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Kinetics of N-substituted phenothiazines and N-substituted phenoxazines oxidation catalyzed by fungal laccases

Research Article

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Abstract: Laccase-catalyzed oxidation of N-substituted phenothiazines and N-substituted phenoxazines was investigated at pH 5.5 and 25°C. The recombinant laccase from *Polyporus pinsitus* (rPpL) and the laccase from *Myceliophthora thermophila* (rMtL) were used. The dependence of initial reaction rate on substrate concentration was analyzed by applying the laccase action scheme in which the laccase native intermediate (NI) reacts with a substrate forming reduced enzyme. The reduced laccase produces peroxide intermediate (PI) which in turn decays to the NI. The calculated constant (k_{ox}) values of the PI formation are $(6.1\pm3.1)\times10^5$ $M^{-1}s^{-1}$ for rPpL and $(2.5\pm0.9)\times10^4$ $M^{-1}s^{-1}$ for rMtL. The bimolecular constants of the reaction of the native intermediate with electron donor (k_{red}) vary in the interval from 2.2×10^5 to 2.1×10^7 $M^{-1}s^{-1}$ for rPpL and from 1.3×10^2 to 1.8×10^5 $M^{-1}s^{-1}$ for rMtL. The larger reactivity of rPpL in comparison to rMtL is associated with the higher redox potential of type I Cu of rPpL. The variation of k_{red} values for both laccases correlates with the change of the redox potential of substrates. Following outer sphere (Marcus) electron transfer mechanism the calculated activationless electron transfer rate and the apparent reorganization energy are 5.0×10^7 $M^{-1}s^{-1}$ and 0.29 eV, respectively.

Keywords: *Laccase • Kinetics • Phenoxazine • Phenothiazine • Oxygen • Bimolecular rate constant • Redox potential*

1. Introduction

Multicopper oxidoreductase - laccase (EC 1.10.3.2) catalyzes the 4 e^- reduction of O_2 to H_2O by using copper centers of three different types. The electrons are taken up at the blue type 1 (T1) Cu site and transferred ~13 Å to the trinuclear Cu cluster, composed of a normal type 2 (T2) and a coupled-binuclear type 3 (T3) site, where the O_2 reduction occurs [1-3]. The T2 Cu site is held in the protein by two His residues and has a water-derived OH^- ligand external to the cluster, whereas the OH^- bridged T3 Cu site is held by three His at each Cu. The reaction of O_2 with the fully reduced enzyme (E_{red}) produces the peroxide intermediate (PI) of laccase following native intermediate (NI) generation [1-3].

The estimated rate of reduced laccase interaction with oxygen is ~2×10^6 $M^{-1}s^{-1}$ and the peroxide intermediate decomposition is more than 350 s^{-1} [2]. In the absence of reducing substrate, the NI slowly decays to the resting enzyme (RE), in which the one remaining oxygen atom of the O_2 is terminally bound as OH^- to the T2 site [1]. The slow rate of NI decay (~0.034 s^{-1}) conflicts with the much higher turnover number, indicating that the resting enzyme is not involved in the catalytic cycle and that the NI is the only catalytically relevant fully oxidized form of the laccase.

The application of laccase in biotechnology is associated with substrate oxidation using molecular oxygen [4]. Typically the laccases are used in concert with other oxidoreductases and in presence of mediators

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[5,6]. To optimize these complex biocatalytic schemes the kinetics of laccase action should be evaluated.

Marcus theory [7] permits the explanation of features of electron transfer in biological systems. Application of the theory allows prediction of the rate of reactions involving redox proteins. The reactivity of laccases and peroxidases with various substrates was analyzed following the Marcus outer sphere electron transfer mechanism [8,9] and the dependence of reactivities on reactants' redox properties were established.

