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MICROCALORIMETRIC STUDIES OF *RHUS VERNICIFERA LACCASE* CATALYTIC OXIDATION REACTIONS

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Abstract

The oxidation of substrates with various function groups (i.e., *ortho*-diphenol, *ortho*-aminophenol, *p*-aminophenol, *ortho*-phenylene diamine and *p*-phenylene diamine) catalyzed by *Laccase* have been studied using LKB-2107 batch microcalorimetry system. The overall reaction enthalpy $\Delta_r H_m$, Michaelis constant K_m, pseudo-first-order rate constant k_2 of the reactions and binding energy ΔG^0 of *Laccase*-substrate complex for each substrate have been determined at 298.15 K, pH 7.4. The binding enthalpy ΔH^0 and binding entropy ΔS^0 of *LS* complex, when the substrate is Gallic acid and 2,3-dicyan hydroquinone respectively, have been also determined. The rate constants of the reaction between *Laccase* and these substrates are essentially identical because the rate of *Laccase*-catalytic reaction is limited by the step of electron transfer from the type 2 Cu(II)-bound substrate to the less exposed type 1 copper site, and the intra-complex electron transfer rate mainly dependent on the amino residues conformational changes. But the rate of the reaction between *Laccase* and substrates with group –OH is slower than group $-NH_2$, because of the higher energy of bounding electron. The stability of *LS* complex depends on ΔS^0, since $\Delta H^0 > 0$, substrate distinguished by *Laccase* depends on the value of allosteric entropy of the amino acid residues induced by substrate. The relationship between *Laccase* and its substrates cannot be regarded as a simple relationship of lock and key, but an induce-fit relationship.

Keywords: enzymatic reaction, microcalorimetry, *Rhus vernicifera Laccase*-substrate

Introduction

Laccases are copper containing metalloprotein enzymes (EC.1.14.18.1). Two *Laccases*, those from the fungus *Polprous versicolor* and the lacquer tree *Rhus vernicifera,* have been the subject of study in the recent decades [1]. *Rhus vernicifera Laccase* catalyzes the four-electron reduction of oxygen molecule by *ortho*- and *para*-aryl diphenols, aminophenols and diamines [2, 3]. Much of the previous research on the *Laccase* focused

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on defining the structure, especially, on the structure of activity copper site, mechanism of the reaction between *Laccase* and substrate, and on the detail information of electron transfer process has been studied as well [4, 5]. Clemmer *et al.* have ever studied the reduction of *Rhus vernicifera Laccase* type 1 copper by 25 mono- and disubstituted hydroquinones [6]. In our previous study on the reaction between *Laccase* and different substrates [7–9], the Michaelis constants and the reaction rate constants are various to a great extent. For better understanding the effects of function group on the stability of enzyme-substrate complex and the rate of reaction, we have now extended our studies to other kinds of substrates of *Laccase*, i.e., *ortho*-diphenol, *o*- and *p*-aminophenol, *o*- and *p*-phenylene diamine and 2,3-dicyan hydroquinone. The free energy change for *Laccase*-catalyzed reaction was described in the previous research [7, 8]. In this paper we have investigated the change of the enthalpy and entropy for the formation process of *Lacasse*-substrate complex.

Microcalorimetric methods are based on the high sensitive and accurate microcalorimeter. This method stands out as one of the most powerful tools for thermodynamic and kinetic studies of both chemical and biochemical processes because it provides in-situ, on-line, quasi-continuous, non-invasive and accurate measurements of thermodynamic and kinetic data of the reaction without constraint on solvent, spectral electrochemical and other properties of the reaction systems involved. Owing to its advantages, microcalorimetric methods have received increasing attention from researchers [10–21].

This paper reported the determinations of overall molar reaction enthalpy $\Delta_r H_m$, Michaelis constant K_m, electron transfer rate constant k_2 and binding Gibbs energy ΔG_0 of *Laccase* reduction by five kind of non-substituent substrates except hydroquinone. Integrating the data published before [7–9] and this experiment, we have analysed the effect of function group and their position and substituent of substrates on the reaction, especially on the stability of *Laccase*-substrate complex and electron transfer rate. Additionally, it reports the molar binding enthalpy and entropy of *Laccase*–Gallic acid and *Laccase*-2,3-dicyan hydroquinone complex. From the point of view of thermodynamics, this study interprets these effect and proves that *Laccase* is a kind of induced-fit theory enzyme.

Reagents and apparatus

The solid *Laccase*, which was extracted from China lacquer of *Rhus vernicifera*, was obtained from the Institute of Resource Chemistry of Wuhan University and was purified. *Laccase* solution was prepared by dissolving the solid *Laccase* in buffer solution and stored in a refrigerator.

