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Studies on single-substrate, enzyme-catalyzed reactions and analysis of transition state by microcalorimetry

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Abstract

The reactions between Laccase and various substrates (3,4-Dihydroxybenzaldehyde, Guaiacol, Pyrogallol, Gallic acid) have been studied using LKB-2107 batch microcalorimetry system. Thermodynamic parameters ($\Delta_r H_m$, ΔG_0, $\Delta G_T^{\neq}$, E_a, $\Delta S_T^{\neq}$) and kinetic parameters (K_m, k_2) have been calculated. The reactions process have been analyzed from free energy changes by using the transition state theory which showed that formation of an enzyme-substrate complex is 'anticatalytic.' The experimental results also indicated that stabilization of enzyme-substrate complex slows down the reaction, whereas stabilization of transition state accelerates it. Two methods are proposed to enhance catalytic power of Laccase. The decrease of activation entropy ($\Delta S_T^{\neq} < 0$) indicated that enzyme-substrate/transition structure is more tightly bound than enzyme-substrate complex structure. © 1997 Elsevier Science B.V.

Keywords: Laccase; Microcalorimetry; Substrate; Thermokinetics; Transition state

1. Introduction

Microcalorimetry is a powerful means of thermochemistry study. Recently it has been widely used in biochemistry, kinetics etc., because thermokinetic research of reaction systems may be undertaken in any solvent, and the spectral, electrochemical or other properties of the substances involved in the reacting system need not be considered. It is a technique that holds much promise for use in the studies of enzyme-catalyzed reactions due to its high sensitivity and accuracy. Since the absorption or production of heat is an intrinsic property of virtually all enzyme-catalyzed reactions, it should be possible to obtain both kinetic and thermodynamic information for this kind of reaction by using this technique [1–4].

Laccases are copper-containing enzymes which catalyze the oxidation of ortho- and para-aryl diamincs and diphenols by oxygen, producing the corresponding quinones and water [5]. Two Laccases, those from the fungus Polprous versicolor and the lacquer tree Rhus vernicifera, have been the subject of much recent study. Both proteins possess four tightly bound copper atoms distributed in the three distinct sites which may be distinguished by light and ESR spectroscopy. In the recent decades, much of the research on the Laccase has focused on defining the structures and reactivity of the dioxygen reduction site [6–8]. Both the thermokinetic properties and analysis of the process using the transition state theory of the reaction

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between Laccase and substrates, however, have seldom been reported. In this paper, studies on thermodynamic parameters ($\Delta_r H_m$, ΔG_0, $\Delta G_T^{\neq}$, E_a, $\Delta S_T^{\neq}$) and kinetic parameters (K_m, k_2) of the Laccase-catalyzed oxidation of various substrates (3,4-Dihydroxybenzaldehyde, Guaiacol, Pyrogallol, Gallic acid). From the analysis of the reactions between each substrate and Laccase it has been found that the positions of the functional group and different substituents in the substrates are connected with the values of thermokinetic parameters.

2. Experimental

2.1. Reagents

Doubly distilled water was used throughout the experiment. Analytical grade sodium orthophosphate (dimetallic) and sodium orthophosphate (monometallic) were needed for the preparation of the buffer solution. The pH of the buffer solution was adjusted 7.4 by means of pH meter, mixing slowly two solutions with concentrations both 0.2 mol l^{-1}.

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 the buffer solution and stored in a refrigerator for use.

Required substrate solutions were made by dissolution of analytical grade Gallic acid and Chemical purity 3,4-Dihydroxybenzaldehyde, Guaiacol, Pyrogallol, (their purity > 98%) in the buffer. In order to prevent oxidation of substrates by dissolved oxygen, purified oxygen-free nitrogen was passed through substrates solution which was gotten before experiment.

All solutions were freshly prepared before each experiment.

2.2. Instrumentation

The heat of reactions of Laccase and substrates were determined using a 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 structure of the instrument, see Ref. [9].

