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STEADY-STATE KINETICS OF LACCASE FROM *RHUS VERNICIFERA*

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Summary

The steady-state kinetics of laccase (monophenol,dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) from the lacquer tree *Rhus vernicifera* is investigated using the respirograph method to produce Lineweaver-Burk plots of oxygen consumption rate against oxygen concentration. A ping-pong mechanism is established. The kinetic constants obtained according to the model is in close agreement with the corresponding values obtained from earlier studies on the transient reactions between the reduced enzyme and oxygen (Andréasson, L.-E., Brändén, R. and Reinhammar, B. (1976) Biochim. Biophys. Acta 438, 370—379) and between the oxidized enzyme and reducing substrates (Andréasson, L.-E. and Reinhammar, B. (1976) Biochim. Biophys. Acta 445, 579—597).

Introduction

The oxidase laccase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1), found in the Japanese lacquer tree *Rhus vernicifera* and in the fungus *Polyporus versicolor* belongs to a very small group of enzymes, including cytochrome *c* oxidase, ceruloplasmin and ascorbate oxidase, which are known to reduce O_2 and H_2O and to contain Cu. Only cytochrome *c* oxidase contains, in addition, heme. It seems reasonable to believe that investigations of the mechanism of the laccase reaction may give clues to the function of Cu in cytochrome *c* oxidase. Like ceruloplasmin and ascorbate oxidase, laccase contains three different types of bound Cu which may be distinguished by light and EPR spectroscopy [1,2]. Another feature common to the three enzymes is a poor specificity for the oxidizable substrate. By anaerobic reduction of the enzyme and subsequent reoxidation by O_2 it has been shown that a Cu^{2+}-Cu^{+} cycle is likely to be a part of the catalytic mechanism [3]. Kinetic work on laccase has so far been concerned mainly with transients (for review see refs. 1 and 2). In the present paper we describe a steady-state kinetic study of the reactions catalyzed by the lacquer tree laccase.

Experimental

Measurements of oxygen uptake rate as a function of oxygen concentration and simultaneous automatic recording of Lineweaver-Burk curves were done by the respirograph technique [4]. We have used an extended version of this original version to which an on-line computer system (NOVA 1200 minicomputer (Data General Corp., Southboro, Mass. U.S.A.)) has been adapted. A short review of this technique and its applications is available [5].

The sample, 4.5 ml, is placed in a cylindrical cell stirred with a synchronous motor. The surface of the liquid is in contact with a flowing gas phase of known composition. At the start of the experiment only nitrogen is present. After anaerobiosis the nitrogen in the gas is gradually replaced by oxygen. The gas composition is determined by gas mixers which are regulated by means of the on-line computer system using feed back control. The replacement is regulated to give a linear increase with time in the oxygen concentration in the liquid. Both this concentration and the oxygen concentration of the gas is measured by means of Clark-type electrodes (Radiometer, Copenhagen). During the experiment the oxygen consumption rate can be calculated from the two electrode signals as previously described [6]. This calculation is performed by the computer during the experiment and reciprocal values of the oxygen concentration and the oxygen consumption rate is generated as the analogue output to an X,Y-recorder. Because initial rates were not measured the concentration of the fixed variable substrate was always so high that the amount used during the measurement did not cause a significant decrease. *R. vernicifera* laccase was purified as described by Reinhammar [7]. The medium contained 0.1 M potassium phosphate and 0.01% serum albumin at pH 7.5. The temperature was 25°C. The following oxidizable substrates were used: hydroquinone (QH_2), potassium ascorbate, *N,N'*-dimethyl *p*-phenylenediamine, *p*-phenylenediamine, *N,N,N',N'*-tetramethyl *p*-phenylenediamine and catechol. All chemicals were best grade commercial products used without further purification. The concentration of enzyme was determined from oxidized minus reduced absorbance differences at 615 nm, assuming a molar absorbance of 5500 $M^{-1} \cdot cm^{-1}$ [8].

