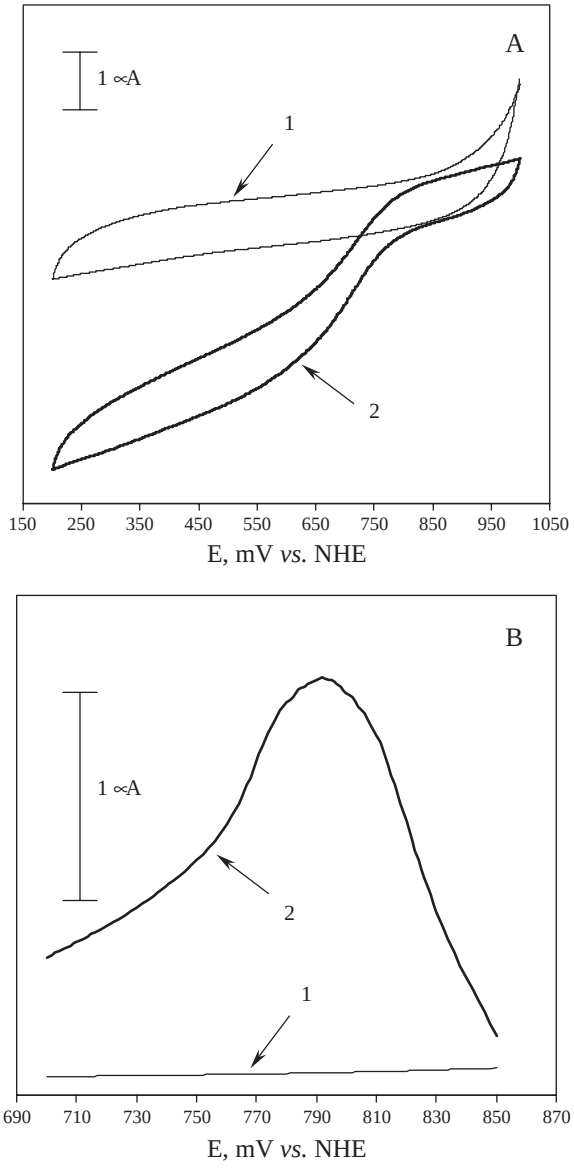

Fig. 2. Voltammograms recorded with HOPG electrodes (1) without and (2) with *T. hirsuta* laccase (≈30 mg/ml laccase solution) entrapped under a dialysis membrane at the electrode surface. (A) Cyclic voltammograms recorded in air-saturated 50 mM citrate-phosphate buffer pH 3.5 containing 100 mM $NaClO_4$; scan rate—10 mVs^{-1}. (B) Osteryoung square wave voltammograms recorded under anaerobic conditions in 100 mM phosphate buffer pH 6.5; amplitude—10 mV, frequency—10 Hz.

square wave voltammograms (SWV) recorded using the HOPG electrode with and without *T. hirsuta* laccase entrapped under the dialysis membrane at the electrode in 0.1 M phosphate buffer solution at pH 6.5 are presented in Fig. 2B. A single wave in the presence of *T. hirsuta* laccase with a peak potential of +796 mV vs. NHE can be seen in this SWV. The maximum of the recorded faradic process is very close to the potential of the T1 site. Similar data were also obtained for *T. ochracea* and *C. maxima* laccases but not for *C. fulvocinerea* laccase under the same conditions as summarised in Table 2.

Fig. 3 summarises the dependence of the electrocatalytic currents on the solution pH. Current densities are plotted versus pH for values ranging from pH 2.6 to pH 7.0. The pH dependence experiments are started after stabilisation of laccases-modified SPG electrodes achieved by waiting for 1 h in order to get a reproducible signal [37,38]. The small decay of the activity of the enzyme during the experiment was corrected by using the value at pH 4.0 recorded at regular intervals. It should be also noticed that the current densities for different pH profile experiments depended on the electrode preparation as well as on batches of the enzyme.

