

Steady-state and Transient-state Kinetic Studies on the Oxidation of 3,4-Dimethoxybenzyl Alcohol Catalyzed by the Ligninase of *Phanerochaete chrysosporium* Burds*

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Catalysis of the H_2O_2 -dependent oxidation of 3,4-dimethoxybenzyl (veratryl) alcohol by the hemopro-tein ligninase isolated from wood-decaying fungus, *Phanerochaete chrysosporium* Burds, is characterized. The reaction yields veratraldehyde and exhibits a stoichiometry of one H_2O_2 consumed per aldehyde formed. Ping-pong steady-state kinetics are observed for H_2O_2 ($K_M = 29 \mu M$) and veratryl alcohol ($K_M = 72 \mu M$) at pH 3.5. The magnitude of the turnover number varies from 2 to 3 s^{-1} at this pH, depending on the preparation of the enzyme. Each preparation of enzyme consists of a mixture of active and inactive enzyme. Extensive steady-state kinetic studies of several different preparations of enzyme, suggest a mechanism in which H_2O_2 reacts with enzyme to form an intermediate that subsequently reacts with the alcohol to return the enzyme to the resting state. The pH dependence of the overall reaction indicates that an ionization occurs having an apparent $pK_a \sim 3.1$. The activity is, thus, nearly zero at pH 5 and increases to a maximum near pH - 2. However, the enzyme is unstable at this low pH.

Transient-state kinetic studies reveal that, upon reaction of ligninase with H_2O_2 , spectral changes occur in the Soret region, which, by analogy to previous studies of horseradish peroxidase, are consistent with formation of Compounds I and II. The active form of the enzyme appears to react rapidly with H_2O_2 ; we observed a positive correlation between the turnover number of the enzyme preparation and the extent of a rapid reaction between H_2O_2 and ligninase to form Compound I. Free radical cations derived from veratryl alcohol do not appear to be released from the enzyme during catalysis; however, other substrates are known to be converted to cation radicals (Kersten, P., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985) *J. Biol. Chem.* 260, 2609-2612). Our results are generally consistent with a classical peroxidase mechanism

for the action of ligninase on lignin-like substrates.

Lignin is an essential component of woody tissues imparting structural rigidity to vascular plants (1). Next to cellulose, it is the most abundant renewable carbon source on Earth. It is synthesized during secondary cell wall thickening by a peroxidase-catalyzed formation of free radicals of substituted phenolic phenylpropane units (2). These radicals couple to form an aromatic polymer which contains more than 12 types of interunit linkages (3). Lignin is remarkably resistant to degradation by most microbes; nevertheless, a few species of white-rot wood-destroying fungi are able to catalyze its oxidation to the level of CO_2 (4, 5). The ability of cultured fungi to degrade lignin is induced by depriving them of nutrient nitrogen, carbon, or sulfur (6). In response to nitrogen starvation, the cells secrete H_2O_2 (7, 8) and an enzyme which, in the presence of H_2O_2 , promotes lignin oxidation (9, 10). The enzyme from the basidiomycete *Phanerochaete chrysosporium* Burds. has been purified and partially characterized as a hemoprotein which requires H_2O_2 as a co-substrate for the oxidation of lignin and related substrates (9-13).

A number of phenolic and nonphenolic lignin-related compounds have been shown to be substrates for this enzyme. The nonphenolic substrates share a structural similarity in being methoxybenzene derivatives (9-13). Recent studies have shown that substrate-centered free radicals are detected during oxidation of methoxybenzene derivatives (14), thereby implicating formation of aryl cation radicals in the mechanism of the enzyme (14, 15). One substrate of ligninase is 3,4-dimethoxybenzyl (veratryl) alcohol, which is oxidized by 2 electrons to the corresponding aldehyde (12). The simplicity of this reaction is in contrast to the reaction with other substrates, which can involve hydroxylation and/or oxidation to yield multiple products, some of which are substrates themselves (12, 13). Preliminary experiments (12) indicated that this reaction exhibited normal Michaelis-Menten saturation kinetics with respect to both substrates, H_2O_2 and veratryl alcohol. Thus, this system is amenable to detailed mechanistic examination. Here we describe the results of steady-state and transient-kinetic studies of the ligninase-catalyzed oxidation of veratryl alcohol by H_2O_2 .

