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Preliminary Communication

Enzymatic oxidation of manganese ions catalysed by laccase

Marina Gorbacheva [a], Olga Morozova [a], Galina Shumakovich [a], Alexander Streltsov [a], Sergey Shleev [a,b,*], Alexander Yaropolov [a]

[a] Laboratory of Chemical Enzymology, A.N. Bach Institute of Biochemistry RAS, 119071 Moscow, Russia
[b] Department of Biomedical Laboratory Science, Faculty of Health and Society, Malmö University, 20506 Malmö, Sweden

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ABSTRACT

The principal possibility of enzymatic oxidation of manganese ions by fungal *Trametes hirsuta* laccase in the presence of oxalate and tartrate ions, whereas not for plant *Rhus vernicifera* laccase, was demonstrated. Detailed kinetic studies of the oxidation of different enzyme substrates along with oxygen reduction by the enzymes show that in air-saturated solutions the rate of oxygen reduction by the T2/T3 cluster of laccases is fast enough not to be a readily noticeable contribution to the overall turnover rate. Indeed, the limiting step of the oxidation of high-redox potential compounds, such as chelated manganese ions, is the electron transfer from the electron donor to the T1 site of the fungal laccase.

1. Introduction

Laccase (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) belongs to the diverse group of blue multicopper oxidases and catalyses one-electron oxidation of different organic substrates, as well as some coordination compounds, coupled to the four-electron reduction of oxygen (O_2) directly to water (H_2O), i.e. without formation of reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and superoxide radical ($O_2^{-\cdot}$) [1–3]. The active centre of laccase contains four copper ions classified into three types: a mononuclear blue copper ion comprising the T1 site and three copper ions forming the trinuclear copper cluster (T2/T3 cluster), containing one ion of type 2 (T2 site) and two copper ions of type 3 (T3 site) [2,4].

Based on the source, laccases can be classified as plant, fungal, bacterial, or insect enzymes [2]. It is generally accepted that plant laccases participate in free-radial reactions during lignin synthesis, whereas fungal enzymes have much broader function, e.g. they participate in lignin degradation, in maturity and morphogenesis of fungi, and in pathogenesis and detoxification processes [2,5,6]. It should be mentioned, however, that not all roles of laccase in different living organisms are yet fully clarified [3,7]. For example, it has been unambiguously demonstrated only recently that laccase-like bacterial enzymes are responsible for the formation of insoluble manganese oxides by enzymatic Mn^{2+} oxidation [8]. Nevertheless, it is a well-known fact that the substrate specificity of laccases depends on the structure and the redox potential of the T1 site (E_{T1}), the first electron acceptor of the enzyme [9]. Depending on E_{T1} laccases can also be classified as low- and high-redox potential enzymes [10–12]. It is believed that only compounds with ionisation potentials below E_{T1} can be efficiently oxidised by laccase [13]. In spite of the very high-redox potential of the Mn^{2+}/Mn^{3+} couple equal to 1510 mV [14], Mn^{2+} can be quite efficiently oxidised by both bacterial (low-redox potential enzymes [15,16]) and fungal (high-redox potential enzymes [11,17]) laccases [8,18]. A possible physiological role of laccase catalysed Mn^{2+} oxidation is to provide H_2O_2 for extracellular reactions performed by ligninolytic enzymes suggesting a novel type of laccase-peroxidases cooperation relevant to biodegradation of lignin [19]. Moreover, enzymatic Mn^{2+} oxidation might play an important role in the function of the ligninolytic complex of wood-degrading fungi since Mn^{3+} is a strong oxidant and can oxidise some non-phenolic substructures of lignin, e.g. guaiacyl (veratryl alcohol and 1-(3-methoxy-4-isopropoxyphenyl)ethanol) and syringyl (3,4,5-trimethoxybenzyl alcohol and 1-(3,5-dimethoxy-4-isopropoxyphenyl)ethanol) model lignin compounds [18–20]. The mechanism of the oxidation of Mn^{2+} by laccase, however, is still not understood in detail. Furthermore, a possible interaction of plant laccases with Mn^{2+} has not been studied so far. Thus, the main objective of this work was to enhance the understanding of a possible enzymatic oxidation of Mn^{2+} catalysed by plant and fungal laccases.