Results

Fig. 1 shows Lineweaver-Burk curves of oxygen uptake rate versus oxygen concentration at different enzyme concentrations with hydroquinone as the oxidizable substrate. The curves are linear and intersect at the abscissa axis. The slope is proportional to the reciprocal enzyme concentration. The same is true for $1/V$.

Experiments with different concentrations of hydroquinone (QH_2) are shown in Fig. 2. The Lineweaver-Burk curves are parallel lines. The secondary plot of intercept against $1/[QH_2]$ is a straight line through the origin. The slope of the Lineweaver-Burk curve does not change when hydroquinone is replaced by other oxidizable substrates. Fig. 3 shows the linear parallel plots obtained with five different substrates.

In the experiments with hydroquinone the enzyme showed no sign of satura-

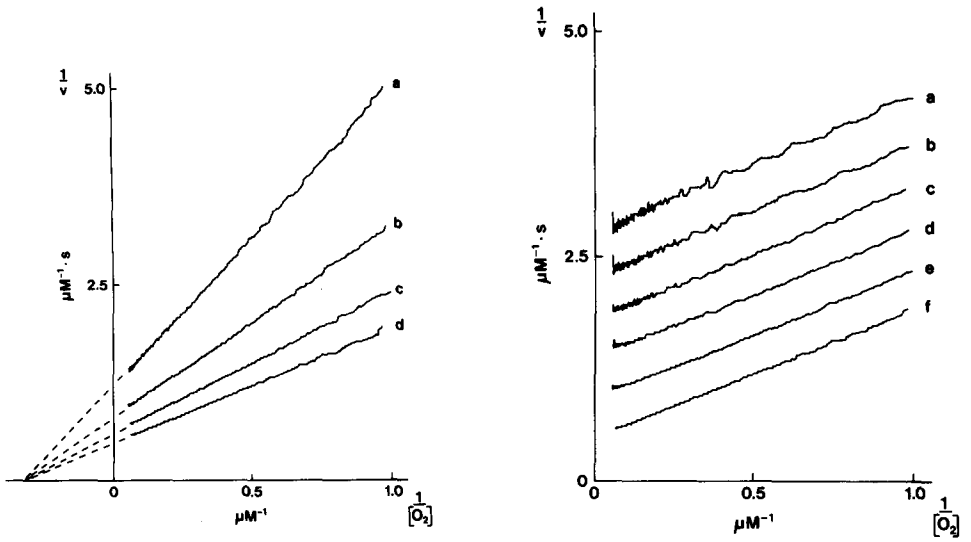


Fig. 1. Lineweaver-Burk curves at different enzyme concentrations. $1/v$ against $1[O_2]$ at: a, 44.5 nM; b, 67 nM; c, 89 nM and d, 111 nM laccase. The medium contained 33.3 mM hydroquinone/0.1 M potassium phosphate/0.01% albumin, pH 7.5.

Fig. 2; Effect of varying the concentration of hydroquinone. $1/v$ against $1/[O_2]$ at: a, 5.5 mM; b, 6.6 mM; c, 8.3 mM; d, 11.1 mM; e, 16.7 mM and f, 33.3 mM hydroquinone. The concentration of laccase was 111 nM. Other conditions were as described in Fig. 1.