3. Results and discussion

3.1. The results

3.1.1. Determination of the overall molar reaction enthalpies

The overall molar reaction enthalpies for the Laccase-catalyzed oxidation of various substrates (3,4-Dihydroxybenzaldehyde, Guaiacol, Pyrogallol, Gallic acid) were determined at pH = 7.4, 298.15 K. The results under the different substrate concentrations and anaerobic conditions are given in Table 1. The uncertainty is 'uncertainty intervals' defined as the final overall standard deviation of the mean. It can be seen from Table 1 that the overall molar reaction enthalpies of the Laccase-catalyzed oxidation of 3,4-Dihydroxybenzaldehyde, Pyrogallol and Gallic acid are slightly different, but these overall molar reaction enthalpy are larger than that of Guaiacol.

3.1.2. Determination of the Michaelis constant

For a single-substrate enzyme-catalyzed reaction, we have [9]

$$1/\Omega_0 = (K_m/\Omega_{max})(1/[S_0]) + 1/\Omega_{max} \quad (1)$$

Table 1
The thermokinetic parameters ($\Delta_r H_m$, K_m, k_2, ΔG_0, $\Delta G_T^{\neq}$) of the reactions between Laccase and different substrate

Substrates	3,4-Dihydroxybenzaldehyde	Guaiacol	Pyrogallol	Gallic acid
$-\Delta_r H_m$/kJ mol^{-1} [a]	321.99 ± 3.40	47.71 ± 0.54	349.70 ± 3.61	330.69 ± 3.07
$K_m \times 10^4$/mol l^{-1} [b]	148.3 ± 0.7	966.6 ± 3.8	7.266 ± 0.160	69.20 ± 1.36
k_2/s^{-1} [b]	4.657 ± 0.175	4.439 ± 0.065	7.909 ± 0.186	2.950 ± 0.031
$-\Delta G_0$/kJ mol^{-1}	10.44	5.791	17.91	12.33
$\Delta G_T^{\neq}$/kJ mol^{-1}	69.21	69.33	67.90	70.34

[a] The mean value of five experiments. [b] The mean value of the data of three experiments.