* Corresponding author. Address: Department of Biomedical Laboratory Science, Faculty of Health and Society, Malmö University, 20506 Malmö, Sweden. Fax: +46 40 665 8100.
E-mail address: sergey.shleev@mah.se (S. Shleev).

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2. Experimental

2.1. Materials

Boric acid, sodium tartrate, 2,2′-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS), nitroblue tetrazolium (NBT), KCl, and $K_4[Mo(CN)_8]$ were purchased from Sigma–Aldrich Chemie GmbH (Germany). Na_2HPO_4, NaOH, KOH, citric acid, 3,4-dimethoxybenzyl alcohol (veratryl alcohol), and 3,4-dimethoxybenzaldehyde (veratryl aldehyde) were from Merck (Germany). $K_4[Fe(CN)_6]$—Alfa Aesar (Germany). $MnSO_4$, $C_2H_2O_4$, and H_3PO_4—ChemMed (Russia). All solutions were prepared using water purified with Milli-Q system (Millipore, USA).

2.2. Enzymes

The basidiomycete *Trametes hirsuta* (Wulfen) Pilát (*Coriolus hirsutus* (Wulfex Fr.) Quél.), strain *T. hirsuta* 56, was obtained from the laboratory collection of the Moscow State University of Engineering Ecology (Russia). The basidiomycete was grown by submerged cultivation [21] and laccase was isolated from the culture medium as described previously [17]. Partly purified *Rhus vernicifera* laccase from the latex of the lacquer tree was kindly provided by Prof. B. Reinhammar (University of Göteborg, Sweden). The final purification for both laccases was performed by means of HPLC on a TSK DEAE-2SW column (LKB, Sweden) using a Stayer HPLC system (Acvilon, Russia). The enzymes were homogeneous as judged from SDS–PAGE. Homogeneous preparations of both enzymes were stored in 0.1 M phosphate buffer, pH 6.5, at −20 °C. Horseradish peroxidase (HRP Type VI, 250–330 U/mg) was purchased from Sigma–Aldrich Chemie GmbH and used without further purification.

2.3. Laccase assay and kinetics studies

Oxidation of Mn^{2+} to Mn^{3+} by purified laccases was monitored spectrophotometrically for up to 2 h by following the formation of Mn^{3+} complexes, in 0.1 M Na-oxalate or 0.1 M Na-tartrate buffer, pH 5.0. The dependence of laccase activity on pH was performed in 0.1 M Na-oxalate buffer. The concentrations of Mn^{3+}-oxalate (ε_{270} = 55,000 mM^{-1} cm^{-1}) and Mn^{3+}-tartrate (ε_{280} = 6500 mM^{-1} cm^{-1}) were determined as described in [19,22]. All spectrophotometric measurements were carried out in air-saturated solutions at 20 °C using a Hitachi-557 spectrophotometer (Japan).

The determination of the standard biocatalytic rate constant (k_{cat}) and the apparent Michaelis constant (K_M) of both laccases towards transition metal complexes and the dependence of their activities on pH were determined in universal Britton–Robinson buffer (40 mM boric acid–40 mM acetic acid–40 mM phosphoric acid adjusted to the necessary pH with 0.2 M NaOH) by estimation of the initial rates of O_2 consumption in a Clark oxygen electrode at 20 °C with constant stirring using voltammetric analyser CV-50W (Bioanalytical systems, USA). The concentration of O_2 in the Britton–Robinson air-saturated buffer at 20 °C was assumed to be 260 μM. K_M-values of both laccases towards O_2 were also determined in universal Britton–Robinson buffer containing 5 mM $K_4Fe(CN)_6$ as electron donor using the Clark O_2 electrode thermostated at 25 °C. In these measurements the concentrations of *R. vernicifera* and *T. hirsuta* laccases were 0.9×10^{-7} M and 3.8×10^{-8} M, respectively, and different concentrations of O_2 inside the Clark electrode were established using pure argon or oxygen from Venta (Russia). All kinetic parameters were calculated using the Michaelis–Menten equation in a Microcal Origin program (version 5.0).

