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Kinetics of Phenol Oxidation by Peroxidase

PALLIGARNAI T. VASUDEVAN* AND LUTING OLIVIA LI

Department of Chemical Engineering, Kingsbury Hall, University of New Hampshire, Durham, NH 03824

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ABSTRACT

Studies of the kinetic behavior of horseradish peroxidase (HRP) at pH 8 and at room temperature indicate that the reaction of phenol with H_2O_2 catalyzed by HRP exhibits normal Michaelis-Menten saturation kinetics. An irreversible reaction mechanism for the steady-state kinetics of HRP, which is consistent with the experimental data, is considered. The second-order rate constants for the reactions of HRP with H_2O_2 and compound II with phenol are $4.14 \times 10^5\ M^{-1}s^{-1}$ and $5.54 \times 10^4\ M^{-1}s^{-1}$, respectively.

Index Entries: Horseradish peroxidase, phenol, hydrogen peroxide.

Nomenclature: •AH, Free radical; AH_2, Phenol or aromatic compound; Compound I, Intermediate oxidized enzymatic form of horseradish peroxidase by H_2O_2; Compound II, Intermediate oxidized enzymatic form of Compound I by phenol; E_{tot}, Total native enzyme horseradish peroxidase; HRP, Native enzyme horseradish peroxidase; k_1, Rate constant for Compound I formation; k_{-1}, Rate constant for Compound I back to the native enzyme; k_2, Rate constant for one-electron reduction of Compound I by phenol; k_3, Rate constant for one-electron reduction of Compound II by phenol; K'_m, Apparent Michaelis constant at constant phenol concentration, mM; K''_m, Apparent Michaelis constant at constant H_2O_2 concentration, mM; V, Initial reaction rate, mM/s; V'_{max}, Apparent maximum reaction rate at constant phenol concentration, mM/s; V''_{max}, Apparent maximum reaction rate at constant H_2O_2 concentration, mM/s.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

A number of investigators (*1–4*) have proposed a novel approach to removing phenols from waste water based on the use of horseradish peroxidase, an oxidoreductase enzyme, with hydrogen peroxide. The treatment results in polymerization and precipitation of phenols. This approach has been tested with more than 20 different phenols (*5,6*). In most cases, 97–99% phenols were removed in a wide range of phenol concentrations (0.0005–5.0 g/L).

Alberti and Klibanov (*3*) have studied the practicability of the enzymatic approach. A number of studies reported in literature have focused on the final removal efficiency of phenols after several hours of treatment. The kinetics of the reaction has been studied by a number of researchers (*7–9*). Steady-state equations for the peroxidase ping-pong mechanism have been developed, and include reversible substrate binding reactions (*10*). Information regarding the mechanism and kinetic behavior of horseradish peroxidase is vital to the development of an enzymatic approach for wastewater treatment.

This paper examines the kinetics of HRP catalyzed oxidation of phenol. Steady-state kinetics is employed in the study of this multisubstrate (H_2O_2 and phenol) enzyme catalyzed reaction. The relevant rate equation includes concentration terms for the two substrates in a variety of functional forms, depending on the reaction mechanism. Various reaction mechanisms (both reversible and irreversible), in terms of the form of the rate equations are considered, and the corresponding kinetic parameters are then obtained. Even though oxidation reactions are considered, the proposed reaction mechanism is consistent with the experimental data.

MATERIALS AND METHODS

Materials

Horseradish peroxidase (Type I) from Sigma Chemical Co. (St. Louis, MO) with a specific activity of 95 purpurogallin U/mg (1 U forms 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C) was used in all the experiments. Hydrogen peroxide (30%, specific gravity 1.1) solution was purchased from Fisher Scientific (Pittsburgh, PA). Phenol (purity 99.9%) was purchased from Mallinckrodt Company, St. Louis, MO. Citric acid (No. C-0759) and sodium monohydrogen phosphate were obtained from Sigma. Methanol (HPLC grade) was purchased from Fisher Scientific.

Preparation of Citric Acid Solution

Eighty grams of citric acid were dissolved in 1000 mL of distilled-deionized water to give 1 L of 8% citric acid solution, which was used to terminate the reaction during steady-state kinetic measurements.

Preparation of Horseradish Peroxidase Solution

A total of 0.0211 g of horseradish peroxidase were dissolved in 50 mL distilled-deionized water to give a 0.422 mg/mL horseradish peroxidase solution, of which 0.01 mL was used in each run.