The difference in the pH-optima for fungal and plant laccases can be easily seen. *Rhus vernicifera* laccase has a pH-optimum of about 6.0 [10], whereas the enzymes from the basidiomycetes have their pH-optima in significantly more acidic regions. Moreover, for all laccases from the basidiomycetes a shoulder in the electrocatalytic current vs. pH can be seen in the pH region 4.0–4.5.

The pH profiles of the activity of *C. fulvocinerea*, *C. maxima*, *T. ochracea*, and *T. hirsuta* laccases for inorganic and organic substrates in homogeneous solution are shown in Fig. 4. It is clear that for the phenolic substrate (catechol in this paper) the profile of the activity vs. pH has a bell-shaped form with a maximum value close to pH 4–4.5 (Fig. 4A). This result is in good agreement with previously published data [19,20,22,39,40]. For the inorganic substrates ($K_4Fe(CN)_6$ in the present study) the profile of the activity vs. pH progressively decreases when pH increases

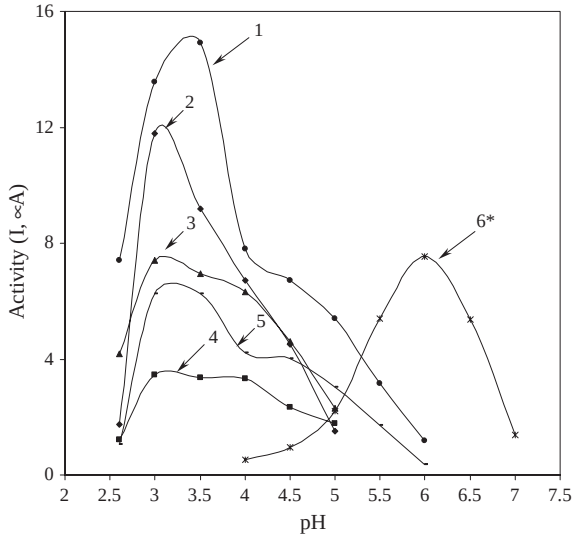

Fig. 3. Oxygen reduction activity versus pH for SPG electrodes modified with adsorbed laccases from different basidiomycetes. 1—*C. unicolor*, 2—*T. hirsuta*, 3—*C. maxima*, 4—*C. fulvocinerea*, 5—*T. ochracea*, 6—*R. vernicifera*; *—literature value [10]. The laccase activity was determined as the oxygen reduction current density of the laccase-modified SPG electrodes at a potential of +350 mV vs. NHE electrode. Citrate-phosphate air-saturated buffer, 50 mM; ionic strength—100 mM $NaClO_4$; scan rate—10 mVs^{-1}; second scan.

(Fig. 4B) in agreement with previously reported data [19,20,22,39,40].

To be able to understand which copper site is the first electron acceptor in the DET reactions, the steady-state redox potentials of the laccase-modified graphite electrodes under aerobic conditions were determined. Initially, the influence of the concentration of the laccase on the redox potential was studied. It was shown previously that the characteristics of the electroreduction of oxygen at laccase-modified graphite/carbon electrodes were dependent on the origin and also on the amount of enzyme on the electrode surface [10,11,36,41]. It was found that the maximum values of the redox potential could be obtained when 10 μg of the enzyme were allowed to adsorb on top of the electrode (Table 3), and thus for further experiments this amount was used for the preparation of the laccase-modified electrodes.