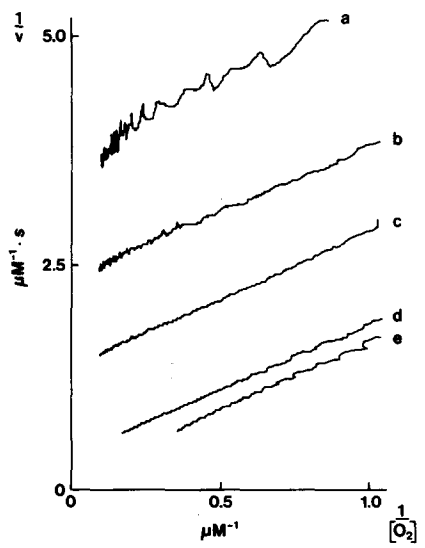


Fig. 3. Oxygen kinetics with different oxidizable substrates. $1/v$ against $1/[O_2]$ at: a, 22.2 mM ascorbate; b, 22.2 mM ascorbate plus 13.3 μM dimethyl phenylenediamine; c, 22.2 mM ascorbate plus 0.28 mM tetramethyl phenylenediamine; d, 33.3 mM by hydroquinone and e, 22.2 mM catechol. The concentration of laccase was 111 nM. Other conditions were as described in Fig. 1.

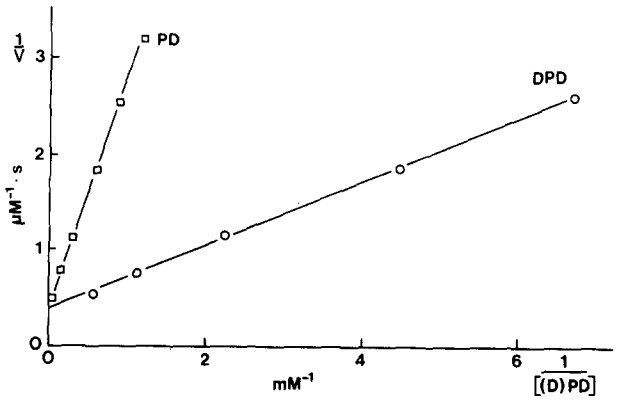


Fig. 4. Secondary plots from experiments with phenylenediamine (PD) and dimethyl phenylenediamine (DPD). Intercepts of Lineweaver-Burk curves, $1/v$ against $1/[O_2]$, are plotted against reciprocal concentrations of phenylenediamine and dimethyl phenylenediamine. The medium contained 22.2 nM laccase and 11.1 mM ascorbate. Other conditions were as described in Fig. 1.

tion. The possibility that saturation might occur at a higher turnover than obtained with hydroquinone was tested by using various fixed concentrations of dimethyl phenylenediamine and phenylenediamine. 11.1 mM ascorbate was added to keep these substances reduced during the experiment. The reaction of ascorbate with the enzyme is too slow to be significant compared to the reaction with phenylenediamine or dimethyl phenylenediamine. Again linear parallel Lineweaver-Burk curves were obtained. The secondary plots of intercept against reciprocal oxidizable substrate concentration are shown in Fig. 4. Unlike the secondary plots obtained with hydroquinone the secondary plots obtained with dimethyl phenylenediamine and phenylenediamine has a positive intercept on the ordinate axis, revealing saturation of the enzyme.

The influence of pH on the oxidation of hydroquinone is shown in Fig. 5. In

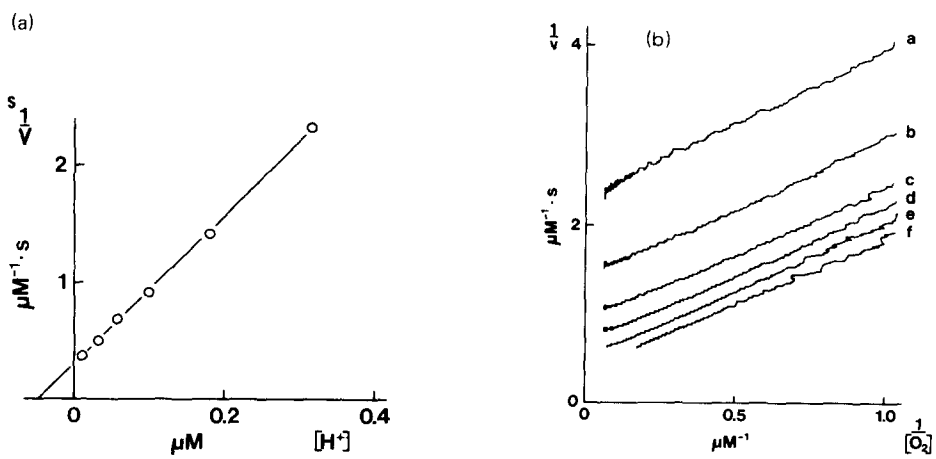
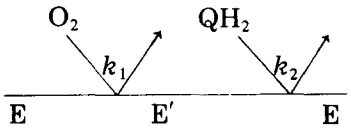