Experimental Procedure

The experiments were carried out in an agitated 50 mL flask (10 mL reaction volume) at room temperature. The reaction mixture contained citric acid-phosphate buffer (0.559*M*, pH 8.0), phenol (0.2494–5.1750 m*M*), H_2O_2 (0.1–10.0 m*M*) and horseradish peroxidase (0.422 mg/L, or 40.09 U/L, or 9.591×10^{-6} m*M*).

In a typical experiment, the reaction was initiated by the addition of horseradish peroxidase, and terminated by the addition of 20 mL of 8% citric acid solution. The initial reaction rate was determined from the consumption of phenol as a function of time. The phenol consumption was quantified by a Hewlett Packard 1050 HPLC with a C18 (octadecylsilica, ODS) reverse-phase column and a variable wavelength detector. Detection was performed at 271 nm. The isocratic mobile phase solvent used was water-methanol (in the ratio 70:30) with a flow rate of 2.25 mL/min. The phenol eluted at 1.92 min.

RESULTS AND DISCUSSIONS

Steady-State Kinetics in the Reaction of Phenol with HRP/H_2O_2

The enzyme reaction involves two substrates (H_2O_2 and phenol). Accordingly, the relevant rate equation will include concentration terms for the two substrates in a variety of functional forms, depending on the reaction mechanism. The rate equation can be modified to include only one substrate as a variable while keeping the initial concentration of the other substrate constant.

Effect of H_2O_2 Concentrations on Initial Rates of the Reaction

The concentrations of HRP was kept sufficiently low (9.591×10^{-6} m*M*) and the assay period was short enough (a few minutes) to ensure that only a small fraction of phenol (less than 5%) was consumed. The time course of phenol disappearance at different concentrations of H_2O_2 is shown in Figs. 1, 2, and 3 at constant initial phenol concentrations of 1.0272 m*M*, 2.5637 m*M*, and 5.1750 m*M*, respectively.

The initial rates for different concentrations of H_2O_2 and three initial concentrations of phenol were obtained on a short time scale (120 s). The

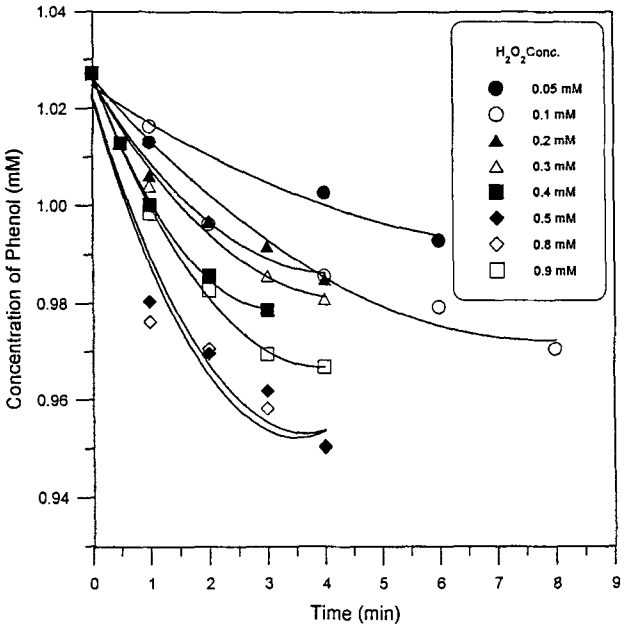

Fig. 1. Time course of phenol disappearance, 9.591×10^{-6} m*M* HRP, 1.0272 m*M* phenol, pH 8.0, 20°C.

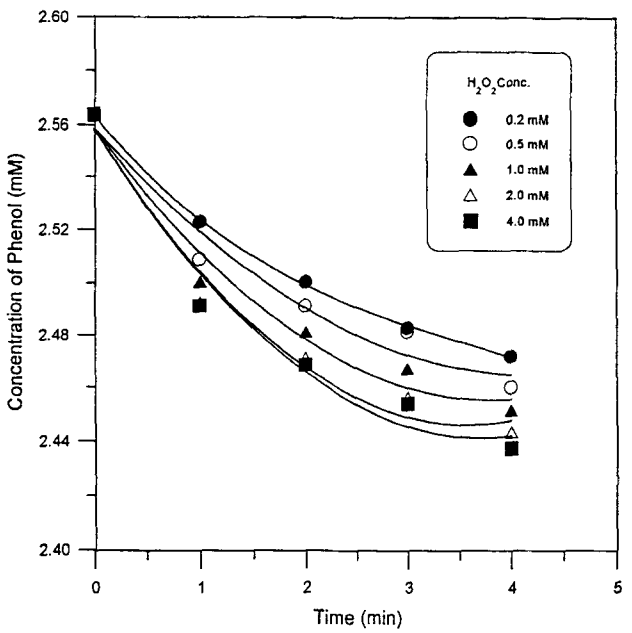

Fig. 2. Time course of phenol disappearance, 9.591×10^{-6} m*M* HRP, 2.5637 m*M* phenol, pH 8.0, 20°C.

