ON THE MECHANISM OF THE REACTION OF THE REDUCED LACCASE WITH OXYGEN

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SUMMARY

1. The kinetics of the reaction of reduced laccase and oxygen have been studied by spectrophotometry and magnetometry, and the results showed that a Cu^{+}–O_2 complex does not exist in the reaction.

2. The second order velocity constant of the above reaction was found to be of comparable value to that of the peroxidase–H_2O_2 reaction. The fact that the velocity constant does not change at different oxygen concentrations supports statement 1 above.

3. The velocity constant is not affected by pH change as long as the enzyme does not denature. For the laccase–O_2 reaction, no copper-linked acid group was found in the laccase molecule.

INTRODUCTION

The following reaction mechanism has been proposed for laccase[1]:

$$2\ Cu^{+} + \tfrac{1}{2}\ O_2 + 2\ H^{+} \longrightarrow 2\ Cu^{++} + H_2O \qquad (1)$$

$$2\ Cu^{++} + \text{hydroquinone} \longrightarrow 2\ Cu^{+} + p\text{-quinone} + 2\ H^{+} \qquad (2)$$

Two points are clear experimentally concerning reaction (1): first, the oxidation from cuprous to cupric is spectrophotometrically[2] and magnetometrically[3,4] operative; second, the stoichiometry of the reaction is as shown in eqn. (1)[1]. These results agree with each other in the economy of the transferred electron, under the condition that, in the reaction, molecular oxygen is reduced to water, not hydrogen peroxide. However, these static methods leave one question unsolved: is a transient enzyme–substrate complex, Cu^{+}–O_2 formed in this reaction before the electron is transferred from copper to oxygen?

Kinetic observations by magnetometric and spectrophotometric approaches under the same conditions may be able to provide a solution to the problem, and some evidence obtained by these methods is presented in this paper.

MATERIALS AND METHODS

Laccase: Laccase was purified from the latex of the Chinese lacquer tree (*Rhus vernicifera*), obtained from Ken Shii, China. The procedures of extraction and purification of the enzyme were essentially the same as those described previously[2].

Ascorbate: Ascorbic acid, purchased from Eastman Organic Chemicals, was freshly neutralized and used for the experiments.

Measurement of oxygen uptake: The platinum microelectrode which was developed for the polarographic estimation of dissolved oxygen[5,6], improved by CHANCE for further biochemical applications[7], was used to record the uptake of oxygen in the laccase reaction.

Spectrophotometric measurements: A double beam spectrophotometer designed by CHANCE[8] provided with a flow apparatus of 10 mm optical path was used to record the change of O.D. of the reaction mixtures. One of the two syringes of the flow apparatus was filled with a laccase solution containing ascorbate, and the other with an oxygen solution of known concentration, obtained by bubbling buffer solution with a gas mixture. The syringe plungers were pushed down and the change of the O.D. of the mixed solution at 615 mμ during flow (accelerated flow method) and after the flow was stopped (stopped flow method) was recorded. The time during flow was calculated from the flow velocity trace. The details of this procedure are given by CHANCE[9]. 500 mμ was used as the reference wavelength.

Magnetometric measurements: BRILL *et al.*[10] developed a magnetic susceptometer of the Rankine balance type for sensitive volume paramagnetic susceptibility measurements. In the flow apparatus[11], combined with the susceptometer, the solution in the main pump-1, after flowing through the half cell-1 and reaching the mixing chamber, is mixed with a solution from the secondary pump; the mixture then flows through the half cell-2. The flow rate is maintained at a constant value during each flow. According to the change of the magnetic susceptibility of the solution caused by mixing with the secondary solution, the magnet which is hung between the two half cells moves toward the cell of the larger paramagnetism, the movement being detected photoelectrically, and the signal obtained being amplified and recorded on chart paper. The signal strength in the record is proportional to the change of magnetic susceptibility of the solution. Because the mixing ratio is as small as 1:82.6, the slight change in the concentration of the components in the main solution can be ignored. Usually, flow noise was observed when the flow was started. After a few seconds, when the noise had ceased, the secondary pump was pushed and the solution in it mixed into the main solution, and the change of magnetic susceptibility recorded. The sensitivity of the susceptometer was calibrated with a $NiCl_2$ solution as standard.

Measurement of laccase concentration: The concentration of laccase was determined by the molecular extinction coefficient of laccase reported previously[2], using a Beckman DU spectrophotometer.

Experimental conditions: All the experiments were performed in $M/30$ phosphate buffer of pH 7.0 at 25° unless notified. The pH was checked with a glass electrode pH meter made by Analytical Measurements Inc.