2.4. Measurements of H_2O_2 and $O_2^{-\cdot}$ production

The formation of H_2O_2 and $O_2^{-\cdot}$ were measured spectrophotometrically using a Hitachi-557 spectrophotometer. In the case of H_2O_2 measurements a slightly modified procedure described in our previous publication was used [23]. An aliquot was taken from the reaction mixture during enzymatic oxidation of Mn^{2+} in the presence of oxalate or tartrate ions, filtered through a 12 kDa membrane filter (Millipore) to remove laccase, and placed into a cuvette. Then, ABTS (9 mM) and HRP (0.5 mg/ml) were added as electron donor and catalyst, respectively, giving a final volume of 1 ml. The concentration of oxidised ABTS was measured at 405 nm (ε_{405} = 36,800 M^{-1} cm^{-1}), which corresponds to the concentration of H_2O_2 according to the following reaction:

$$ABTS^{2-} + H_2O_2 + 2H^+ = ABTS^{-\cdot} + 2H_2O$$

The formation of $O_2^{-\cdot}$ was confirmed by addition of nitroblue tetrazolium, a compound sensitive to $O_2^{-\cdot}$, to the reaction medium already containing *T. hirsuta* laccase as catalyst.

2.5. Cyclic voltammetry measurements

Cyclic voltammograms (CVs) were recorded in a three-electrode electrochemical cell using a three-electrode potentiostat (BAS CV-50W) and a one-compartment electrochemical cell with a total volume of 10 ml. A glassy carbon electrode (BAS), an Ag|AgCl|3 M NaCl electrode (BAS, 210 mV vs. NHE), and a platinum wire (1 mm diameter) served as working, reference, and counter electrodes, respectively. The surface of the working electrode was polished with alumina FF slurry (0.1 μm, Stuers, Copenhagen, Denmark), rinsed with double-distilled water, and allowed to dry. CVs were recorded in 0.1 M Na-tartrate buffer, pH 5.0 in the potential range of 0–1200 mV vs. NHE and scan rates varied from 10 up to 100 mV/s. All potentials in the present work are given vs. NHE.

2.6. Enzymatic oxidation of veratryl alcohol

2.6.1. Spectrophotometric assay

The initial velocity of veratryl aldehyde formation was measured by the increase in 310 nm absorbance using ε_{310} = 9300 M^{-1} cm^{-1} [24].

2.6.2. HPLC studies

Analysis of veratryl alcohol oxidation products was performed by reverse phase hydrophobic HPLC on a Luna C18 column (250 × 4.6 mm) (Phenomenex, USA) with a linear gradient of acetonitrile (5–95%) in 0.086% H_3PO_4 at a 1 mL min^{-1} flow rate using a Stayer-system (Acvilon, Russia). The incubation mixture contained 1 mM veratryl alcohol, 1 mM $MnSO_4$, and 10^{-7} M of the laccases in 0.1 M Na-oxalate buffer, pH 5.0. The control mixture had either laccases or Mn^{2+} missing. The sample preparation included ultrafiltration through PM-10 membrane in an Amicon cell (USA) to remove all proteins followed by acidification of the filtrate with 0.086% H_3PO_4 (v/v). The products were identified in accordance with their retention times. Veratryl alcohol and veratryl aldehyde were used as standards. Quantification of the products in eluate was performed integrating the elution peak using the Multichrom software (Russia).