The goal of our work was to investigate the kinetics of N-substituted phenothiazines (PT) and N-substituted phenoxazines (PX) oxidation in the presence of fungal laccases. This is an extension of our previous investigation of laccase action [8] with special emphasis on determination of kinetic parameters considering detailed schemes of laccase action [1-3,10]. Two different recombinant laccases – with low and high formal redox potential – were used. Review of recent literature dealing with the kinetic mechanism of laccase action [1-3,8-10] allowed us to develop a framework to analyze the experimental data and to calculate the bimolecular constants of laccase reactivity with oxygen and investigated substrates – electron donors. The investigated substrates act as single electron donors possessing moderate redox potential. PTs are involved in laccase catalyzed recalcitrants conversion, dye oxidation and lignin degradation [4]. The oxidized forms of PXs show remarkable stability [11,12] therefore they may find new applications in laccase catalyzed processes.

2. Experimental Procedures

2.1 Enzymes and chemicals

Recombinant laccases from *Polyporus pinsitus* (rPpL) and *Myceliophthora thermophila* (rMtL) were expressed in Aspergillus oryzae and purified as described in [13-15]. These were received from Novozymes A/S (Copenhagen, Denmark). The concentration of rPpL was determined as described in [8], whereas the concentration of rMtL was determined spectrophotometrically by using the extinction coefficient 1.34×10^5 $M^{-1}cm^{-1}$ at 276 nm [15]. *Coprinus cinereus* peroxidase (rCiP) was used as received from Novozymes A/S (Copenhagen, Denmark).

3-(10*H*-phenoxazin-10-yl)-1-propanesufonic acid sodium salt (PPSA), 3-(10*H*-phenoxazin-10-yl)propanoic acid (PPA), 2-(10*H*-phenoxazin-10-yl)ethanol (PET) and 3-(10*H*-phenoxazin-10-yl)propylamine (PPAM) were synthesized as described in [12]. 3-(10*H*-phenothiazin-10-yl)-1-propanesulfonic acid sodium salt (PTPSA), 10-methyl-10*H*-phenothiazine-1-carboxylic acid (MPC1), 10-methyl-10*H*-phenothiazine-2-carboxylic acid (MPC2), 3-(10*H*-phenothiazin-10-yl)propan-1-ol (HPP) and 10-ethyl-10*H*-phenothiazine-4-carboxylic acid (EPC4) were obtained from Novozymes A/S (Copenhagen, Denmark). Promazine hydrochloride (PZ), buffer reagents and other chemicals were purchased from Sigma. Solutions of the investigated substrates were made in triple distilled water (PPSA, PTPSA, and PZ) or methanol (other substrates). The final concentration of methanol in the reaction mixture did not exceed 1% (v/v). Methanol was from Fluka.

2.2 Electrochemical measurements

A formal redox potential of PTPSA was established by cyclic voltammetry (CV). The CV was performed using an electroanalytical system (Cypress Systems, USA) equipped with a glassy carbon electrode (model CS-1087, Cypress Systems, USA). Glassy carbon electrode was freshly polished with aluminum oxide before the measurements and treated ultrasonically in water for 10 min. A saturated calomel electrode (SCE, saturated with KCl, model K-401, Radiometer, Denmark) was used as reference electrode. A Pt wire (diameter 0.2 mm, length 4 cm) was mounted on the end of the reference electrode and served as an auxiliary electrode. The measurements were performed in 50 mM acetate buffer solution, pH 5.5 and room temperature. Potential scan rate (v) varied in the range from 12 to 100 mV/s. The formal redox potential was calculated as the midpoint potential of the reduction and oxidation peaks potentials. The values of the redox potentials of the other investigated compounds were taken from references [8] and [12]. In [8] the redox potential values were measured at pH 7.0. The pH change only slightly influences formal redox potential of PT's and PX's since oxidation is associated with a single electron transfer.