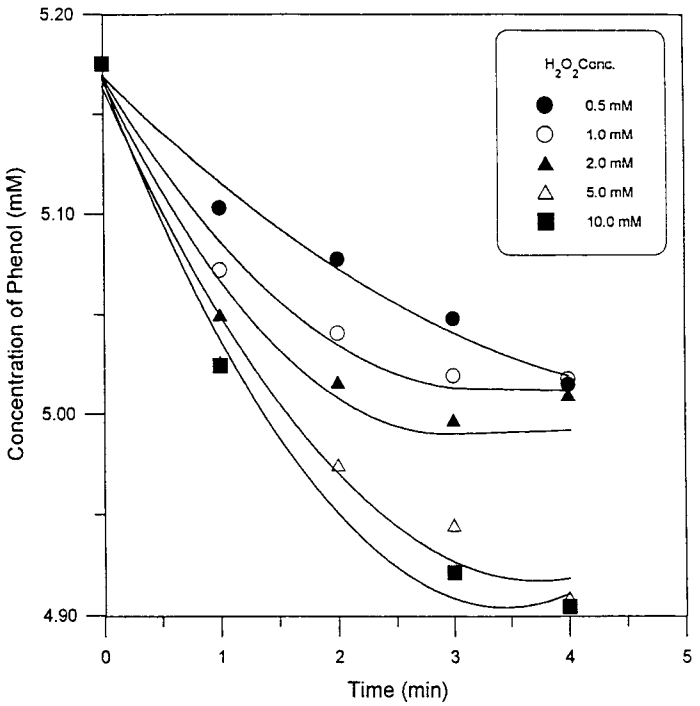

Fig. 3. Time course of phenol disappearance, 9.591×10^{-6} m*M* HRP, 5.1750 m*M* phenol, pH 8.0, 20°C.

data obtained takes into consideration the inactivation of peroxidase during the initial stages of oxidation, and thus represent steady-state values. The parameters obtained therefore represent steady-state parameters (*11*). The kinetic data as a function of H_2O_2 concentration are presented in Figs. 4, 5, and 6. It is clear from the figures that the initial reaction rate increases with an increase in H_2O_2 concentration. The initial rate (V) vs substrate concentration was fitted to the Michaelis-Menten equation:

$$V = (V'_{max}\,[H_2O_2]) / (K'_m + [H_2O_2]) \quad (1)$$

The variables V and $[H_2O_2]$ are the rate of phenol consumption and H_2O_2 concentration, respectively. Parameters V'_{max} and K'_m, the apparent maximum reaction rate and apparent Michaelis constant, respectively, were estimated by non-linear regression. From Figs. 4, 5, and 6, it is clear that these kinetic data fit well with the theoretical curve obtained from the Michaelis-Menten equation, and therefore the oxidation of phenol by HRP and H_2O_2 appears to follow Michaelis-Menten kinetics.

The values of the fitted parameters for different phenol concentrations are shown in Table 1. It is clear that both V'_{max} and K'_m increase with initial concentration of phenol increasing. The reasons for this will be explained later (last paragraph in Discussions).