3. Results and discussion

One of the basic parameters of laccase catalysed redox reactions is the difference in the standard redox potentials of the initial electron donor (enzyme substrate, E_S) and the first electron acceptor

(the T1 site of the enzyme, E_{T1}). The E_{T1}-values of high-redox potential *T. hirsuta* and low potential plant *R. vernicifera* laccases, used in the present work as catalysts, are 780 mV (pH 6.0) [25] and 430 mV (pH 6.8) [26], respectively. The redox potential of the Mn^{2+}/Mn^{3+} couple (1510 mV at pH 7.0 [14]), is significantly higher than the E_{T1}-values of both fungal and plant blue multicopper oxidases [11]. Thus, one cannot expect direct enzymatic oxidation of Mn^{2+} catalysed by laccases. The direct electrochemical method used in this work showed that the formation of manganese complexes with chelators significantly decreased the redox potential of the Mn^{2+}/Mn^{3+} couple and makes it potentially oxidiseable by the high-redox potential enzyme. Dicarboxylic and oxycarboxylic acids are good chelators of both Mn^{2+} and Mn^{3+} and they also provide the needed pH level for the enzymatic reaction to occur [27]. In the CV of a glassy carbon electrode submerged in 0.1 M Na-tartrate buffer, pH 5.0, containing 0.2 mM Mn^{2+}, both the anodic and cathodic peaks with potentials of 990 and 890 mV, respectively, could be observed (Fig. 1). The peak potential difference of 100 mV suggests a quasi-reversible redox transformation of chelated manganese ions. The midpoint potential of the redox process was found to be 940 mV vs. NHE. This value corresponds to the redox potential of the tartrate chelated Mn^{2+}/Mn^{3+} couple.

Because E_{T1} of *T. hirsuta* laccase at pH 5.0 is only 130 mV lower compared to the midpoint potential of the Mn^{2+}/Mn^{3+} tartrate couple (Table 1), thermodynamically it can be suggested that the enzyme would be able to slowly oxidise Mn^{2+} in the tartrate complex. The E_{T1}-values of the laccases at pH 5.0 presented in Table 1 were calculated taking into account the previously investigated variation with pH (ca. 16 mV/pH) of E_{T1} on pH for *R. vernicifera* [28] and *T. hirsuta* [29] laccases. Contrary to the situation with fungal laccases, a very large difference between E_{T1} of *R. vernicifera* Lc and the Mn^{2+}/Mn^{3+} tartrate complex (470 mV) should result in the absence of Mn^{2+} oxidation by the enzyme. This simple theoretical reasoning was experimentally observed in our studies as discussed below.

As expected, no enzymatic oxidation of Mn^{2+}-oxalate and Mn^{2+}-tartrate complexes by low-redox potential *R. vernicifera* laccase was registered (Table 1), whereas the formation of Mn^{3+}-complexes by *T. hirsuta* laccase was clearly shown (e.g. oxalate in Fig. 2). However, the rate of enzymatic oxidation of Mn^{2+} by *T. hirsuta* laccase is not so high. Nevertheless, the enzyme increase the rate of manganese oxidation to approximately 10 times that of non-biological oxidation at pH 5, in good agreement with previously published data concerning biological rates of manganese oxidation [8,30]. The sublinear dependence of the amount of Mn^{3+}-oxalate complex formed over the extended time period is explained by the low stability of the product of enzymatic reaction, viz. the Mn^{3+}-oxalate complex (Fig. 2, inset). Kinetic studies of enzymatic oxidation of Mn^{2+} by *T. hirsuta* laccase showed the classical Michaelis–Menten dependence. However, the efficiency of the enzymatic process was very low compared to the oxidation of other substrates of the enzymes, e.g. cyanide transition metal complexes (Table 1), which have much lower values of midpoint potentials compared to the manganese ions (940 mV).

The pH dependences of the oxidation of artificial and putative natural substrates of *T. hirsuta* laccase, $K_4[Mo(CN)_8]$ and Mn^{2+}, respectively, are compared in Fig. 3. Contrary to the cyanide complexes, which have very acidic pH optima, the pH-optimum of the oxidation of the chelated Mn^{2+} is shifted to subacid values (pH around 5.0), the natural pH level of the ligninolytic enzymes of wood-degrading fungi. The rate of the oxidation of the artificial enzyme substrates at pH 5.0, however, is still three orders of magnitude higher than the rate of the oxidation of the Mn^{2+}-oxalate complex, one of the possible natural substrates of *T. hirsuta* laccase (Table 1).