2.3 Kinetic measurements and calculations

The oxidation of the substrates was monitored spectrophotometrically using a computer assisted spectrophotometer (Gilford Instrument 2600) in 50 mM acetate buffer solution, pH 5.5 at 25°C. The kinetic curves were recorded at the wavelength which corresponds to the maximum absorbance of radical cations of N-substituted phenoxazines and N-substituted phenothiazines. The concentration of oxidized substrates was calculated using the extinction coefficients of 1.6×10^4 $M^{-1}cm^{-1}$ at 530 nm for radical cations of phenoxazine derivatives and 8.9×10^3 $M^{-1}cm^{-1}$ at 514 nm for the radical cations of phenothiazine derivatives. The values of extinction coefficients of the radical cations were determined experimentally by titration of

the corresponding substrates with hydrogen peroxide in the presence of rCiP. The concentration of hydrogen peroxide was determined spectrophotometrically by using the extinction coefficient of 39.4 $M^{-1}cm^{-1}$ at 240 nm of wavelength [16].

The initial rate (V_0) of substrates oxidation was calculated by fitting the kinetic curves by exponential or linear functions. In the case of exponential function the initial reaction rate was calculated as a product of k and C_0, where k is the first order reaction constant and C_0 is an initial concentration of substrate. For linear dependence the initial rate was calculated as a slope.

To calculate kinetic parameters a complex scheme of laccase action was considered. The scheme includes the reaction of native intermediate (NI) with a molecule of electron donor (ED). Single reduced enzyme (E_1) reacts consecutively with three substrate molecules producing reduced enzyme E_{red}. The E_{red} produces peroxide intermediate (PI) during reaction with oxygen. The PI in turn decays to the NI [1-3,10]. The scheme of catalytically relevant laccase action can be described:

$$NI + ED \rightarrow E_1 + P \qquad k_1 \qquad (1)$$
$$E_1 + ED \rightarrow E_2 + P \qquad k_2 \qquad (2)$$
$$E_2 + ED \rightarrow E_3 + P \qquad k_3 \qquad (3)$$
$$E_3 + ED \rightarrow E_{red} + P \qquad k_4 \qquad (4)$$
$$E_{red} + O_2 \rightarrow PI \qquad k_{ox} \qquad (5)$$
$$PI \rightarrow NI + 2\,H_2O \qquad k_d \qquad (6)$$

where ED - single electron donor, E_1, E_2, E_3 and E_4 corresponds to laccase reduced with 1, 2, 3 and 4 electrons , respectively.

Following this scheme the expression of k_{red} can be derived:

$$k_{red} = k_1 k_2 k_3 k_4/(k_1 k_2 k_3 + k_1 k_2 k_4 + k_1 k_3 k_4 + k_2 k_3 k_4) \quad (7)$$

If one of the constant of laccase reduction (k_1, k_2, k_3 or k_4) is significantly less than others, k_{red} approaches a value of this constant. Therefore the simplified scheme of laccase action can be rewritten:

$$NI + 4\,ED \rightarrow E_{red} + 4\,P \qquad k_{red} \qquad (8)$$
$$E_{red} + O_2 \rightarrow PI \qquad k_{ox} \qquad (9)$$
$$PI \rightarrow NI + 2\,H_2O \qquad k_d \qquad (10)$$

In this scheme k_{red} corresponds to the slowest rate of T1 center reduction of oxidized and catalytically relevant form of the enzyme.

Following this scheme the initial steady state rate of ED oxidation can be expressed:

$$V_{ED} = 4\,k_d\,k_{red}\,k_{ox}\,[ED]\,[O_2]\,[E]_{tot}/(k_{red}\,k_{ox}\,[ED]\,[O_2] + k_d\,k_{ox}\,[O_2] + k_d\,k_{red}\,[ED]) \quad (11)$$

where $[E]_{tot}$ – total catalytically relevant enzyme concentration.

The reaction rate constants were calculated by using the dependence of V_0 on substrates concentration and derived kinetic equation 11. For data fitting the programs GraFit (Erithacus Software LTD.) and MathCad 2001(MathSoft, Inc.) were used.