Analytical grade *o*-diphenol (purity>98%) was used without further purification. Chemical grade *o*-, *p*-aminophenol and *o*-, *p*-diamino were purified by vacuum sublimation before used. Required concentration of substrate solution was made by dissolving each of them in the buffer solution. Purified oxygen-free nitrogen was used for prevention non-enzymatic oxidation of substrates and anaerobic condition

by passing it through substrate solution in a reaction cell of calorimeter for 30 min before each experiment.

Doubly distilled water was used throughout the experiment. Analytical grade sodium orthophosphate (dimetallic) and sodium orthophosphate (monometallic) were used for the preparation of the buffer solution, and both their concentrations were 0.2 mol L^{-1}. The pH value of the buffer solution was adjusted to 7.4 by means of pH meter.

The heat of *Laccase* catalytic reaction was determined using an LKB-2107 batch microcalorimeter system. The voltage signal was recorded by means of LKB-2210 dual-pen integrating recorder. For details of the performance and the structure of instrument [9] can be consulted.

Results and discussion

Determination of the overall molar reaction enthalpies

For a single-substrate enzymatic reaction, if the reaction can finish completely, the overall molar reaction enthalpies ($\Delta_r H_m$) can be expressed as the equation shown below:

$$\Delta_r H_m = Q_{tot} / n \tag{1}$$

in which Q_{tot} is the total quantity of heat for n mol substrate that have been oxidized completely. For exothermic reaction the $\Delta_r H_m$ is minus and for endothermic reaction is positive. For the *Laccase*-catalyzed oxidation of *ortho*-diphenol, *o*- and *p*-aminophenol, *o*- and *p*-phenylene diamine, the molar enthalpies were determined at pH=7.4, 298.15 K, under much lower different substrate concentration. The mean value of $\Delta_r H_m$ for each substrate was obtained from five experiments given in Table 1.

Table 1 The biochemical constants and thermodynamic parameters of the reactions between *Laccase* and different substrates at 298.15 K, pH=7.4

Substrate	$-\Delta_r H_m$ /kJ mol^{-1}	$10^2 K_m$/mol L^{-1}	$10^2 k_2/s^{-1}$	$-\Delta G^0$/kJ mol^{-1}
o-diphenol	257.70	5.49	1.25	7.19
o-aminophenol	252.21	0.78	1.71	12.04
p-aminophenol	86.19	2.07	2.75	9.61
o-phenylene diamine	293.56	10.30	0.33	5.63
p-phenylene diamine	163.36	0.56	1.23	12.86

Determination of the Michaelis constants and velocity constants

According to Michaelis–Menten model, the reaction between *Laccase* (L) and substrate (S) at anaerobic condition can be written as:

$$L+S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} LS \xrightarrow{k_2} L+P \tag{2}$$

Michaelis constant K_m is definite as:

$$K_m = \frac{k_{-1}+k_2}{k_1} \tag{3}$$

while k_1 is the formation rate constant of *LS*, k_{-1} is the dissociation rate constant of *LS*, k_2 is the rate constant of product (*P*) production.

Laccase-catalyzed reaction obeys Michaelis mechanism. The Lineweaver–Burk double reciprocal velocity equation can be written as:

$$\frac{1}{v_0} = \frac{1}{v_{max}} + \frac{K_m}{v_{max}} \frac{1}{[S_0]} \tag{4}$$

The Michaelis constant K_m was obtained according to Eq. (4) in terms of a plot of reciprocal initial heat production rate ($1/v_0$) *vs*. reciprocal initial concentration of substrate ($1/[S_0]$). Figure 1 shows the Lineweaver–Burk plot of *Laccase*-catalytic oxidation of *o*-, *p*-aminophenol, and *o*-, *p*-phenylene diamine. From Fig. 1 we can see that all of the curves for each substrate are linear and intersect the abscissa in a point. The point stands for $-1/K_m$. The mean value of K_m for each substrate from three experiments at different initial concentration of *Laccase* was given in Table 1. A good lin-