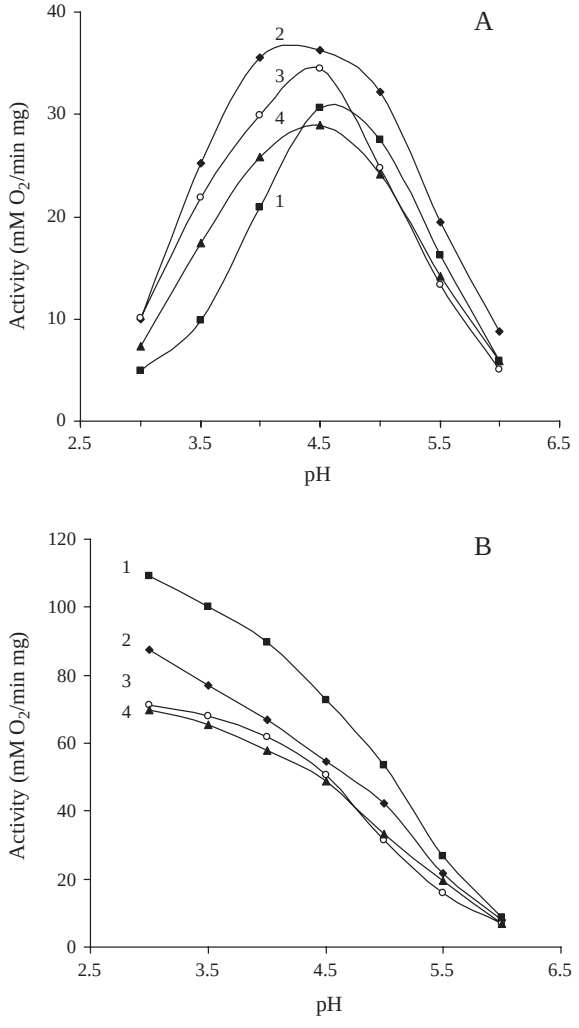

Fig. 4. Oxygen reduction activity versus pH for *C. fulvocinerea* (1), *C. maxima* (2), *T. ochracea* (3), and *T. hirsuta* (4) laccases. One unit of activity is defined as the change in mM O_2/min per mg of protein; all measurements are performed in air-saturated 100 mM citrate-phosphate buffer. The activity of the laccases in homogeneous solution was measured with a Clark electrode using (A) catechol and (B) $K_4[Fe(CN)_6]$ as substrates.

Table 3
Steady-state potentials of *Trametes hirsuta* laccase-modified SPG electrodes at different pHs and amount of laccase adsorbed on the electrode surface

Laccase	3 μg	6 μg	10 μg	20 μg	50 μg
pH 3.0	810 mV	820 mV	910 mV	910 mV	890 mV
pH 6.0	670 mV	690 mV	780 mV	770 mV	760 mV

The reported potentials are average values determined from 6 independent experiments. The deviations from the mean values were not higher than 20 mV. All potentials are given vs. NHE.

For *T. hirsuta* laccase the maximum value of the steady-state potential (open circuit potential) of the laccase-modified graphite electrode (+910 mV vs. NHE) was received at pH 3.0 (Table 4). In spite of the fact that the enzyme showed a high activity for electroreduction of oxygen at pH 3.0 (Fig. 3), this value was too far away from the value of the redox potential of the O_2/H_2O couple under the same conditions (1050 mV vs. NHE). The standard redox potential of the O_2/H_2O couple is +1.23 V at pH 0 and known to be established only at specially pre-treated platinum electrodes and in extremely pure solutions [42]. In contrast, the lowest value of the steady-state potential of the *T. hirsuta* laccase-modified electrode was exhibited at pH 6.0 (780 mV, Table 3), where only traces of the enzyme activity could be found. However, this value is close to the redox potential of the O_2/H_2O couple (870 mV) at the same pH. Even closer values of the steady-state potential were obtained for electrodes modified with *T. ochracea* and *C. fulvocinerea* laccases (Table 4). Moreover, the steady-state potentials of the laccase-modified SPG electrodes changed with 20–30 mV/pH between pH 4.5 and 6.0 (Table 4). However, in the pH region, where all studied enzymes showed a high electrochemical activity, the steady-state potentials of the laccase-modified electrodes changed with 65 mV/pH (*T. hirsuta*), 33 mV/pH (*T. ochracea*, *C. fulvocinerea*), and 13 mV/pH (*C. maxima*) (Table 4).