To test the physiological role of enzymatic oxidation of chelated manganese ions, the oxidation of a model lignin compound, veratryl alcohol, by *T. hirsuta* laccase in the presence of chelated Mn^{2+} was performed. Indeed, this possibly natural biocatalytic process was observed spectrophotometrically at a quite significant rate, whereas there was a complete absence of such enzymatic reaction in the case of *R. vernicifera* laccase. Additional HPLC studies, however, did not confirmed without a doubt the formation of veratryl aldehyde during alcohol oxidation. Instead, the formation of veratryl acid was clearly observed. Anyhow, the rapid and deep oxidation of veratryl alcohol observed in our studies might be explained by the co-formation of H_2O_2 and $O_2^{-\cdot}$ during the enzymatic oxidation of Mn^{2+} in the presence of oxalate or tartrate ions, which was confirmed by the enzymatic oxidation of ABTS in the presence of HRP (showing H_2O_2 formation) or nitroblue tetrazolium (indicating the presence of $O_2^{-\cdot}$) in the presence of *T. hirsuta* laccase. The participation of $O_2^{-\cdot}$ in secondary chemical reactions results in the formation of H_2O_2, a substrate of lignin peroxidases, another type of enzyme forming the ligninolytic complex of white-rot fungi.

In order to understand the limiting step of the enzymatic oxidation of chelated Mn^{2+} the basic kinetic parameters of *T. hirsuta* and *R. vernicifera* laccases towards different compounds were accurately measured and the results are summarised in Table 1. The apparent Michaelis constants (K_M) of both enzymes towards O_2, the second natural substrate (electron acceptor) in the catalytic cycle of all blue multicopper oxidases, are the lowest K_M-values obtained in our studies. In contrast, the standard biocatalytic rate constant (k_{cat}) and the efficiency of O_2 reduction by the T2/T3 cluster (k_{cat}/K_M) for both laccases are the highest values (Table 1). Approximate numerical values of the second-order rate constant of O_2 reduction by the T2/T3 cluster of plant and fungal laccases are $5 \times 10^6\ M^{-1}\ s^{-1}$ and $5 \times 10^7\ M^{-1}\ s^{-1}$, respectively [31,32]. Thus, in air-saturated solutions the rate of O_2 reduction by the T2/T3 cluster of laccases is very high and fast enough not to be a readily noticeable contribution to the overall turnover rate (Table 1). Importantly, previously estimated second-order rate constants for plant and fungal laccases are in good agreement with the effi-

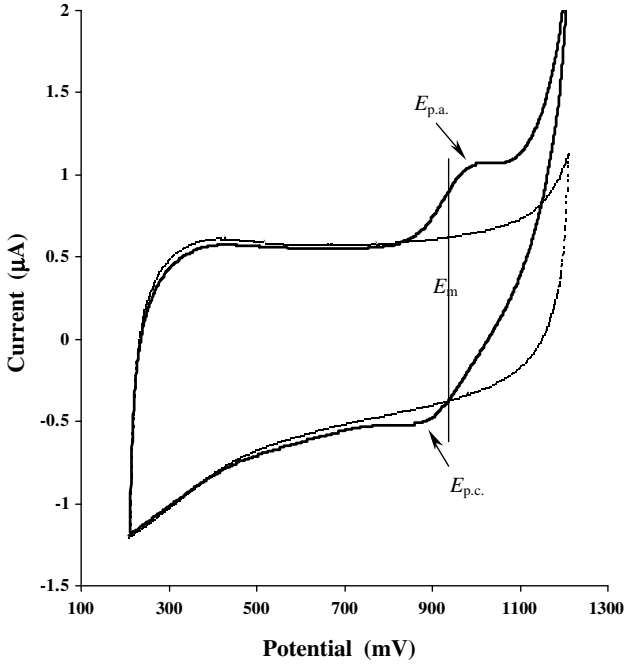

Fig. 1. Cyclic voltammograms of a glassy carbon electrode recorded in 0.1 M Na-tartrate buffer pH 5.0 in the presence (bolded line) and absence (solid line) of 0.2 mM of $MnSO_4$ (scan rate, 50 mV/s; starting potential, 200 mV).