EXPERIMENTAL PROCEDURES

Enzyme Preparations—Ligninase was isolated and purified from *P. chrysosporium* strain BKM1767 (ATCC 24725) as reported previously (11), except that 5 mM sodium succinate, pH 5.5, was used as the buffer throughout the procedure. Purification at this pH yields better resolution on the DEAE Bio-Gel A column. Purity of the

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ligninase was assessed by four methods: fast performance liquid chromatography (Mono-Q ion exchange column; Pharmacia) (16), native gel electrophoresis (16), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17), and isoelectric focusing (11). By these criteria, the protein preparations were greater than 95% pure. Recent reports have demonstrated that multiple ligninase are produced by *P. chrysosporium* (16). The enzyme used in this study is the same as that described by the initial report of Tien and Kirk (9, 11), and recently designated ligninase H8 by Kirk et al. (16).

The extinction coefficient at 409 nm initially reported for this ligninase, which contains one heme/enzyme, was $102 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (11). This value was based on biuret determination of the protein content and calculated using a M_r of 42,000. We have subsequently demonstrated that the ligninase is a glycoprotein (at least 14% by weight). Thus, the M_r , as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11), is high. Recalculation of the 409 nm extinction coefficient by quantitation of the heme by the pyridine hemochromogen method (*cf.* Ref. 18) yielded a new value of $168 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. The extinction coefficient of myoglobin was determined by this procedure and yielded a value of $165 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, in close agreement with the literature value of $157 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (19). The value of $168 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ was used in all calculations in the present study. This value is higher than $130 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ reported by Gold et al. (12) for a ligninase also isolated from *P. chrysosporium*.

Materials—Veratryl alcohol and tartaric acid were obtained from Aldrich Chemical Co. Prior to use, veratryl alcohol was vacuum distilled to free it of the trace contaminant methyl-3-methoxy-4-hydroxybenzoate (identified by gas chromatography/mass spectrometry). Unless removed, this phenolic contaminant caused distinct lag periods in the initial velocity experiments (see below). The concentration of the stock H_2O_2 (Mallinckrodt) solutions, prepared daily, was determined by titration with potassium permanganate in 5.4% sulfuric acid. 5,5'-Dimethyl-1-pyrroline-*N*-oxide was a product of Sigma and vacuum distilled prior to use. All other chemicals were reagent grade and were used without further purification.

Stopped Flow Apparatus—The stopped flow apparatus and the associated computer systems used were those described by Bull et al. (20). The apparatus uses three driving syringes of 0.25, 2.5, and 2.5 ml capacity, delivering volumes of 0.02, 0.2, and 0.2 ml, respectively. The small syringe contained the H_2O_2 solution, whereas one of the large syringes contained enzyme, weakly buffered with 1 mM sodium tartrate, pH 4.5, to maintain stability. The other large syringe contained a stronger buffer (usually 105 mM sodium tartrate, pH 3.5) and, when used, veratryl alcohol. The apparatus uses two mixers: with the H_2O_2 and the buffered substrate solution mixed first. The resulting solution is combined with the enzyme in the second mixer. The dead time from the first mixer to the observation cell is 5 ms; from the second mixer it is only 2 ms. The observation path length is 2 cm, and the light has a nominal band width of 3 nm.