Fig. 5. Effect on hydroquinone oxidation of varying pH, $1/v$ against $1/[O_2]$ at various pH. a, pH 6.5; b, pH 6.75; c, pH 7.0; d, pH 7.25; e, pH 7.5 and f, pH 8.0. The medium contained 111 nM laccase and 33.3 mM hydroquinone. Other conditions were as described in Fig. 1. a, secondary plot of data from b.

the pH range 6.5—8 the Lineweaver-Burk curves are parallel lines. The secondary plot intercept versus $[H^+]$ is linear (Fig. 5a). Similar experiments with other oxidizable substrates did not give linear secondary plots.

Discussion

The experiments with hydroquinone as the oxidizable substrate (Figs. 1 and 2) are consistent with the ping-pong di Theorell-Chance mechanism:



which has the rate equation:

$$v = \frac{k_1 k_2 e[O_2][QH_2]}{2k_1[O_2] + k_2[QH_2]} \quad (1)$$

The reciprocal form of Eqn. 1 is

$$\frac{e}{v} = \frac{1}{k_1[O_2]} + \frac{2}{k_2[QH_2]}$$

The factor 2 accounts for the fact that 4 electrons are involved to reduce oxygen to water, while only 2 electrons are transferred in the reaction with hydroquinone.

It is a characteristic feature of the above model that neither substrate can saturate the enzyme. Thus there are no limiting K_m values.

According to the model the rate constant for the reaction between O_2 and the enzyme, k_1, is inversely proportional to the slope of the $1/v$ against $1/[O_2]$ plot. The value obtained $6.1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, is in excellent agreement with the corresponding value found from transient kinetics [9]. The rate constant for the reaction between hydroquinone and the enzyme, k_2, can be determined from the intercept. The value obtained, $1.2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$, also agrees with that obtained from transient kinetics [10]. In Table I the kinetic constants found from steady-state kinetics (this paper and ref. 11) is compared to those obtained from transient kinetics [9,10].

It is seen from Fig. 3 that the value of k_1 obtained from the slope is identical when different oxidizable substrates are used. This observation supports the ping-pong mechanism as it indicates that these substrates or their corresponding oxidation products are not bound to the enzyme when the reaction with O_2 takes place.

At the high turnover rates obtained with phenylenediamine and dimethyl phenylenediamine the secondary plot (Fig. 4) revealed a saturation of the enzyme which was not visible with hydroquinone. In order to account for this effect, an expansion of Eqn. 1 is necessary. A term which is independent of the concentration has to be included in the reciprocal rate equation:

$$\frac{e}{v} = \frac{4}{k'} + \frac{2}{k_2[PD]} + \frac{1}{k_1[O_2]} \quad (2)$$

TABLE I

KINETIC CONSTANTS OF THE *RHUS VERNICIFERA* LACCASE-CATALYZED REACTIONS

Substrate	Kinetic constants *	Steady-state kinetics		Transient kinetics (refs. 8 and 9)
		This study **	Ref. 10	
Oxygen	K_m	21.3 μM ***		
	k_1	$6.1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$		$\approx 6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$
Hydroquinone	K_m	0.2 M ***	0.17 M	
	k_2	$1.17 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ †		$1.58 \cdot 10^3$ $M^{-1} \cdot s^{-1}$
Ascorbate	k_2	259 $M^{-1} \cdot s^{-1}$		250 $M^{-1} \cdot s^{-1}$
p-Phenylenediamine (PD)	K_m	6.7 mM	6.6 mM	
	k_2	3.7×10^4 $M^{-1} \cdot s^{-1}$		
N,N'-Dimethyl *p*-phenylene diamine (DPD)	K_m	0.82 mM	3.9 mM	
	k_2	$2.7 \cdot 10^5$ $M^{-1} \cdot s^{-1}$		
PD and DPD	k (maximal turnover)	560 s^{-1}		