Table 1
Basic kinetic parameters of redox reactions catalysed by plant and fungal laccases

Substrate	E_S(mV)	*Trametes hirsuta* ($E_{T1} \sim 800$ mV)			*Rhus vernicifera* ($E_{T1} \sim 460$ mV)		
		k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
O_2	940	13,000*	~0.15	$\sim 87 \times 10^6$	1300*	~0.2	$\sim 6.5 \times 10^6$
Mn^{2+}-oxalate complex	n.d.	0.04	0.43	93	0	0	0
Mn^{2+}-tartrate complex	940	0.08	0.11	727	0	0	0
$K_4[Mo(CN)_8]$	780	78	0.67	$0.12 \cdot 10^6$	0	0	0
$K_4[Fe(CN)_6]$	430	114	0.18	$0.63 \cdot 10^6$	64	0.46	$0.14 \cdot 10^6$

Notes: *, literature values [31,32,35]; n.d., not determined; redox potentials are given vs. NHE.

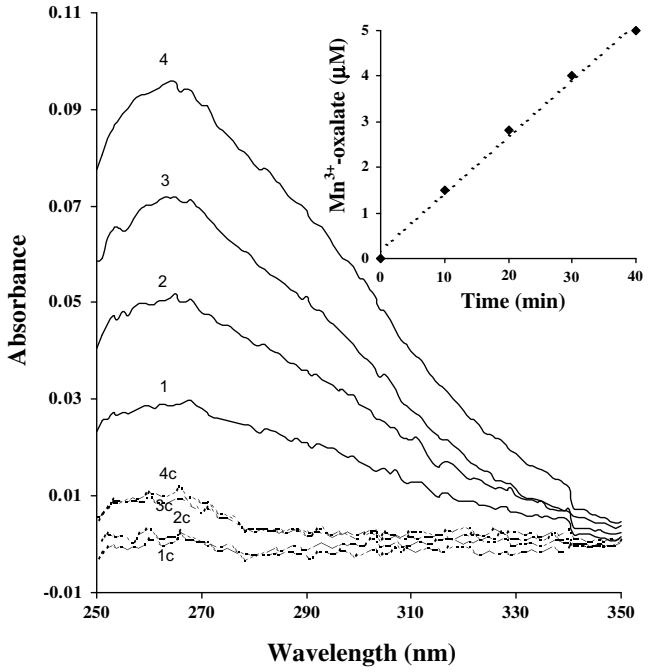

Fig. 2. Spectral monitoring of the formation of Mn^{3+}-oxalate complex in an enzymatic reaction catalysed by *T. hirsuta* laccase (0.1 M Na-oxalate buffer, pH 5.0 containing 8×10^{-8} M of laccase and 1.0 mM $MnSO_4$). Curves 1, 2, 3, and 4—UV spectra recorded after 10, 20, 30, and 40 min of the enzymatic reaction; curves 1c, 2c, 3c, and 4c—control spectra recorded after 10, 20, 30, and 40 min without addition of *T. hirsuta* laccase into the reaction media. Insets: the concentration of Mn^{3+}-oxalate complex vs. time during the enzymatic oxidation of Mn^{2+}.