The initial velocity of veratraldehyde formation was measured by the increase in 310 nm absorbance (veratraldehyde extinction at $310 \text{ nm} = 9300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (11)). Each kinetic trace consisted of 151 data points. In certain experiments three traces were averaged to reduce noise. In single wavelength experiments where the Soret region of the ligninase was monitored, each trace consisted of 401 data points divided into two time ranges; three traces were averaged. Rapid-scan experiments were performed with the same stopped flow apparatus and computer system. The rapid-scan monochromator (model RSS-1; Harrick Inc., Ossining, NY) was controlled by software from OLIS Inc., Athens, GA. Light from a Canrad-Hanovia 150 watt short-arc Xenon lamp powered by an OLIS XE-150 highly regulated supply was imaged on the entrance slit at 2-fold magnification. Additional reflective optics constructed at the University of Michigan cast a one-third magnified image of the exit slit on the UV fiber optic leading to the stopped flow. A quartz plate directed about 10% of the radiation from the exit slit into a reference photomultiplier. Instability caused by arc fluctuations was cancelled by dividing the stopped flow photomultiplier tube signal by the reference photomultiplier tube signal using an analog Devices AD535K divider chip (1 MHz small signal band width). Typically, 101 data points were collected from 350 to 450 nm at $20 \mu\text{s}/\text{datum}$; scans could be initiated as little as 5 ms apart. Tests as high heme concentration indicated that stray light in the Soret region was less than 1%. Overall resolution was estimated to be about 5 nm based on the shape of holmium oxide wavelength calibration spectra.

ESR Measurements—The formation of free radicals during enzyme-catalyzed veratryl alcohol oxidation was investigated using ESR

spectroscopy. Measurements were performed with a Varian E-109 spectrometer operating at 9.5 GHz. Reaction mixtures were examined at room temperature in a quartz flat cell. A typical ESR reaction solution contained $0.5 \mu\text{M}$ ligninase, 0.4 mM H_2O_2 , 2 mM veratryl alcohol, and 10 mM 5,5'-dimethyl-1-pyrroline-*N*-oxide in 50 mM sodium tartrate at pH 3.5. Oxygen was removed in some experiments by purging with nitrogen.

RESULTS

Stoichiometry and Inhibitors—The ligninase-catalyzed oxidation of veratryl alcohol to veratraldehyde exhibits a stoichiometry of 1 H_2O_2 utilized per aldehyde formed (Table I). This stoichiometry prevailed at all H_2O_2 concentrations examined (2.2–260 μM) in the presence of 2 mM veratryl alcohol (Table I), and no other products were observed. When the ratio of H_2O_2 to alcohol was raised to 2:1, other unidentified products (less than 5%) were detected (data not shown). The presence of dioxygen had no effect on the stoichiometry at low H_2O_2 concentrations. At high H_2O_2 concentrations, less than stoichiometric amounts of veratraldehyde were formed in aerobic incubations. Therefore, under the conditions of our kinetic experiments, the reaction is occurring with a well defined stoichiometry, and O_2 is probably not a reactant. In other experiments, for which we present no data, it was found that the enzyme is inhibited by F^- ($K_i = 0.8 \text{ mM}$).¹ Because CO was found to form a complex with the reduced form of the protein (12), we tested for CO inhibition of the reaction and found none. Therefore, the reduced form of the enzyme does not appear to form during catalysis.

pH Optimum and Activation Energy—The time course of veratraldehyde formation is shown in Fig. 1. The traces shown were obtained from stopped flow shots at various pH values under the condition that substrate concentration is much greater than K_M for both substrates; the traces show linear rates of veratraldehyde formation over a period of at least 45 s. The turnover number derived from each trace is plotted as a function of pH in the inset of Fig. 1. The reaction exhibits an apparent optimum near pH 2 with activity rapidly decreasing at higher acidities. This decrease is probably caused by denaturation of the protein because the kinetic traces have a much shorter period of linearity under these conditions. Above pH 2, the decrease in activity seems to be caused by the deprotonation of a group required for catalysis. Indeed, the data shown in the inset of Fig. 1 are nicely fit by a single ionization having $\text{p}K_a \sim 3.1$ and using turnover number_{max} $\sim 7.8 \text{ s}^{-1}$ is indicated by the solid line. No appreciable activity was observed at pH values greater than 5.

The overall reaction velocity, under the condition that substrate concentration is much greater than K_M for both substrates, was measured over the temperature range 4–27 °C at pH 3.5 in tartrate buffer. The Arrhenius plot was linear in this temperature range (data not shown) and yielded an activation energy of 14.7 kcal/mol. These results suggest that a single mechanism is operative under the conditions of our experiments.