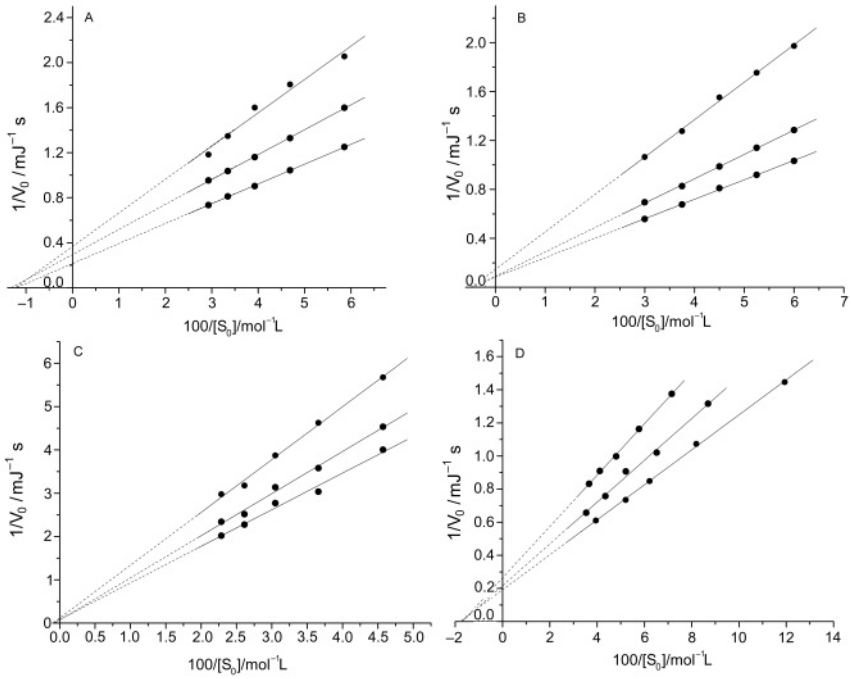

Fig. 1 The Lineweaver–Burk plot of the reaction between *Laccase* and substrates: A – *ortho*-aminophenol; B – *para*-aminophenol; C – *ortho*-phenylene diamine; D – *para*-phenylene diamine

ear relationship of $(1/v_0)$ *vs*. $(1/[S_0])$, in which the relationship coefficient is larger than 0.99 for each substrate, represents that the reaction between *Laccase* and each substrate satisfied the Michaelis–Menten model.

From the steady state kinetic of a single-substrate, single-intermediate enzymatic reaction, the maximum velocity v_{max} is expressed as:

$$v_{max} = -k_2 \Delta_r H_m V[E_0] \tag{5}$$

In Eq. (5) v_{max} is the exothermic velocity of the reaction, V is the volume of the reaction solution, k_2 is the velocity constant and $[E_0]$ is the initial concentration of *Laccase*.

The following equation is obtained:

$$k_2 = -\frac{v_{max}}{\Delta_r H_m V[E_0]} \tag{6}$$

The pseudo-first-order rate constant k_2 of the reactions at 298.15 K were calculated according to Eq. (6). The results were listed in Table 1. The binding free energies for *Laccase* complex with each substrate were listed also in Table 1.

It can be seen from Table 1 that the rate constants of the reaction between *Laccase* and these substrates are essentially identical. This would not be surprising since the rate of *Laccase*-catalytic reaction was limited by the step of electron transfer from the type 2 Cu(II)-bound substrate to the less exposed type 1 copper site. This intra-complex electron transfer rate mainly depends on the amino residues conformational changes as suggested by Holwerda and Gray [22]. But the function group and their position affect the rate somewhat. Generally, the rate of the reaction between *Laccase* and substrates with group –OH is slower than group $–NH_2$, because of the stranger bounding of electron. The rate of substrates with group at *para*- position are faster than *ortho*-position because of this structure is capable of formation of bridge bond between substrates and two type copper site (type 2 and type 1). K_m of these substrates is slightly different from each other. Because, as we discuss in set 3.3, the stability of *Laccase*-substrate complex depends mainly on the entropy changes of complex formation, but the energy factors contributed to the interactions between the group of substrates and the amino residues or copper site of *Laccase* effects the stability of *LS* complex. These interactions conclude ligand bond between Lewis basicity of substrate and copper site, hydrogen bond, hydrophobic interaction and other interaction between the group of substrate and amino residues of *Laccase*.

Enthalpy and entropy of Laccase-substrate complex

Generally, $k_{-1} >> k_2$, the formation constant (K_s) of *LS* can be derived as

$$K_s = \frac{[LS]}{[L][S]} = \frac{k_1}{k_{-1}} = \frac{1}{K_m} \tag{7}$$

According to the fundamental thermodynamic relation below:

$$\Delta G^0 = -RT\ln K_s \tag{8}$$

and

$$\frac{\mathrm{d}(\Delta G^0/T)}{\mathrm{d}T} = -\frac{\Delta H^0}{T^2} \tag{9}$$

The following relation can be obtained:

$$\frac{\mathrm{d}\ln K_s}{\mathrm{d}T} = \frac{\Delta H^0}{RT^2} \tag{10}$$

Integrated form of the equation is

$$\ln K_s = -\frac{\Delta H^0}{RT} + I \tag{11}$$

then

$$\ln K_m = \frac{\Delta H^0}{RT} + I' \tag{12}$$

where ΔG^0, ΔH^0 is binding free energy and binding enthalpy respectively, I and I' are integrate constant.