RESULTS

Kinetic measurements of laccase reaction by magnetometry and spectrophotometry

Magnetometry: An anaerobic solution of the reduced laccase with an excess amount of ascorbate was placed in the main pump of the flow apparatus of the susceptometer, and a saturated solution of oxygen at one atmospheric pressure was placed in the secondary pump. The laccase concentration was adjusted so that the

amount of the oxygen mixed in is in stoichiometric proportion to laccase. A typical record from this experiment is shown in Fig. 1. By changing the speed of flow and the concentration of ascorbate, the kinetic curves of the magnetic susceptibility

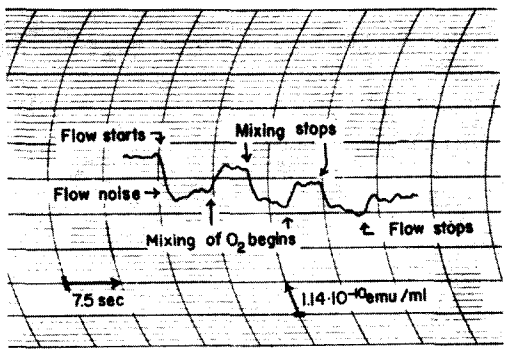

Fig. 1. A magnetometric record of the laccase reaction obtained by the susceptometer at 22°. Initial concentrations of laccase, oxygen and ascorbate are 60 μM (on the basis of copper concentration), 15 μM and 9.8 mM respectively.

change of laccase solution can be obtained (see Fig. 2). These curves show a rapid oxidation of copper by molecular oxygen followed by slow reduction by ascorbate. As will be shown in a later section of this paper, the second order velocity constant of the reaction of reduced laccase and oxygen (k_1) is $2.8 \cdot 10^6\ M^{-1}\ \text{sec}^{-1}$.

$$\text{Reduced laccase} + O_2 \xrightarrow{k_1} \text{oxidized laccase} + O_2^{----} \qquad (3)$$

so the amount of oxygen, remaining unreacted in the reaction mixture after a given period, can be calculated. As molecular oxygen itself has a paramagnetic susceptibility of $3390 \cdot 10^{-6}$ emu/mole at 20° (see ref. 12), the susceptibility of the remaining oxygen must be subtracted from the overall increase of the paramagnetic susceptibility. The plots in Fig. 2 show the corrected values. The curve I in Fig. 2 shows that the maximum change in magnetic susceptibility of the laccase copper corresponds to the cuprous to cupric change. This result is coincident with the previous experiment with a Gouy magnetic balance[3]. In these curves, no evidence appeared to indicate that a transient Cu^+–O_2 complex exists.

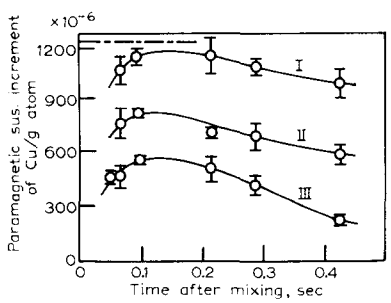

Fig. 2. Kinetics of the laccase reaction by magnetometry at pH 7.0. ——·——, represents the theoretical value of the magnetic susceptibility of cupric copper at 22°. For details, see Table I. Points are shown with root-mean-square error.

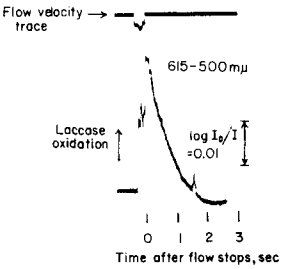

Fig 3. A stopped flow record of the laccase reaction by spectrophotometry. For details, see Table I.

Spectrophotometry: The spectrophotometric record of the reaction under the same experimental condition as that of curve III of Fig. 2 is shown in Fig. 3. This record is a stopped flow experiment. The observed maximum value of cupric copper ($[Cu^{++}]_{max}$) and $t_{\frac{1}{2}off}$* of this curve are in good agreement with those obtained from magnetic measurement (see Table I). It means that the blue color is necessarily accompanied by the cupric state of the copper atom.

TABLE I

SUMMARY OF THE EXPERIMENTS IN FIGS. 2 AND 3

Experiment	*[Laccase]* μM*	*$[O_2]$** μM*	*Temperature*	*[Ascorbate] mM*	*$[Cu^{++}]_{max}$* μM*	*$t_{\frac{1}{2}off}$ (sec)*
Magnetometric I***	60	16	21	1.3	57	—
Magnetometric II	60	15	22	9.8	40	—
Magnetometric III	60	15	22.5	20.0	28	0.35
Spectrophotometric	60	15	25	20.0	27	0.4

* On the basis of coppper concentration.
** Initial concentration.
*** Magnetometric I, II and III correspond to the curves I, II and III in Fig. 2.