3. Results and Discussion

In presence of oxygen the rPpL catalyzed production of radical cations of PT and PX (Figure 1). The oxidation rate saturated at high substrate concentrations.

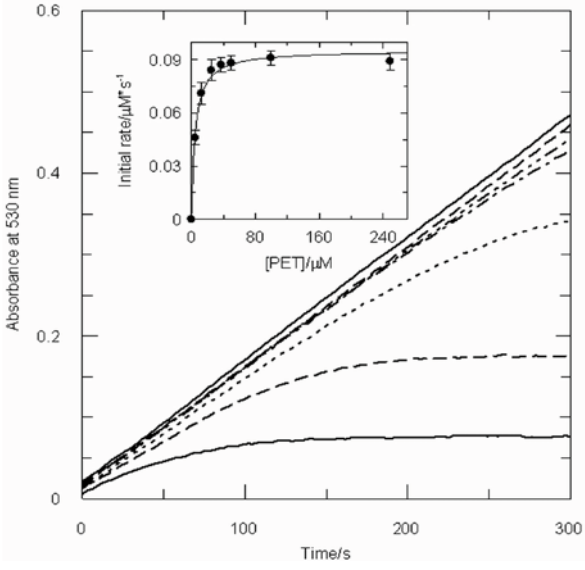

Figure 1. Kinetics of $PET^{+\cdot}$ production at various PET concentrations. The concentration of PET varied in the interval from 5 μM to 250 μM, the concentration of rPpL was 27 nM, 50 mM acetate buffer solution, pH 5.5, and 25°C. The insert shows the dependence of the initial rate on the PET concentration and the curve is an approximation of the data by equation 11.

Thermostable laccase (rMtL) also catalyzed PX and PT oxidation with the formation of radical cations (Figure 2). The dependence of the initial rate on substrate concentrations showed saturation shape like as rPpL, but for PZ and MPC1 almost linear dependence was established (Figure 2).

For the calculations of bimolecular constants k_{red} and k_{ox} the value of k_d equal to 350 s^{-1} was used as for other laccases [3]. The values of k_{ox} and k_{red} almost did not change if k_d during approximation varied between 100 to 1000 s^{-1}. Low sensitivity of k_{red} and k_{ox} to k_d variation indicates that the reaction of PI decay (eq. 10) is not rate limiting process at the substrates concentration used. The calculated values of k_{red} and k_{ox} are presented in Table 1. In the case of MPC1 and PZ the dependences

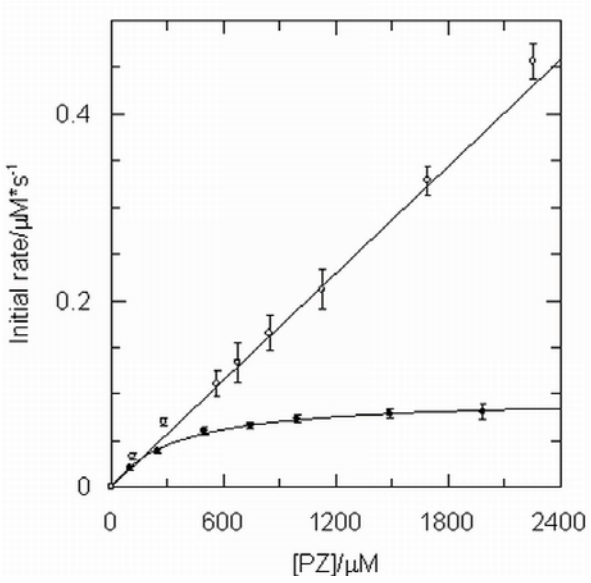

Figure 2. The dependence of the initial rate of PZ oxidation on substrate concentration in the presence of 0.32 nM of rPpL (●) and 376 nM of rMtL (o) at pH 5.5 and 25°C. Curves are the approximations of the data by equation 11.