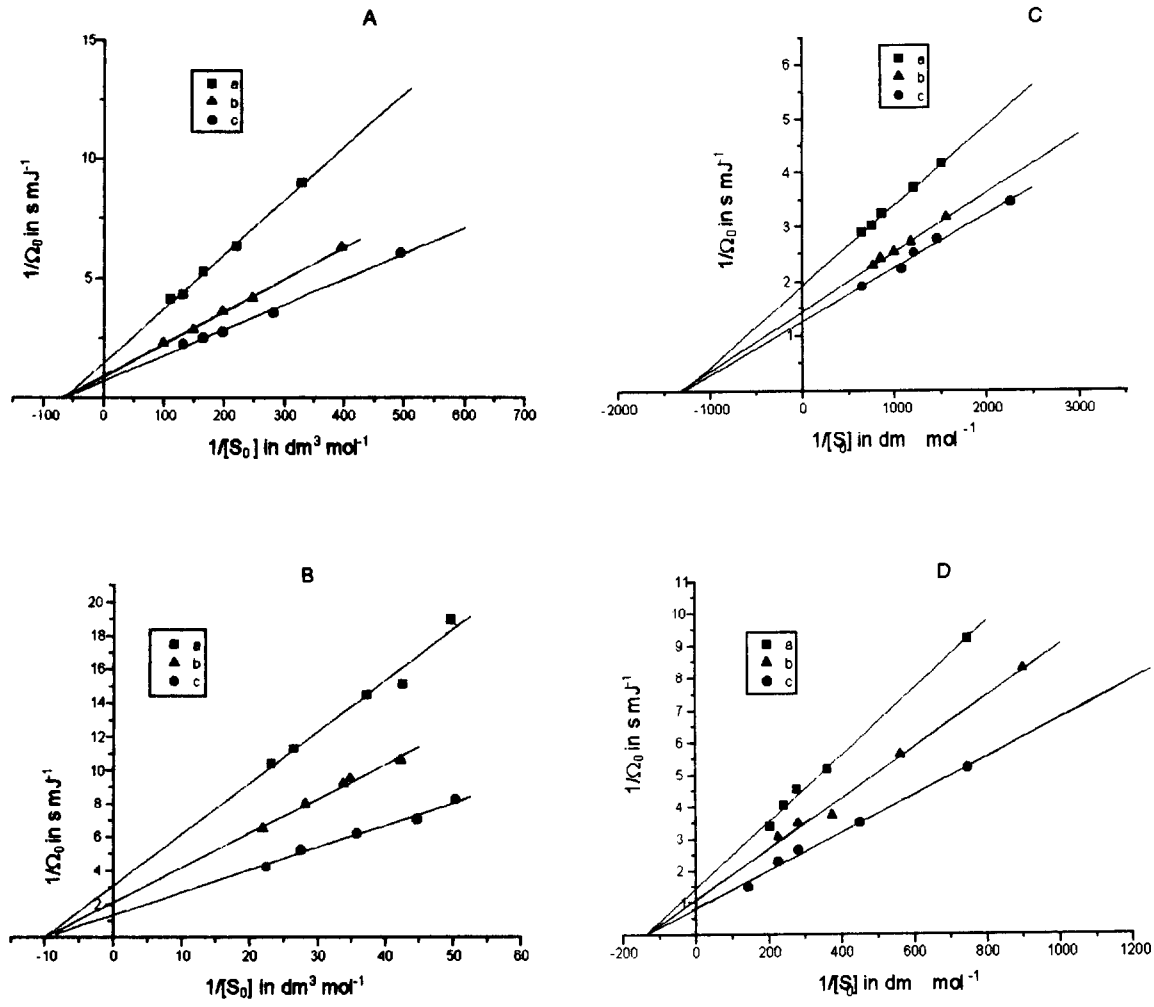

Fig. 1. Lineweaver–Burk plot of $1/\Omega_0$ vs. $1/[S_0]$ for various substrates at different Laccase concentrations. A: 3,4-Dihydroxybenzaldehyde; B: Guaiacol; C: Pyrogallol; D: Gallic acid.

This is a linear equation called the Lineweaver–Burk equation in which Ω_0 is the initial exothermic rate of the reaction (represented by mJ s^{-1} in the enthalpic determination) and $[S_0]$ is the substrate initial concentration. The maximum exothermic rate, Ω_{max}, and the Michaelis constant K_m can be calculated from the intercept of $1/\Omega_{max}$ and the slope K_m/Ω_{max}, respectively. Fig. 1 shows a plots of $1/\Omega_0$ vs. $1/[S_0]$ at different enzyme concentrations. It can be seen from Fig. 1 that the curves are linear and intersect the abscissa in a point. The point stands for $-1/K_m$. The slope is proportional to the reciprocal of the enzyme concentration. The results are shown in Table 1 and revealed that the sequence of the values of K_m is Guaiacol > 3,4-Dihydroxybenzaldehyde > Pyrogallol > Gallic acid. While the K_m values are often used to stand for the relative affinities of series of substrates for a particular enzyme.

3.1.3. Determination of the rate constant

Let us investigate the steady-state kinetic of a simple single-substrate, single-intermediate enzyme-catalyzed reaction.

$$v_{max} = k_2[E_0] \quad (2)$$

where v_{max} is the maximum reaction rate (represented

by mol l^{-1} s^{-1} in the equation), k_2 is the rate constant and $[E_0]$ is the initial concentration of Laccase.