Velocity constant and mechanism of the reaction of the reduced laccase and oxygen

Preliminary experiment: By using the platinum microelectrode and the double beam spectrophotometer, a simultaneous measurement of the change of oxygen concentration and O.D. at 615 mμ upon mixing ascorbate into the air-saturated laccase solution was undertaken (Fig. 4). In this experiment, ascorbate was added to

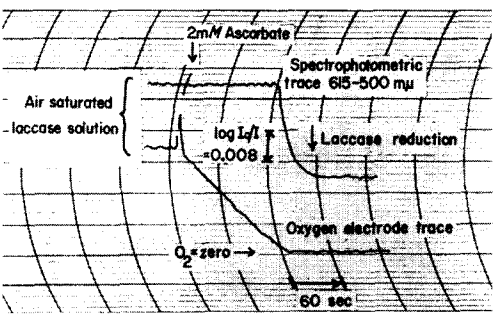

Fig. 4. Oxygen uptake and the spectrophotometric change of the laccase solution. The concentration of laccase is 6 μM.

the laccase solution in the optical cuvette by a small glass rod. As seen in the figure, the oxygen concentration decreases linearly with time, and only after the oxygen is almost exhausted does the absorption at 615 mμ begin to disappear. No detectable change of the steady state level of the oxidized enzyme was observed upon mixing with ascorbate. This means that the reaction velocity of the reduced laccase with oxygen is fast enough to keep the oxidized level of the enzyme constant even with a very low level of oxygen.

* The time interval from half-maximal formation of cupric copper until its concentration has fallen to half its maximal value.

k_1 measurement by spectrophotometry: The accelerated flow method was employed for measurement of the k_1 of eqn. (3) by mixing an anaerobic solution of the reduced laccase with an oxygen solution in the flow apparatus. A typical record is shown in Fig. 5. Dilute ascorbate was used to reduce the enzyme so that complete oxidation of laccase copper was obtained when the flow stopped. In order to check the order of the reaction, the values of

$$\frac{[\text{Oxidized Laccase}]}{[\text{Total Laccase}]\,([\text{Total Laccase}] - [\text{Oxidized Laccase}])} (= k_1 \cdot t)$$

for the reaction of reduced laccase with a stoichiometric amount of oxygen were plotted against time (Fig. 6). In this formula [Oxidized Laccase] and [Total Laccase] are the amount of oxidized and total laccase at time t; t is the time during flow, *i.e.* time after mixing, and k_1 is based on the molecular oxygen and laccase (not copper) concentration. As can be seen, the plots drop on a straight line which indicates that the reaction follows a second order mechanism. The value obtained

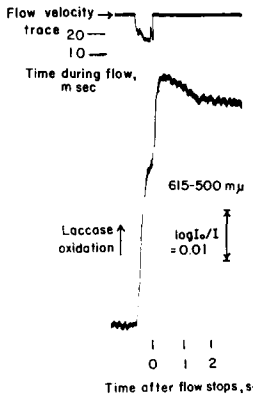

Fig. 5. An accelerated flow record of the laccase reaction by spectrophotometry. Initial concentrations of laccase, oxygen and ascorbate are 11 μM, 25 μM and 2 mM respectively.

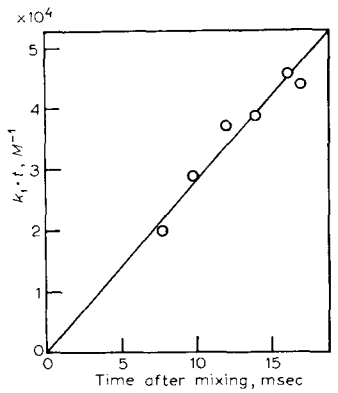

Fig. 6. Plot of $k_1 \cdot t$ against t. Initial concentrations of laccase, oxygen and ascorbate are 14 μM, 14 μM and 2 mM respectively.

for k_1 is $2.8 \cdot 10^6$ M^{-1} sec^{-1} at 25° and pH 7.0. This experiment was repeated at different initial oxygen concentrations with a constant concentration of laccase. The calculated second order velocity constant did not change as shown in Fig. 7. These facts mean that the reduced laccase reacts with oxygen molecule by a simple second order mechanism.