4. Discussion

In our recent publications a new classification of laccases based on the value of the redox potential of the T1 copper site has been proposed [11,21]. Recently we also extended such a classification towards all "blue" multicopper oxidases [12]. Based on the primary structures of the copper enzymes, all "blue" multicopper oxidases can be divided into three groups: low, middle and high potential enzymes [12]. The low redox potential enzymes (340–490 mV) have methionine as the axial ligand of the T1 site, the middle redox potential enzymes (470–710 mV) have leucine, and the high potential "blue" oxidases (730–780 mV) have phenylalanine. From Table 2 it is clearly seen that the laccases from *T. hirsuta*, *T. ochracea*, *C. maxima*, and *C. fulvocinerea* are high redox potential enzymes. Data about the electrochemistry of the low redox potential laccase from

Table 4
Steady-state potentials (ssp) of laccase-modified SPG electrodes at different pHs and the difference between the steady-state potential of the laccase-modified SPG electrode and the equilibrium redox potential of the O_2/H_2O couple under the same conditions (ΔE)

Laccase	pH 3.0		pH 4.5		pH 6.0	
	ssp	ΔE	ssp	ΔE	ssp	ΔE
Trametes ochracea	870 mV	−180 mV	820 mV	−140 mV	790 mV	−80 mV
Trametes hirsuta	910 mV	−140 mV	810 mV	−150 mV	780 mV	−90 mV
Coriolopsis fulvocinerea	870 mV	−180 mV	820 mV	−140 mV	790 mV	−80 mV
Cerrena maxima	850 mV	−200 mV	830 mV	−130 mV	780 mV	−90 mV

The reported potentials are average values determined from 6 independent experiments. The deviations from mean values were not higher than 20 mV. All potentials are given vs. NHE.

R. vernicifera adsorbed on carbon electrodes are available in the literature [10,43]. In the present work the electrochemical behaviour (specifically, DET) of laccases with significantly different biochemical characteristics (e.g., carbohydrate content, pH-optimum, catalytic activity, redox potential of the T1 site, etc.) was compared.

As was mentioned above in the presence of molecular oxygen (enzyme substrate) a reduction current can be recorded at the laccase-modified carbon electrode due to DET between the electrode and the adsorbed laccase [9,10,28,29,31,41,43]. However, the voltammograms of the SPG-modified electrodes did not reveal any redox transformation under anaerobic conditions. Few papers report on a clear electrochemical response of laccase on carbon electrodes under anaerobic conditions using cyclic voltammetry [28,31]. In these publications highly ordered pyrolytic graphite electrodes were used and the CVs exhibited minimal electrochemical response and in Ref. [28] only in the presence of a promoter. For the high redox potential laccases (*T. hirsuta*, *T. ochracea*, and *C. maxima*) clear electrochemical response could only be observed in the case of PME configuration on HOPG electrodes under anaerobic conditions using SWV (Fig. 2B). Probably the relatively high capacitive current of the SPG electrode, approximately 10 times than that for the HOPG electrode, see Figs. 1 and 2, hinders such observation of the same process with the more conventional CV measurements. Similarly, such a transformation was recorded with high concentrations of bilirubin oxidase at SPG or HOPG electrodes [12,38].

The absence of any electrochemical activity of *C. fulvocinerea*-modified electrodes under anaerobic conditions even when using SWV can probably be explained by the isolating effect caused by the carbohydrates on the enzyme surface. The laccases from *T. hirsuta*, *T. ochracea*, and *C. maxima* have less than 13%, whereas the enzyme from *C. fulvocinerea* has about 32% carbohydrates (Table 1) [21]. Glycosylation of horseradish peroxidase (HRP) was previously found to drastically hinder DET between the enzyme and graphite electrodes revealed when comparing the glycosylated wild type HRP with its non-glycosylated counterpart, recombinant HRP expressed in *E. coli* [44]. The absence of glycosylation for recombinant HRP resulted in a much higher rate of heterogeneous DET between the enzyme and the electrode, and also a much higher percentage of adsorbed enzyme molecules oriented for DET compared to the glycosylated wild type HRP. Thus, the absence of any electrochemical response from *C. fulvocinerea* laccase under anaerobic conditions is probably due to the high level of glycosylation, which increases the electron tunnelling distance between this laccase and conducting carbon. The same explanation can be put forward to reason the lowest electrocatalytic activity of the reduction of oxygen for SPG electrodes modified with *C. fulvocinerea* laccase, whereas the activity of this enzyme in homogeneous solution is comparable or higher than the activities of the other laccases.