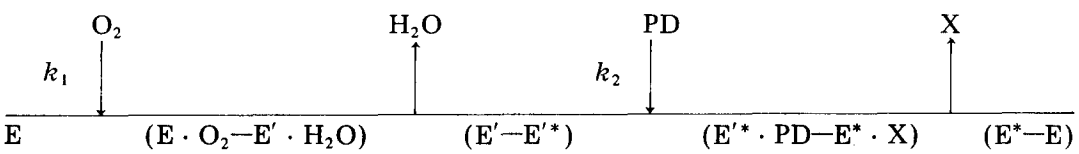
* The rate constants are defined by Eqn. 2.

** At 0.1 M potassium phosphate pH 7.5, 25°C.

*** Extrapolated values obtained at infinite concentration of the substrate which was not varied, assuming a maximal turnover equal to k_3.

† $k_2 = 1.9 \cdot 10^3/(1 + [H^+]/K)$ where $K = 5.0 \cdot 10^{-8}$ M^{-1}.

The term $k'/4$ is the maximal turnover of the enzyme at saturation of substrates. The factor 4 is included to express k' in electrons per s per laccase molecule. The appearance of this term in the kinetics of phenylenediamine (PD) and dimethyl phenylenediamine indicates the existence of one or more rate-limiting steps in the conversion of central complexes and/or release of products. The rate of this (these) step(s) is however too fast to be noticed in the kinetics of the slower substrates, hydroquinone and ascorbate. A model which illustrates some of the possible locations of the rate limitation is:



The following apparent first-order reactions might be rate limiting and contribute to the $4/k'$ term: (i) The electron transfer within the enzyme · oxygen complex, $E \cdot O_2 \rightleftharpoons E' \cdot H_2O$, and the enzyme · phenylenediamine complex, $E'^* \cdot$ phenylenediamine $\rightleftharpoons E^* \cdot X$, (ii) rearrangements within the oxidized and reduced enzyme, $E' \rightleftharpoons E'^*$ and $E^* \rightleftharpoons E$, and (iii) release of products, H_2O and X.

The fact that two molecules of phenylenediamine have to react with the enzyme in order to transfer the four electrons, which are necessary for the reduction of O_2 to H_2O, will complicate the scheme further. Only the conversion of enzyme complexes with phenylenediamine or its oxidation products seems not likely to be rate limiting because the same value for k' is

obtained when phenylenediamine is replaced by dimethyl phenylenediamine.

In order to account for the observed kinetics, the simplest assumption concerning the reaction of the substrates with the enzyme, is that they all react in a practically irreversible reaction. For O_2, ascorbate and hydroquinone, this assumption is supported by the close agreement between the values of the steady-state constants k_1 and k_2 reported here (Table I) and the on-constants measured by means of transient kinetics [9,10].

The experiments on the pH dependency of the oxidation of hydroquinone (Fig. 5) show that the H^+ can be considered an uncompetitive inhibitor with respect to oxygen and a competitive inhibitor with respect to hydroquinone (Fig. 5a). The simplest model reproducing this behaviour is