Effect of pH on k_1: The effect of pH on k_1 was studied by mixing reduced laccase in 0.01 M phosphate, pH 7.0 with 0.1 M buffers of different pH's, which contain 50 μM of oxygen. The final pH was checked. Results are shown in Fig. 8. It is seen that k_1 is constant in the region of pH 4–9, and k_1 decreases outside of this region. At pH 2 or 10, irreversible decolorization and inactivation of laccase occurs in the absence of substrate at room temperature. So, this decrease of k_1 can be attributed to the denaturation of the enzyme. It can be said that any acidic group which dissociates hydrogen ion in this pH region (4–9) in laccase molecule does not affect the reaction (1) by dissociating its proton. The overall activity of laccase has the maximum

at pH 7.4 (see ref. 2) and the activity decreases markedly on both sides of pH 7.4: this means that only the reduction step of laccase by substrate is pH dependent.

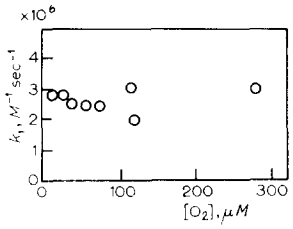

Fig. 7. Effect of the concentration of oxygen on k_1. The concentration of laccase is 20 μM. The concentration of ascorbate is 2 mM or 5 mM for experiments with O_2 concentration of under 100 μM or above 100 μM, respectively. For experiments with O_2 concentrations between 40–80 μM and 120–300 μM, flow apparatuses of 2 mm and 1 mm optical path[13] respectively were used in order to obtain higher flow speed (time during the flow was 4–8 msec and 0.8–2.0 msec respectively) which is required because of the higher speed of the reaction under these conditions.

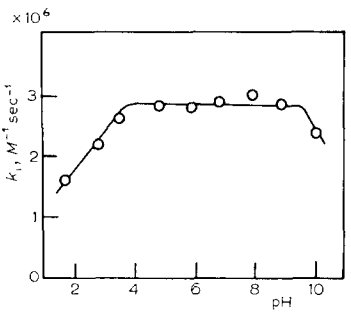

Fig. 8. Effect of pH on k_1. Glycine, acetate and the KOLTHOFF's buffers were used for pH ranges of 1.9–3, 4–5 and 6–10, respectively. The concentration of ascorbate is 2 mM.

DISCUSSION

The following simple mechanism for the oxidation of laccase

$$\text{reduced laccase} + O_2 \rightarrow \text{oxidized laccase} + O_2^{----} \qquad (3)$$

which excludes a possible enzyme–substrate complex of the type of Cu^{+}–O_2 as proposed by WARBURG[14] in its intermediate step, is ascertained by spectrophotometric and magnetometric surveys. First, no transient complex was observed either magnetometrically or spectrophotometrically, and second, the lack of dependency of the second order velocity constant of this reaction on the oxygen concentration means that the speed of utilization of oxygen by the reduced laccase is proportional to oxygen concentration throughout the range studied (14–280 μM).

Laccase and ascorbic acid oxidase have similar characteristics. They are both oxygen-linked oxidases with copper atoms as the prosthetic group, they are both deep blue, and both are reduced to leuco-form by ascorbic acid. THIMANN *et al.*[15] reported that the ascorbic acid oxidase has a weak affinity for oxygen. In contrast to this, in the case of laccase, the oxidation of the enzyme by molecular oxygen proceeds very quickly, and the value obtained for the second order velocity constant is comparable to that of the reaction of the formation of enzyme–substrate complex between horse radish peroxidase and hydrogen peroxide[16], which means a high affinity of laccase copper for oxygen.

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NOUVELLE METHODE DE PURIFICATION DE LA GONADOTROPINE CHORIALE HUMAINE

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SUMMARY

A new method of purification of human chorionic gonadotropin

Human chorionic gonadotropin is purified up to 12,000 IU/mg, from urine of pregnant women, by a new method of preparation. After benzoic acid adsorption and extraction in an acetate buffer of pH 4.8, the method involves a fractionation with calcium in 50 % ethanol and a precipitation at the pHi. Kaolin adsorption and chromatography on Decalso columns yield a fraction at a titre of 7,000 IU/mg.

A further purification by chromatography on Dowex 2 columns or starch electrophoresis gives a final preparation of 10,000 to 12,000 IU/mg, which behaves as a homogenous substance in a physicochemical test for purity.

INTRODUCTION

L'étude des caractéristiques physico-chimiques d'une hormone protéique est subordonnée à l'obtention d'une substance pure. Aussi, les méthodes d'extraction et de purification de la gonadotropine choriale humaine se sont-elles succédées depuis trente ans.