According to the theory of thermokinetics, the following equation is obtained:

$$\Omega_{max} = -\Delta_r H_m V v_{max} \quad (3)$$

Combining Eq. (2) with Eq. (3), we obtain

$$k_2 = -\Omega_{max}/(\Delta_r H_m V[E_0]) \quad (4)$$

The values of k_2 were calculated from Eq. (4) and listed in Table 1. A molecular weight of Laccase is 120 000 [10]. The volume of solution (V) is 6.00 ml.

Slight differences of the rate constants (in Table 1) showed that the position of the functional group and different substituents in the substrates have little influence on the rate constants.

3.1.4. Determination of the binding energies and the activation Gibbs free energies

The binding energies and the activation Gibbs free energies were calculated in accordance with the following Eqs. (5) and (6) [11].

$$\Delta G_0 = -RT \ln(1/K_m) \quad (5)$$

$$\Delta G_T^{\neq} = -RT \ln(k_2 h/k_b T) \quad (6)$$

Where k_b is Boltzmann constant, h Plank constant, k_2 the rate constant and K_m is the Michaelis constant. The values (ΔG_0 and $\Delta G_T^{\neq}$) of the Laccase-catalyzed oxidation of the substrates (3,4-Dihydroxybenzaldehyde, Guaiacol, Pyrogallol, Gallic acid) are listed in Table 1. The results revealed that the higher the value of $\Delta G_T^{\neq}$, the slower the reaction rate at a constant temperature.

3.1.5. The effect of temperature on Laccase-catalyzed reactions

According to above-mentioned methods, at different temperature and pH = 7.4, the Michaelis constant, the rate constant, the binding energies and the activation Gibbs free energies of the Laccase-catalyzed oxidation of Gallic acid were determined and presented in Table 2.

Changes in temperature bring about modifications in the catalytic oxidation behavior of Laccase. The rate constants increase with the increase in temperature, but the Michaelis constants decrease with the increase in temperature. The activation Gibbs free energies ($\Delta G_T^{\neq}$) are independent of changes in temperature.

3.1.6. Determination of the activation energy and the activation entropies

The simple single-substrate, single-intermediate enzyme-catalyzed reaction can be written as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \quad (7)$$

During enzyme-catalyzed reactions, enzyme-substrate complex (ES) decomposes to form the products (P). This step is the slowest in the whole process, and so it controls the rate of enzyme-catalyzed reaction.

According to transition state theory, the following Eqs. (8) and (9) are written as:

$$k_2 = (RT/Nh)\exp(\Delta S_T^{\neq}/R)\exp(-E_a/RT) \quad (8)$$

$$\Delta G_T^{\neq} = \Delta H^{\neq} - T\Delta S_T^{\neq} = E_a - T\Delta S_T^{\neq} \quad (9)$$

If values of k_2 and E_a have been determined, the activation entropy $\Delta S_T^{\neq}$ can be calculated. According to Arrhenius equation, we usually plot $\ln k_2$ vs. $1/T$ to obtain E_a [Eq. (10)].

$$\ln k_2 = -E_a/RT + A \quad (10)$$

E_a value was calculated using data of k_2 and T in Table 2. (Activation energy E_a = 59.83 kJ mol^{-1}, correlation coefficient r = 0.9946.)

Table 2
The thermokinetic parameters (K_m, k_2 ΔG_0, ΔG_T, $\Delta S_T^{\neq}$) of the reaction between Laccase and Gallic acid

T/K	$K_m \times 10^3$/mol l^{-1} [a]	k_2/s^{-1} [a]	$-\Delta G_0$/kJ mol^{-1}	$\Delta G_T^{\neq}$/kJ mol^{-1}	$\Delta S_T^{\neq}$/J K^{-1} mol^{-1}
295.15	9.531 ± 0.021	2.053 ± 0.024	−11.42	70.50	−36.15
298.15	6.920 ± 0.136	2.950 ± 0.031	−12.33	70.34	−35.25
303.15	4.720 ± 0.066	3.915 ± 0.068	−13.50	70.84	−36.22
308.15	4.169 ± 0.199	6.033 ± 0.080	−14.04	70.95	−36.09

[a] The mean value of the data of three experiments.