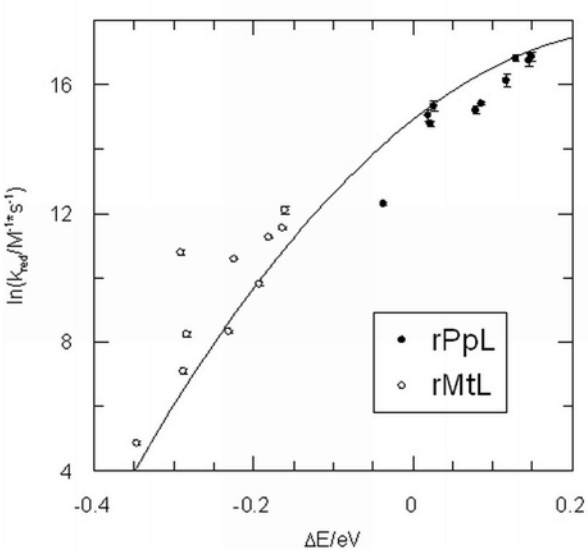

Figure 3. The dependence of k_{red} of PT and PX oxidation on the difference of redox potential of reactants. Curve was drawn by using the equation 12 with the parameters $k_{red}^0 = 5.0\times10^7\ M^{-1}s^{-1}$ and $\lambda = 0.29$ eV [8].

of initial rate on the substrates concentration for rMtL were almost linear, and therefore did not allow the calculation of k_{ox}.

It is possible to notice that k_{ox} varies slightly for both laccases. The mean values of k_{ox} derived for all substrates are $(6.1\pm3.1)\times10^5\ M^{-1}s^{-1}$ for rPpL and $(2.5\pm0.9)\times10^4\ M^{-1}s^{-1}$ for rMtL. The obtained values of k_{ox} for both laccases are lower in comparison to the values of bimolecular constant of PI formation ($\sim2\times10^6\ M^{-1}s^{-1}$) calculated for lacquer tree (*Rhus vernicifera*) laccase [10]. The recombinant laccases and *R. vernicifera* laccase have different amino acid sequences, active site environment and glycosylation. The largest difference of constants, possibly, is caused by differing rate of NI decay. The lower redox potential of rMtL copper type I (0.47 V [14]) in comparison to redox potential of rPpL (0.78 V [14]) is possibly a consequence of faster NI decay with formation of resting enzyme.

The calculated k_{red} values for rPpL were larger than k_{red} for rMtL. The k_{red} varies in the interval from 2.2×10^5 to $2.1\times10^7\ M^{-1}s^{-1}$ and from 1.3×10^2 to $1.8\times10^5\ M^{-1}s^{-1}$ for rPpL and rMtL, respectively. The lower reactivity of rMtL correlates with lower redox potential type I Cu redox

Substrate	E, mV	rPpL		rMtL	
		k_{ox}, $M^{-1}s^{-1}$	k_{red}, $M^{-1}s^{-1}$	k_{ox}, $M^{-1}s^{-1}$	k_{red}, $M^{-1}s^{-1}$
PPSA	631	$(5.2\pm0.1)\times10^5$	$(2.1\pm0.3)\times10^7$	$(2.9\pm0.1)\times10^4$	$(1.8\pm0.2)\times10^5$
PPA	634	$(1.2\pm0.1)\times10^6$	$(1.9\pm0.4)\times10^7$	$(2.8\pm0.1)\times10^4$	$(1.03\pm0.03)\times10^5$
PET	651	$(4.8\pm0.1)\times10^5$	$(2.0\pm0.2)\times10^7$	$(3.1\pm0.1)\times10^4$	$(7.7\pm0.2)\times10^4$
PPAM	694	$(3.4\pm0.1)\times10^5$	$(4.9\pm0.2)\times10^6$	$(2.6\pm0.1)\times10^4$	$(3.90\pm0.02)\times10^4$
EPC4	663	$(2.9\pm0.1)\times10^5$	$(1.0\pm0.2)\times10^7$	$(2.6\pm0.1)\times10^4$	$(1.8\pm0.1)\times10^4$
PTPSA	701	$(4.3\pm0.2)\times10^5$	$(4.0\pm0.5)\times10^6$	$(3.7\pm0.3)\times10^4$	$(4.1\pm0.2)\times10^3$
HPP	754	$(1.0\pm0.2)\times10^6$	$(4.5\pm0.7)\times10^6$	$(1.3\pm1.0)\times10^4$	$(3.8\pm0.3)\times10^3$
MPC1	758	$(4.6\pm0.5)\times10^5$	$(2.6\pm0.2)\times10^6$	-	$(1.2\pm0.1)\times10^3$
MPC2	761	$(1.0\pm0.6)\times10^6$	$(3.4\pm0.6)\times10^6$	$(1.1\pm0.2)\times10^4$	$(4.8\pm0.4)\times10^4$
PZ	817	$(3.8\pm0.1)\times10^5$	$(2.2\pm0.1)\times10^5$	-	$(1.3\pm0.1)\times10^2$