All laccase substrates can in principle be divided in two groups: (i) electron-no-proton donors and (ii) electron-proton donors [18,22,23]. The first group (i) includes simple inorganic redox compounds, e.g., ferrocyanide, and organic substrates, which are oxidised by the enzyme through a cation radical mechanism, e.g., ABTS [45]. For these substrates the decrease in laccase activity with an increase in pH is explained by conversion of the active enzyme into an inactive form due to complex formation between the T2 Cu^{2+} and OH^- [14,46]. The second group (ii) includes phenols and aromatic amines, for which the formal potentials have a strong dependence on the solution pH. Thus, both processes, i.e., inhibition by OH^- at alkaline pHs and a decrease in the thermodynamic driving force at acidic pHs (the difference between the formal potential of the substrate and the redox potential of the T1 site of laccase) affect the activity–pH profile of the enzyme resulting into a bell-shaped dependence. This classification explains the two types of pH dependencies (see Fig. 4) for oxidation of different substrates by fungal laccases. The pH profiles for the electroreduction of oxygen by adsorbed high redox potential laccases correlate with the pH profiles for the oxidation of the first group of substrates (ABTS, $K_4Fe(CN)_6$). The electrode works, thus, like an electron donor and the protons necessary for reduction of oxygen to water are taken from the surrounding solution. Concomitantly, the decrease in the activities in the low pH region (from 3 and lower) is due to stability loss of the laccases at low pH values.

It should be mentioned that for all studied fungal laccases, a shoulder in the heterogeneous activity versus

pH profile was found between pH 4 and 4.5 (Fig. 3). This pH region corresponds to the maxima of the activities of the enzymes in homogeneous solution for the second group of substrates (electron-proton donors) (cf. Figs. 3 and 4). A similar profile was previously obtained for the pH dependence of oxygen electroreduction for bilirubin oxidase-modified SPG electrodes [12,38]. At this moment it is not possible to clearly explain the shoulder in these activities versus pH plots. However, it is well known that there exist a series of oxygen functionalities on the surface of carbon, especially at the ends of the edge planes of graphite [47]. It has been suggested previously that such oxygen functionalities, e.g., phenolic and quinoic groups, could work as surface tethered mediators facilitating the electron transfer from the electrode to surface bound redox enzymes, e.g., HRP [48,49] and *Pycnoporus cinnabarinus* laccase [50]. It could thus be that at close to neutral pH the graphite electrode behaves primarily as an electron-no-proton donor, similar to group (i), but additionally as an electron-proton donor, group (ii), caused by the phenolic surface functionalities. This could explain that the activity versus pH profile for adsorbed laccases on graphite (Fig. 3) resembles both the activity versus pH profile for laccase in solution using group (i) electron donors, Fig. 4B, and group (ii) donors, Fig. 4A. Moreover, the pH-optimum of *R. vernicifera* laccase for most substrates is close to neutral pH [51], which is in good agreement with the pH profile of electroreduction of oxygen by the enzyme (Fig. 3). This similarity of the pH dependencies between the homogeneous and heterogeneous catalytic processes for different laccases probably indicates that it is primarily the properties of the enzyme that determine the characteristics for oxygen electrocatalysis, however, the enzyme–graphite interface plays some but a minor role. This suggestion was previously proposed for the low and high redox potential laccases [10–12,36] as well as confirmed for another multicopper oxidase, bilirubin oxidase [12,38].

The list of facts presented below also indicates that the heterogeneous ET characteristics of the laccases on graphite are very similar to their homogeneous properties. As can be seen from Table 2 the half wave potential for the electroreduction of oxygen as well as the steady-state potentials of the laccase-modified SPG electrodes are very close to the redox potentials of the T1 site of the enzymes. The steady-state potentials of the laccase-modified SPG electrodes changed with 20 mV/pH between pH 4.5 and 6.0 (Table 4) and the half wave potential for bioelectroreduction of oxygen changed from 5 to 30 mV/pH (Table 2). These values are too far away from the 60 mV/pH dependence of the redox potential for the O_2/H_2O couple, whereas they are close to the values, which were demonstrated by Nakamura and Reinhammar for the pH dependence of the potential of the T1 site for the low and high redox potential laccases [17,52].