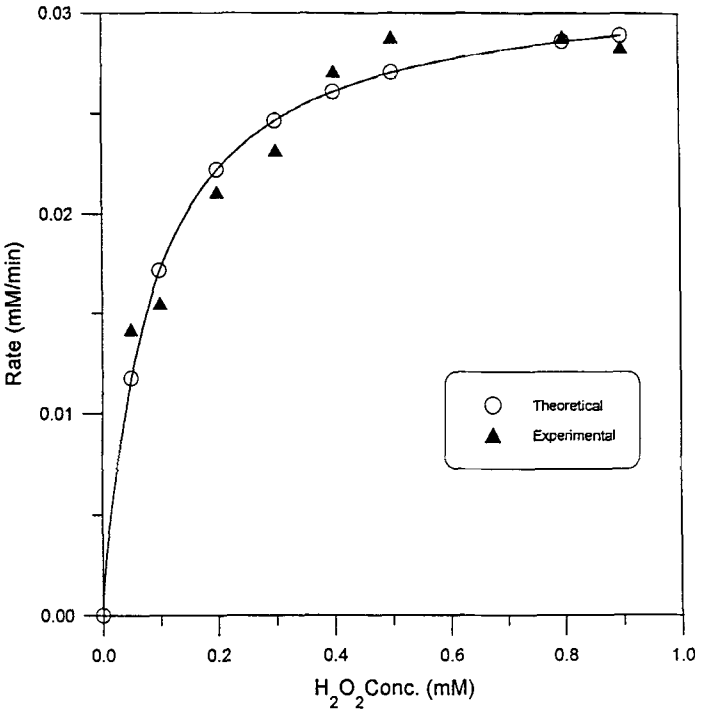

Fig. 4. Steady-state initial rates of oxidation of phenol as a function of H_2O_2 concentrations. 9.591 × 10^{-6} m*M* HRP, 1.0272 m*M* phenol, pH 8.0, 20°C.

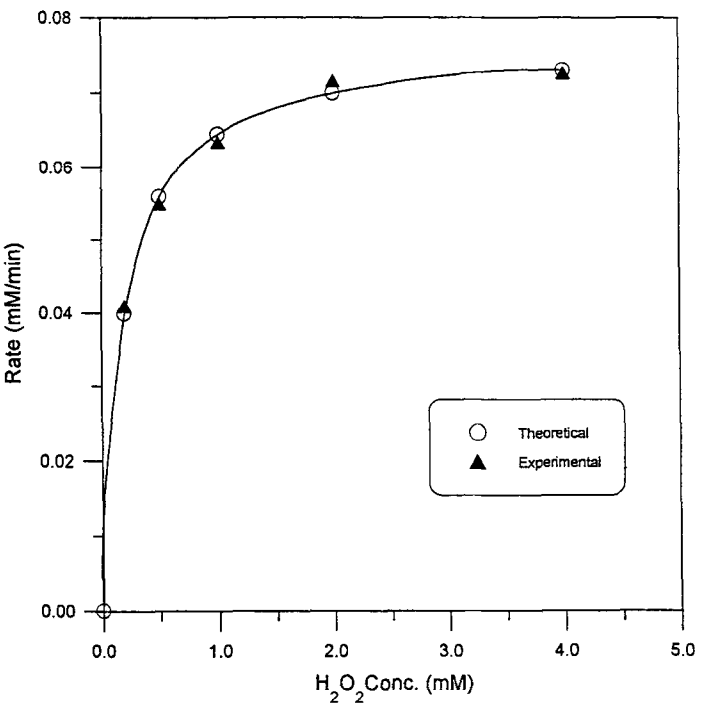

Fig. 5. Steady-state initial rates of oxidation of phenol as a function of H_2O_2 concentrations. 9.591 × 10^{-6} m*M* HRP, 2.5637 m*M* phenol, pH 8.0, 20°C.

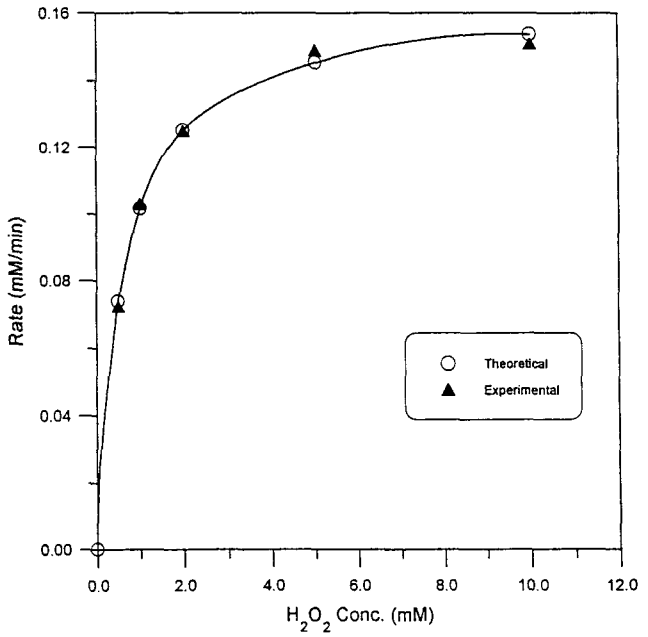

Fig. 6. Steady-state initial rates of oxidation of phenol as a function of H_2O_2 concentrations. 9.591×10^{-6} m*M* HRP, 5.1750 m*M* phenol, pH 8.0, 20°C.