$$O_2 + E \overset{k_1}{\rightarrow} E'$$

$$QH_2 + E' \overset{k_2}{\rightarrow} E$$

$$H^+ + E' \rightleftharpoons E'H^+ \ (K)$$

which yields

$$v = \frac{k_1 k_2 e[O_2][QH_2]}{2k_1[O_2](1 + [H^+]/K) + K_2[QH_2]} \tag{3}$$

the reciprocal form of which is

$$\frac{e}{v} = \frac{2(1 + [H^+]/K)}{k_2[QH_2]} + \frac{1}{k_1[O_2]}$$

The same result may also be obtained from other simple models. Any useful model must be based on the rather unusual assumption that only one ionizable group contributes to the pH dependency of the reaction. The pK of this group is 7.3 as determined from Fig. 5a. This rules out, that the protonization of hydroquinone (pK_1 = 9.9 and pK_2 = 11.6) is responsible for the inhibition by H^+ as proposed by Holwerda and Gray [12]. The ionizable group could be an imidazole group which has p$K \approx 7$. Laccase contains 17 histidine residues [7]. The non-linearity of the secondary plots from the measurements of the pH dependence of the oxidation of other substrates, indicates that more than one ionizable group is involved in these cases.

Considering the properties of the ping-pong di Theorell-Chance mechanism found for the oxidation of hydroquinone one would expect the enzyme to occur only in the reduced form E and the oxidized form E′ in experiments with hydroquinone. Therefore spectroscopic forms of the enzyme observed in stopped flow experiments and not identifiable with E or E′ are not likely to be involved in the turnover. The maximal turnover of the enzyme found with phenylenediamine and dimethyl phenylenediamine correspond to a half-life of the enzyme form converted in the rate-limiting step of about 2 ms. This gives little hope that stopped flow experiments with the faster substrates will reveal other active forms of the enzyme than E and E′.

We have recently studied the steady-state kinetics of cytochrome *c* oxidase which can also be described with a ping-pong model involving a practically irreversible reaction between oxygen and the reduced enzyme [13]. The

maximal turnover of cytochrome *c* oxidase was found to be 400 electrons per s, and the on-constant for oxygen, k_1 was $9 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. Whereas the maximal turnover for laccase, 560 s^{-1}, is nearly the same as for cytochrome *c* oxidase, the on-constant for oxygen of laccase is about 15-fold lower than that of cytochrome *c* oxidase. One might speculate that laccase is an evolutionary ancestor of cytochrome *c* oxidase. Accordingly the replacement of two coppers with two hemes is seen to cause a considerable increase in the affinity for oxygen without the maximal turnover being affected.

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References

1 Malmström, B.G., Andréasson, L.-E. and Reinhammar, B. (1975) in The Enzymes (Boyer, P.D., ed.), Vol. XII, pp. 507—579, Academic Press, New York
2 Fee, J.A. (1975) in Structure and Bonding (Dunitz, J.D., Hemmerich, P., Holm, R.H., Ibers, J.A., Jørgensen, C.K., Neilands, J.B., Reinen, D. and Williams, R.J.P., eds.), Vol. 23, pp. 1—60, Springer-Verlag, Berlin
3 Nakamura, T. (1958) Biochim. Biophys. Acta 30, 44—52
4 Degn, H. and Wohlrab, H. (1971) Biochim. Biophys. Acta 245, 347—355
5 Degn, H. (1977) Trends Biochem. Sci. 2, 171—173
6 Petersen, L.C. (1978) Eur. J. Biochem. 85, 339—344
7 Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35—47
8 Malmström, B.G., Reinhammar, B. and Vänngård, T. (1970) Biochim. Biophys. Acta 205, 48—57
9 Andréasson, L.-E., Brändén, R. and Reinhammar, B. (1976) Biochim. Biophys. Acta 438, 370—379
10 Andréasson, L.-E. and Reinhammar, B. (1976) Biochim. Biophys. Acta 445, 579—597
11 Peisach, J. and Levine, W.G. (1965) J. Biol. Chem. 240, 2284—2289
12 Holwerda, R.A. and Gray, H.B. (1974) J. Am. Chem. Soc. 96, 6008—6022
13 Petersen, L.C., Nicholls, P. and Degn, H. (1976) Biochim. Biophys. Acta 452, 59—65