The facts mentioned above, the similarities discussed between the pH dependencies for the heterogeneous and homogeneous electrocatalysis of oxygen reduction in combination with the characteristics of the enzyme (activity vs. pH, formal potential of the T1 site vs. pH, steady-state potential vs. pH) point to the fact that the T1 site is the first electron acceptor in DET from a carbon electrode to the enzyme. This conclusion is also based on the fact that the observed dependencies of the electrocatalytic currents on pH for adsorbed laccase correlate with those obtained with the corresponding dissolved enzymes for the majority of organic and inorganic substrates, where the T1 site dictates these properties and has been determined to be the first electron acceptor [13,14,22]. An additional fact that supports this conclusion is that the peak potentials of the SWVs recorded under anaerobic conditions, the half wave potentials for oxygen electroreduction at graphite electrodes modified with laccases from different origins as well as the steady-state redox potentials are always close to the redox potentials of the T1 sites for the corresponding laccases (Tables 2 and 4). Thus, the conclusion that the T1 site is the first electron acceptor in the enzyme when adsorbed on carbon electrodes under both aerobic and anaerobic condition is well supported by our results in combination with data reported by others.

In summary, the electrochemical (heterogeneous) ET properties of high and low redox potential laccases from different sources were analysed both under anaerobic and aerobic conditions. Based on both the obtained results as well as taking into account previously published results [9–12,28,36] the mechanism of the electroreduction of oxygen at carbon electrodes modified with laccase is proposed and presented in Fig. 5. The electrons are transferred from the electrode to the T1 site of the adsorbed enzyme. The closest distance between the surface of the enzyme and the T1 site is about 8 Å [14] and if properly oriented on the electrode surface such a distance would allow efficient electron tunnelling [53]. The electron is then transferred through an internal ET mechanism to the T2/T3 cluster (the distance is

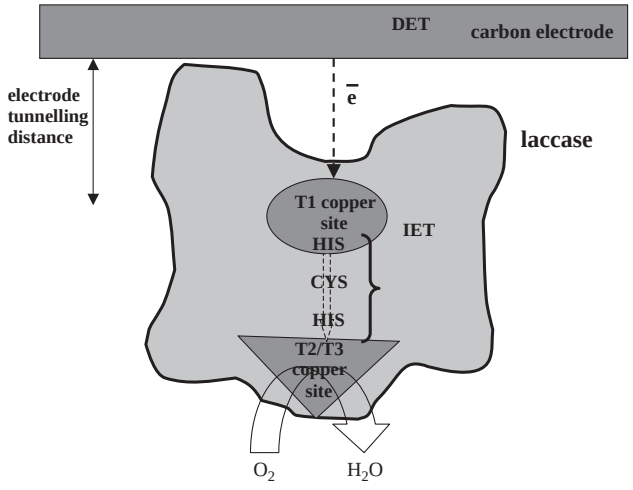

Fig. 5. A possible mechanism of the direct electron transfer for laccase adsorbed on a carbon electrode in agreement with recent reviews [11,12].

12–13 Å), where molecular oxygen is reduced to water. This mechanism is in good agreement with the previously proposed mechanism for laccase and also for another "blue" multicopper oxidase, bilirubin oxidase [11,12,54]. The heterogeneous ET between carbon and the T1 site can proceed at anaerobic conditions (Fig. 2B), which indicates that the carbon-T1 copper ET process is possibly independent on the redox state of the T2/T3 copper cluster. It can also be suggested that the mechanism presented in Fig. 5 is similar for all "blue" multicopper oxidases at carbon electrodes.

Acknowledgement

The authors thank Dr. E. Gorshina for the cultivation of basidiomycetes *T. hirsuta* and *T. ochracea*, Drs. O. Koroleva and V. Gavrilova for the preparation of *C. fulvocinerea* and *C. maxima* laccases. The work was financially supported by The European Commission (contract No ICA2-CT-2000-10050), the Russian Foundation for Basic Research (project No 03-04-48937), the FNTP of Russian Ministry of Science and Technology, and The Swedish Research Council.

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