Table 1
Apparent Michaelis-Menten Parameters
for the Reaction of HRP with Phenol and H_2O_2

Phenol (m*M*)	V'_{max} (*mM*/s)	K'_m (m*M*)
1.0272	5.27×10^{-4}	0.0844
2.5637	1.27×10^{-3}	0.182
5.1750	2.72×10^{-3}	0.605

Effect of Phenol Concentrations on Initial Rates of the Reaction

Experiments were also conducted in which the time course of phenol disappearance for different initial concentrations of phenol was obtained at a constant initial H_2O_2 concentration of 0.1 m*M* (Fig. 7).

The initial rates at different concentrations of phenol, but constant initial concentration of H_2O_2, were obtained on a short time scale (within first 120 s). The kinetic data as a function of phenol concentration, but constant initial concentration of H_2O_2, are illustrated in Fig. 8. It is clear that the initial reaction rate increases with an increase in phenol concentration. The initial rate (V) vs substrate concentration was fitted to the Michaelis-Menten equation

$$V = (V''_{max}[AH_2]) / (K''_m + [AH_2]) \quad (2)$$

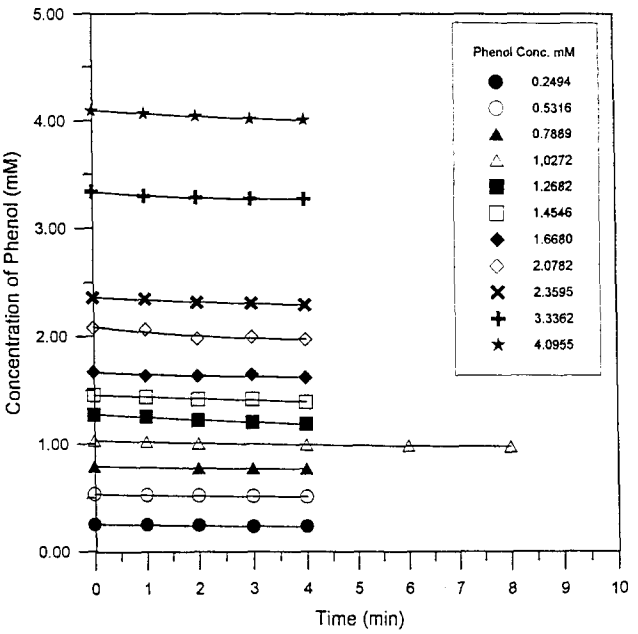

Fig. 7. Time course of phenol disappearance, 9.591×10^{-6} m*M* HRP, 0.1 m*M* H_2O_2, pH 8.0, 20°C and 11 different initial phenol concentrations.

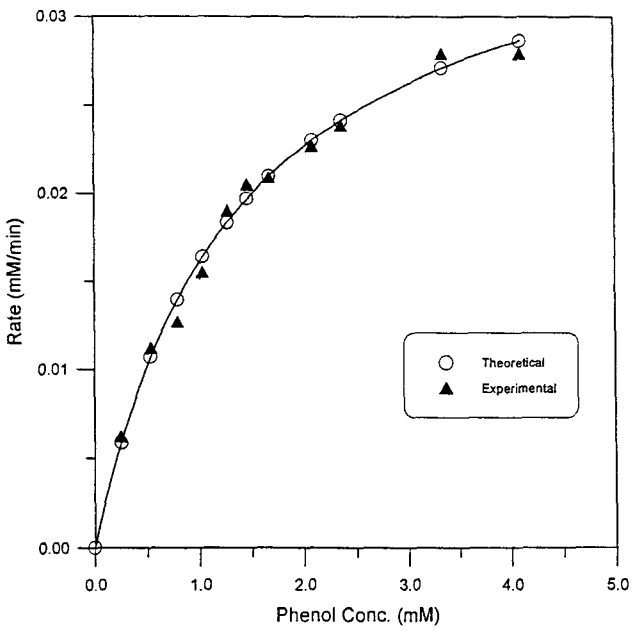

Fig. 8. Steady-state initial rates of oxidation of phenol as a function of phenol concentrations. 9.591×10^{-6} m*M* HRP, 0.1 m*M* H_2O_2, pH 8.0, 20°C.