Steady-state Kinetics—The mechanism of the H_2O_2 -dependent, ligninase-catalyzed oxidation of veratryl alcohol was investigated using initial velocity studies. The concentrations of both substrates were varied in a systematic manner and the results analyzed assuming steady-state conditions. Fig. 2A shows the double reciprocal plot from an experiment in which the concentration of veratryl alcohol was varied in the presence of several different fixed concentrations of H_2O_2 at pH 2.5. The results show essentially parallel lines, suggesting a

¹The mode of inhibition was competitive with respect to H_2O_2 . The K_i is approximately equal to the K_D for F^- (1.0 mM).

TABLE I

Stoichiometry of veratryl alcohol oxidation

Experimental conditions were as described under "Experimental Procedures."* Reaction mixtures contained 1.6 μM ligninase, 2 mM veratryl alcohol, 50 mM sodium tartrate, pH 3.5, and the specified amount of H_2O_2 . Reactions were initiated by H_2O_2 addition. After completion, veratraldehyde was quantitated at 310 nm.

[H_2O_2]	[Veratraldehyde]	Aldehyde/ H_2O_2
	μM	
2.15	2.14	0.99
12.9	13.4	1.04
21.5	21.4	0.99
43.0	44.7	1.04
86.0	91.8	1.07
129	135	1.06
258	267	1.04

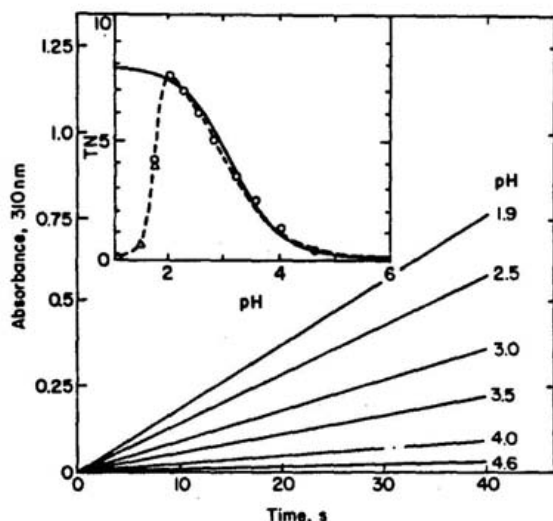


FIG. 1. Time course of veratraldehyde formation as a function of pH. The increase in 310 nm absorbance is plotted versus time at various pH values. Reaction mixtures contained saturating H_2O_2 (0.4 mM) and veratryl alcohol (2 mM) with ligninase (0.17 μM) in 50 mM sodium tartrate (O) or sodium phosphate (Δ) at the specified pH values. Each trace resulted from a stopped flow shot. The inset is a plot of the turnover number (TN) versus pH derived from these and other curves. The solid line corresponds to a controlling ionization with $\text{p}K_a = 3.1$ and turnover number $_{\text{max}} = 7.8 \text{ s}^{-1}$.

ping-pong mechanism. Fig. 2B is the secondary replot of the slopes from Fig. 2A versus the reciprocal of H_2O_2 concentrations. This plot yielded a line with zero slope and an intercept of $7.8 \times 10^6 \text{ M}\cdot\text{s}$ (K_M (veratryl alcohol)/turnover number). A replot of the y intercepts from Fig. 2A versus the reciprocal of H_2O_2 concentrations indicated a K_M for H_2O_2 of 83 μM and a turnover number of 8.4 s^{-1} (Fig. 2C). The K_M for veratryl alcohol was then calculated to be 57 μM . Kinetic parameters at three values of pH using a single preparation of enzyme are presented in Table II. The K_M for veratryl alcohol is not affected by pH, whereas that of H_2O_2 decreases in approximate accord with a single ionization. As already shown in Fig. 1, turnover number decreases as the pH is raised. The productive binding rate for H_2O_2 (turnover number/ K_M) does not change significantly with pH, whereas for veratryl alcohol this parameter decreases.