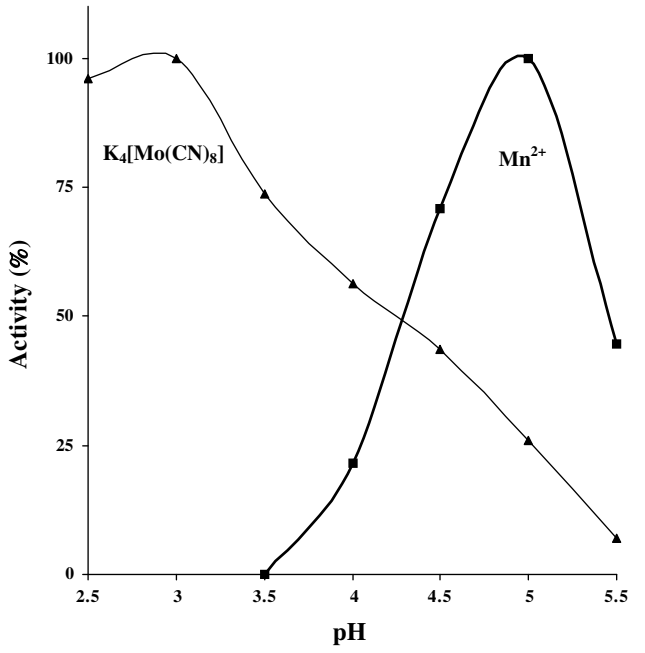

Fig. 3. pH dependence of the oxidation of $K_4[Mo(CN)_8]$ in 40 mM Britton–Robinson buffer containing 1 mM $K_4[Mo(CN)_8]$ and 0.074 μM laccase (solid curve; triangles) and Mn^{2+} in 0.1 M Na-oxalate buffer containing 1 mM $MnSO_4$ and 0.37 μM laccase (bolded curve, squares) by *T. hirsuta* laccase.

ciency of O_2 reduction by the T2/T3 clusters of both enzymes calculated using the K_M-values determined in the present study (Table 1).

The k_{cat} values and the efficiency of the oxidation of both Mn^{2+}-oxalate and Mn^{2+}-tartrate complexes by *T. hirsuta* laccase are the lowest values obtained in our studies. These results are in good agreement with previously published kinetic data about the enzymatic oxidation of Mn^{2+}-pyrophosphate complex by high-redox potential *T. versicolor* laccase [18].

Based on the available spectral and kinetic investigations of laccases it is widely held that neither the internal electron transfer nor the binding of O_2 is limiting the turnover rate of the enzyme during its natural function [32]. Thus, in all likelihood, the limiting step of the oxidation of the laccase substrate is the electron transfer from the electron donor to the T1 site of the laccases. To test this hypothesis for enzymatic oxidation of the chelated Mn^{2+}, the effect of the redox potential on the efficiency of enzymatic catalysis of plant and fungal laccases was investigated. For this purpose electron donors with quite similar structure, size, and molecular weight, but very different redox potentials, were chosen (Table 1). The kinetic studies showed that both k_{cat} and k_{cat}/K_M were highly dependent on the difference between the one-electron redox potential E_{T1} and E_S (Table 1), i.e. similar to the previously obtained correlation between E_{T1} of fungal laccases and E_S of phenolic, energy-rich compounds, and other natural substrates of laccases [33]. Thus, our data confirm the previously proposed hypothesis that the electron transfer rate constant (k_{ET}) between substrate and the T1 site of laccases is one of the major components of k_{cat} of blue multicopper oxidases [34]. Indeed, the limiting step of the oxidation of very high-redox potential, energetically poor compounds, such as chelated Mn^{2+} ions, is the electron transfer from the electron donor to the T1 site of the fungal laccase.

In summary, both plant and fungal laccases can directly oxidise phenolic lignin substructures, which usually have quite low-redox potentials. However, due to the very high E_{T1}-values, fungal laccases can also indirectly oxidise non-phenolic lignin compounds, e.g. veratryl alcohol, with the help of chelated Mn^{3+} and reactive oxygen species, which are formed during the enzymatic reactions catalysed by the high-redox potential enzymes. The dramatic difference in biocatalytic behaviour between high- and low-redox potential laccases is, in all likelihood, related to the different physiological roles of these enzymes in nature, viz. polymerisation of building blocks of lignin in the case of plant laccase and lignin degradation by fungal laccase, one of the enzymes forming the ligninolytic complex of wood-degrading fungi.

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