The variable, V, represents the rate of phenol consumption, and $[AH_2]$ the concentration of phenol. Parameters V''_{max} and K''_m, which are also apparent Michaelis-Menten parameters, were estimated by nonlinear regression. From Fig. 8, it is clear that the experimental data match well with the theoretical curve obtained by fitting the experimental data to Eq. 2. It appears that the oxidation of phenol by HRP and H_2O_2 follows Michaelis-Menten kinetics. The values of the parameters V''_{max} and K''_m are 6.37×10^{-4} mM/s and 1.37 mM, respectively. The theoretical initial reaction rate, at 9.591×10^{-6} HRP, 1.0272 mM phenol, and 0.1 mM H_2O_2, is 2.720×10^{-4} mM/s, which is close to the theoretical value of 2.857×10^{-4} mM/s obtained by fitting the corresponding experimental data at a constant initial phenol concentration of 1.0272 mM to Eq. 1.

Proposal of Reaction Mechanisms and Estimation of Kinetic Parameters

In this paper, three possible mechanisms are considered:

Mechanism I (*12,13*)

$$E + H_2O_2 \xrightarrow{k_1} \text{Compound I} + H_2O \quad (3)$$

$$\text{Compound I} + AH_2 \xrightarrow{k_2} \text{Compound II} + \bullet AH \quad (4)$$

$$\text{Compound II} + AH_2 \xrightarrow{k_3} E + \bullet AH \quad (5)$$

Mechanism II

$$E + H_2O_2 \overset{k_1}{\rightleftharpoons} \text{Compound I} + H_2O \quad (6)$$

$$\text{Compound I} + AH_2 \xrightarrow{k_2} \text{Compound II} + \bullet AH \quad (7)$$

$$\text{Compound II} + AH_2 \xrightarrow{k_3} E + \bullet AH \quad (8)$$

Mechanism III

$$E + H_2O_2 \overset{k_1}{\rightleftharpoons} \text{Compound I} + H_2O \quad (9)$$

$$\text{Compound I} + AH_2 \overset{k_2}{\rightleftharpoons} \text{Compound II} + \bullet AH \quad (10)$$

$$\text{Compound II} + AH_2 \overset{k_3}{\rightleftharpoons} E + \bullet AH \quad (11)$$

In each mechanism, hydrogen peroxide oxidizes the native HRP to an active intermediate form, Compound I. Compound I then reacts with a phenol molecule, to produce a free radical (•AH), and the enzyme complex, Compound II. Compound II then oxidizes a second phenol molecule producing another free radical, and the native HRP. The free radicals formed are released into the solution where they can combine to form polyphenolic products. These polymers are less soluble in water and can

precipitate from solution. If precipitation does not occur, the larger polyphenolic compound can react with HRP (Compound I) resulting in the formation of a still larger polymer, which will eventually precipitate. The significant feature of these final polymers is that in contrast to their monomeric precursors, these polymers are practically insoluble in water.

For Mechanism I, using a quasi-steady-state approximation (Reaction [5] is assumed to be the rate limiting step of the entire reaction cycle), the following rate expression is obtained:

$$V = -\frac{d[AH_2]}{dt} \tag{12}$$

$$= k_3[AH_2][\text{compound II}] \tag{13}$$

$$= \frac{\frac{k_2k_3}{k_2+k_3}[E]_{tot}[AH_2][H_2O_2]}{\frac{k_2k_3}{k_1(k_2+k_3)}[AH_2] + [H_2O_2]} \tag{14}$$

$$= \frac{k_1[E]_{tot}[H_2O_2][AH_2]}{\frac{k_1(k_2+k_3)}{k_2k_3}[H_2O_2] + [AH_2]} \tag{15}$$

$$= \frac{V'_{max}[H_2O_2]}{K'_m + [H_2O_2]} \tag{16}$$

$$= \frac{V''_{max}[AH_2]}{K''_m + [AH_2]} \tag{17}$$

where

$$V'_{max} = \frac{k_2k_3}{k_2+k_3}[E]_{tot}[AH_2], \quad K'_m = \frac{k_2k_3}{k_1(k_2+k_3)}[AH_2], \quad \frac{V'_{max}}{K'_m} = k_1[E]_{tot} \tag{18}$$

$$V''_{max} = k_1[E]_{tot}[H_2O_2], \quad K''_m = \frac{k_1(k_2+k_3)}{k_2k_3}[H_2O_2], \quad \frac{V''_{max}}{K''_m} = \frac{k_2k_3}{(k_2+k_3)}[E]_{tot} \tag{19}$$

Both V'_{max} and K'_m are linearly related to $[AH_2]$. The ratio V'_{max}/K'_m is constant and independent of the AH_2 (phenol) concentration. Also, the ratio V''_{max}/K''_m is constant and independent of H_2O_2 concentration.