Table 1. Redox potential values and kinetic constants of N-substituted phenothiazines and phenoxazines oxidation with oxygen catalyzed by recombinant laccases at pH 5.5 and 25°C. PTPSA redox potential is determined in this work, redox potentials of N-substituted phenoxazines and of N-substituted phenothiazines are taken from references [8,12].

center. The k_{red} values obtained for rPpL and some substrates (PPA, EPC4, HPP, MPC1, MPC2, and PZ) were similar to the constants depicted in [8].

The k_{red} depends on substrate redox potentials for both laccases (Table 1). As the redox potential increases, the k_{red} decreases. The reactivity of laccases can be modeled by the Marcus outer sphere electron transfer mechanism [7] as it was done for other enzymes [8-9,14]. Following this mechanism the reactivity is related to substrates' redox potentials as described:

$$\ln(k_{red})=\ln(k_{red}^{0})-(\lambda-\Delta E)^2/4\lambda k_B T \quad (12)$$

where k_{red}^{0} is the rate constant of activationless electron transfer, λ is the reorganization energy, which reflects the energy necessary to bring the reactants into the transition state, ΔE is the difference in redox potentials for reactants expressed in eV (1 eV=96.5 kJ mol^{-1}), k_B is Boltzmann's constant, and T is the thermodynamic temperature.

The dependence of k_{red} on ΔE is shown in Figure 3.

It is interesting that the data of both laccases fit the same curve. This indicates that reactivity of laccases (type I Cu reduction) is determined mainly by free energy of reaction. Both dependences follow equation 12 with good approximation using parameters $k_{red}^{0}=5.0\times10^{7}$ $M^{-1}s^{-1}$ and λ=0.29 eV, values calculated for other laccases substrates [8].

4. Concluding Remarks

The aim of this work was to investigate the kinetics of the oxidation of N-substituted phenothiazines and N-substituted phenoxazines catalyzed by recombinant laccases rPpL and rMtL. The analysis of complex scheme of laccase action permitted the calculation of both oxidative (k_{ox}) and reductive (k_{red}) constants of laccases. At pH 5.5 k_{ox} = 6.1×10^{5} $M^{-1}s^{-1}$ and k_{ox} = 2.5×10^{4} $M^{-1}s^{-1}$ for rPpL and rMtL, respectively. The k_{red} varied from 2.2×10^{5} to 2.1×10^{7} $M^{-1}s^{-1}$ for rPpL and from 1.3×10^{2} to 1.8×10^{5} $M^{-1}s^{-1}$ for rMtL. The variation of k_{red} for both laccases correlated with the change of the redox potential of substrates. The calculated activationless electron transfer rate (following the Marcus outer sphere electron transfer mechanism) and the apparent reorganization energy were 5.0×10^{7} $M^{-1}s^{-1}$ and 0.29 eV, respectively.

Acknowledgments

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