In a small temperature internal (in this experiment, ΔT<15 K), ΔH^0 can be considered as a constant. The linear relation in plot $\ln K_m$ *vs.* $1/T$ can be obtained. The value ΔH^0 can be calculated by R·slop. So the binding entropy ΔS^0 can be calculated according to Eq. (13):

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T} \tag{13}$$

The Michaelis constants of the reaction between *Laccase* and Gallic acid at different temperature have been reported [7]. In this experiment we have determined the K_m of oxidation of 2, 3-dicyan hydroquinone by *Laccase* at different temperature, the results were shown in Table 2. Figure 2 is the plot of K_m *vs.* $1/T$ of those two substrates. The calculating value of ΔH^0 and ΔS^0 are listed in Table 3.

Table 2 Michaelis constant of reaction between *Laccase* and 2,3-dicyan hydroquinone at different temperature, while pH=6.0

T/K	303.15	308.15	310.65	313.15
$K_m \cdot 10^4$/mol L^{-1}	8.80	5.54	3.72	2.58

Table 3 Binding enthalpy and binding entropy of *Laccase*-substrate complexes

Substrate	ΔH^0/ kJ mol^{-1}	ΔS^0 /J mol^{-1} K^{-1}
Gallic acid*	50.56	210.47
2,3-dicyan hydroquinone	92.71	364.09

*The original data from [7]

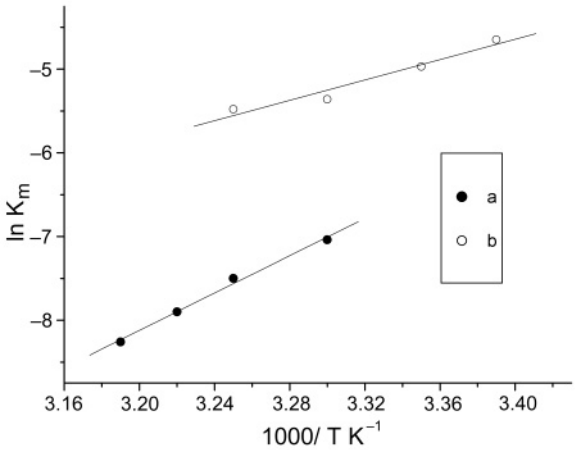

Fig. 2 The plot of $\ln K_m$ *vs.* $1/T$ for *Laccase* catalyzed reaction, the substrate a – 2,3-dicyan hydroquinone, $r = 0.9964$; b – Gallic acid, $r = 0.9754$

Since the binding enthalpy of *LS* complex $\Delta H^0 > 0$, the minus value ΔG^0, depends on $-T\Delta S^0$. This means that the stability of complex *LS* depends on ΔS^0. Considering ΔS^0 is attributed to two parts: one is the decrease of total movement entropy of substrate in solution, which decreases the total entropy of reaction system; the other is the determining factor to increase entropy of system which is attributed to the allosteric effect of the amino acid residues around the activity copper site of *Laccase*. This allosteric reaction of residues is induced by substrate. Since the formation of LS complex is an essential step of *Laccase* catalytic reaction, whether a compound is a substrate of *Laccase* dependents on this compound's capability of inducing the allosteric reaction of residues and combining with the copper site of *Laccase*. In other words, recognizing the substrate by *Laccase* depends on the value of allosteric entropy of the amino acid residues induced by substrate.

Conclusions

Rhus vernicifera Laccase is one of less selectivity enzymes, which can catalyze the oxidation of many substrates. The *Laccase* catalyzed reaction accords in with Michaelis–Menten reaction model. The formation of *LS* complex is an essential step in the reaction process and is driven mainly by entropy which is attributed by the conformation changes of the amino acid residues around the activity copper site of *Laccase*. The allosteric effect of protein in *Laccase* is induced by substrate anion in reaction system. So, the relationship between *Laccase* and its substrates cannot be regarded as a simple relationship of lock and key, but an induce-fit relationship.

The rate of *Laccase*-catalytic reaction is limited by intra-complex electron transfer rate, which is protein-dependent but may be influenced by the function group and their position. The stability of *LS* complex depends on the value of entropy changes of the allosteric effect of protein and the intensity or form of the interaction between

substrate and the copper site or residues of *Laccase*. The steadier the *LS* complex, the slower the rate of *Laccase*-catalytic reaction can be detected.

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