For Mechanism II, using a quasi-steady-state approximation, the following rate expression is obtained:

$$V = -\frac{d[AH_2]}{dt} \tag{20}$$

$$= k_3[AH_2][\text{compound II}] \tag{21}$$

$$= \frac{\frac{k_2k_3}{k_2+k_3}[E]_{tot}[AH_2][H_2O_2]}{\frac{k_3(k_{-1}+k_2[AH_2])}{k_1(k_2+k_3)} + [H_2O_2]} \tag{22}$$

$$= \frac{k_1[E]_{tot}[H_2O_2][AH_2]}{\frac{k_{-1}k_3+k_1(k_2+k_3)[H_2O_2]}{k_2k_3} + [AH_2]} \tag{23}$$

$$= \frac{V'_{max}[H_2O_2]}{K'_m + [H_2O_2]} \tag{24}$$

$$= \frac{V''_{max}[AH_2]}{K''_m + [AH_2]} \tag{25}$$

where

$$V'_{max} = \frac{k_2 k_3}{k_2 + k_3}[E]_{tot}[AH_2],\ K'_m = \frac{k_3(k_{-1} + k_2[AH_2])}{k_1(k_2 + k_3)},\ \frac{V'_{max}}{K'_m} = \frac{k_1[E]_{tot}[AH_2]}{\frac{k_{-1}}{k_2} + [AH_2]} \quad (26)$$

$$V''_{max} = k_1[E]_{tot}[H_2O_2],\ K''_m = \frac{k_{-1}k_3 + k_1(k_2 + k_3)[H_2O_2]}{k_2 k_3},\ \frac{V''_{max}}{K''_m} = \frac{\frac{k_2 k_3}{k_2 + k_3}[E]_{tot}[H_2O_2]}{\frac{k_{-1}k_3}{k_1(k_2+k_3)} + [H_2O_2]} \quad (27)$$

Both V'_{max} and K'_m are linear functions of $[AH_2]$. The ratio V'_{max}/K'_m, however, is a hyperbolic function of AH_2 (phenol) concentration. Also, the ratio V''_{max}/K''_m is a hyperbolic function of H_2O_2 concentration.

For Mechanism III, using a quasi-steady-state approximation as well as the schematic method of King and Altman (which provides a shortcut to expressions for the relative distribution of enzyme species in terms of the substrate concentrations and the various rate constants), we can obtain the distribution of enzyme species. The rate expression then is:

$$V = -\frac{d[AH_2]}{dt} \quad (28)$$

$$= k_3[\text{compound II}][AH_2] - k_{-3}[E][\bullet AH] \quad (29)$$

$$= \frac{[E]_{tot}(k_1 k_2 k_3[H_2O_2][AH_2]^2 - k_{-1}k_{-2}k_{-3}[\bullet AH]^2)}{\text{denominator}} \quad (30)$$

where

$$\begin{aligned}\text{denominator} = {} & (k_{-2} + k_{-3})k_{-1}[\bullet AH] + k_{-1}k_3[AH_2] \\ & + k_2 k_3[AH_2]^2 + k_1 k_{-2}[H_2O_2][\bullet AH] \\ & + (k_2 + k_3)k_1[AH_2][H_2O_2] + k_{-2}k_{-3}[\bullet AH]^2 \\ & + k_2 k_{-3}[\bullet AH][AH_2]\end{aligned}$$

In the absence of products ($[\bullet AH] = 0$ since polymerizations is very fast relative to the rate of radical formation), the rate equation becomes:

$$V = \frac{k_1 k_2 k_3[E]_{tot}[AH_2][H_2O_2]}{k_3(k_{-1} + k_2[AH_2]) + k_1(k_2 + k_3)[H_2O_2]} \quad (31)$$

The preceding equation is the same as that obtained from Mechanism II. Therefore, only Mechanisms I and II are now considered.

Based on the results obtained in the study, the plausible mechanism for the oxidation of phenol by H_2O_2 catalyzed by HRP appears to be an irreversible reaction mechanism (Mechanism I). The distinction between the two mechanisms is based upon the ratio V'_{max}/K'_m. In Mechanism I, this

ratio is equal to $k_1[E]_{tot}$ and is independent of $[AH_2]$. For Mechanism II, the ratio of V'_{max}/K'_m is a hyperbolic function of $[AH_2]$, which is contrary to the experimental results. A plot of V'_{max} vs K'_m (Table 1) is linear with a slope of $k_1[E]_{tot} = 3.97 \times 10^{-3}\ s^{-1}$ ($[E]_{tot} = 9.591 \times 10^{-6}$ m*M*) and an intercept not significantly different from zero. From this the rate constant $k_1 = 4.14 \times 10^5\ M^{-1}s^{-1}$.