At different temperature, k_2 and E_a values determined in experiment have been substituted in Eqs. (8) and (9) to give activation entropy ($\Delta S_T^{\neq}$) and activation free energy ($\Delta G_T^{\neq}$) values. These data are shown in Table 2. The entropy ($\Delta S_T^{\neq}$) values of these experiments are all below zero. According to the thermodynamic principle a large decrease of entropy in a reaction means that products are more order than reactants. Thus enzyme-substrate complex and enzyme-substrate/transition-structure complex being a process of entropy decrease is a change in which the system goes from less orderly to more orderly. The results indicated that enzyme-substrate/transition structure is more tightly bound than enzyme-substrate complex.

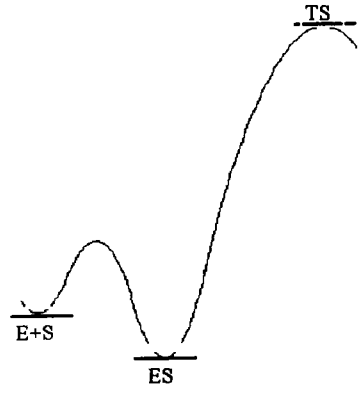

Fig. 2. Free energy diagram for enzyme-catalyzed reactions in which E = enzyme, S = substrate, ES = enzyme /substrate complex, and TS = transition state.

3.2. Discussion

Even if a true enzyme-substrate complex (with a typical lifetime in the neighborhood of 1 ms) could be subjected to examination of X-ray diffraction, it would be likely to differ considerably in structure from the transition state for the reaction (with a typical half-life of 10^{-10} ms) [12]. Rapid reaction techniques, which detect dynamic changes during catalysis, generally convey little detailed structural information about the protein. However, it is important for enzyme mechanisms to analyze enzyme-substrate/transition-structure. The transition state structures of enzyme-catalyzed reactions provide fundamental information about the interactions used to catalyzed biological reactions and also provide a blueprint for the design of transition state inhibitors. In this paper, the structure of transition state is investigated from changes in energy. The binding energies of the Laccase-catalyzed oxidation of various substrates (3,4-Dihydroxybenzaldehyde, Guaiacol, Pyrogallol, Gallic acid) are all below zero, these indicate that the Gibbs free energies decrease in the process of Laccase binding substrates in the condition described above (pH = 7.4, T = 298.15 K). The Gibbs free energy of the Laccase-substrate complex increases in the process of forming an activated complex. Changes in the free energy can be seen in Fig. 2 [13].

Inspection of Fig. 2 leads to conclusions. The forward rate of an enzymatic reaction reflects the free energy difference between the highest and lowest points. According to Fig. 2 in which ES is always below ($E + S$), a lowering of ES slows down the rate by increasing the TS/ES differential. In other words, formation of an enzyme/substrate complex is 'anticatalytic.' The tighter the complex, the lower the ES free energy level, the more the enzymatic reaction is inhibited. Stabilization of ES slows down the reaction, whereas stabilization of TS accelerates it. If both ES and TS are lowered by the same amount, then the rate remains unchanged. Clearly, the benefit of stabilizing TS is canceled by an equivalent stabilization of ES. Of course, an enzymatic rate can be accelerated, despite an ES lowering, as long as the TS is stabilized to an even greater extent. Thus, two methods are proposed to enhance catalytic power of Laccase, one is the enhancement of the binding energy (ΔG_0), the other is the enhancement of the stabilization of the enzyme/transition-structure.

The free energy of enzyme-substrate complex and enzyme-substrate/transition structure are related to solvents. Thus, appropriate solvents are chosen in order to enhance activity of enzymes. Yurii L. Khmelnitsk et al. [14] reported that salts dramatically enhance the activity of enzymes suspended in organic solvents. At present, scientists had laid stress on Laccase-catalyzed reactions in non-water media [15–17].

Acknowledgements

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