Recovery of the native HRP from Compound II is the rate-controlling step. Consequently, it is assumed that $k_2 >> k_3$ and a simplified rate equation is obtained from Equation 14. Other researchers (*14,15*) have shown that k_2 is about 10 times larger than k_3.

$$V = \frac{k_3[E]_{tot}[AH_2][H_2O_2]}{\frac{k_3}{k_1}[AH_2] + [H_2O_2]} \tag{32}$$

The parameters V'_{max} and K'_m become:

$$V'_{max} = k_3[E]_{tot}[AH_2] \tag{33}$$

$$K'_m = \frac{k_3}{k_1}[AH_2] \tag{34}$$

Plots of parameters V'_{max} and K'_m as functions of $[AH_2]$ thus yield straight lines with slopes of $k_3[E]_{tot} = 5.32 \times 10^{-4}\ s^{-1}$ and $k_3/k_1 = 0.130$, respectively. These values of the rate constants k_1 and k_3 are $4.26 \times 10^5\ M^{-1}s^{-1}$ and $5.54 \times 10^4\ M^{-1}s^{-1}$, respectively. The values of k_1 determined directly from Mechanism I (Eq. 18) or by using the simplified form of the rate equation (Eqs. 33 and 34) are in good agreement. Based on this study, it is impossible to draw any conclusions about the relative magnitude of the rate of Compound I conversion to Compound II. Finally, the reason that V'_{max} and K'_m increase with an increase in phenol concentration, as illustrated in Table 1, can now be explained by considering Eq. 18 or Eqs. 33 and 34. It is clear from these equations that V'_{max} and K'_m are directly proportional to the phenol concentration.

CONCLUSIONS

The oxidation of phenol by HRP and H_2O_2 follows Michaelis-Menten kinetics. The experimental data strongly suggests that the reaction mechanism is irreversible. The rate constant, k_3, for the rate-controlling step (conversion of Compound II to the native enzyme) is $5.54 \times 10^4\ M^{-1}s^{-1}$, and the rate constant, k_1, for the formation of Compound I, is $4.14 \times 10^5\ M^{-1}s^{-1}$.

REFERENCES

1. Klibanov, A. M., Tu, T. -M., and Scott, K. P. (1986), *Science* **221,** 259–261.
2. Maloney, S. W., Manem, J., Mallevialle, J., and Fiessinger, S. (1986), *Environ. Sci. Technol.* **20,** 249–253.

3. Alberti, B. N. and Klibanov, A. M. (1981), *Biotechnol. Bioeng. Symp.* **11,** 373–379.
4. Nicell, J. A., Bewtra, J. K., Taylor, K. E., Biswas, N., and St. Pierre, C. (1992), *Water Sci. Technol.* **25,** 157–164.
5. Klibanov, A. M. and Morris, E. D. (1981), *Enzyme Microb. Technol.* **3,** 119–122.
6. Klibanov, A. M., Alberti, B. N., Morris, E. D., and Felshin, L. M. (1980), *J. Appl. Biochem.* **2,** 414–421.
7. Nicell, J. A. (1994), *J. Chem. Tech. Biotech.* **60,** 203–215.
8. Danner, D. J., Brignaz, P. J., Arceneaux, Jr., D., and Patel, V. (1973), *Arch. Biochem. Biophys.* **156,** 759–763.
9. Ryu, K., McEldoon, J. P., Pokora, A. R., Lyms, W., and Dordick, J. S. (1993), *Biotechnol. Bioeng.* **42,** 807–814.
10. Dundord, H. B. (1991), in Peroxidases in Chemistry and Biology, Vol. II, Everse, J., Everse, K. E., and Grisham, M. B. eds., CRC, Boca Raton, FL, pp. 1–24.
11. Vasudevan, P. T. and Weiland, R. H. (1990), *Biotechnol. Bioeng.* **36,** 783–789.
12. Chance, B. (1952), *Arch. Biochem. Biophys.* **41,** 404–415.
13. George, P. (1953), *J. Biol. Chem.* **201,** 427–434.
14. Job, D. and Dunford, H. B. (1976), *Eur. J. Biochem.* **66,** 607–614.
15. Dunford, H. B. and Adedrain, A. J. (1986), *Arch. Biochem. Biophys.* **251,** 536–542.