Different preparations of enzyme have rather different values of turnover number, with the highest activity being 3.4 s^{-1} at pH 3.5. However, we observed little variation in the K_M values for veratryl alcohol and for H_2O_2 among different preparations (Table III). These differences could not be attributed to variation in protein purity as assessed by four different chromatographic techniques (see "Experimental

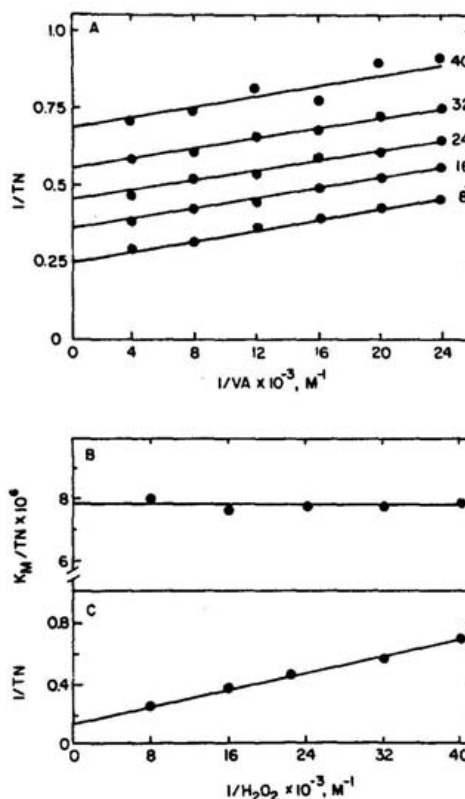


FIG. 2. Determination of the steady-state kinetic parameters for ligninase at pH 2.5. Panel A, double reciprocal plot of the turnover number (TN) versus veratryl alcohol (VA) concentration at different levels of H_2O_2 . Numbers to the right of the lines indicate the H_2O_2 concentration as inverse millimolar units. The ligninase concentration was 0.17 μM and the buffer was 50 mM sodium tartrate, pH 2.5. Each point is the average of three stopped flow traces. Panel B, the slopes from panel A are plotted against the inverse H_2O_2 concentrations. The value of K_M /turnover number for veratryl alcohol is calculated from the y intercept. Panel C, the intercepts from panel A are plotted against inverse H_2O_2 concentrations. Turnover number and K_M are calculated from the y and x intercepts, respectively.

TABLE II

Steady-state kinetic parameters of ligninase at different pH values

The experimental conditions were as described under "Experimental Procedures." The kinetic parameters were determined from initial velocity studies where the concentration of veratryl alcohol (VA) and H_2O_2 were varied as described in the legend to Fig. 2A. Note that the values for turnover number (TN) are somewhat higher than those reported in Fig. 1. This variation undoubtedly arises from differences among enzyme preparations. While the variation prevents a proper analysis of error, we estimate that the internal error is less than 10%.

pH	K_M	K_M	TN	TN/ $K_M(\text{H}_2\text{O}_2)$	TN/ $K_M(\text{VA})$
	$\mu\text{M VA}$	$\mu\text{M H}_2\text{O}_2$			
2.5	57	83	8.4	10.1×10^4	14.7×10^4
3.5	72	29	2.9	9.9×10^4	4.0×10^4
4.5	54	11	0.8	6.9×10^4	1.4×10^4

Procedures"); neither were any differences in optical or ESR spectral properties noted among different preparations. As noted below, there are differences in the kinetic reactivity of different preparations toward H_2O_2 .

H_2O_2 Inhibition—At concentrations of $\sim 3 \text{ mM}$, H_2O_2 inhibits the reaction. When the concentration of veratryl alcohol was varied in the presence of several inhibitory concentrations of H_2O_2 , a competitive pattern of inhibition was observed (Fig. 3A). The secondary replot of the slopes of the double reciprocal lines versus the corresponding concentrations of H_2O_2 (Fig. 3B) was linear and indicated a K_I of $\sim 2.8 \text{ mM}$.

TABLE III

Steady-state kinetic parameters of two different preparations of ligninase

The experimental conditions were as described under "Experimental Procedures." The kinetic parameters were determined from initial velocity studies at pH 3.5 where the concentration of veratryl alcohol (VA) and H_2O_2 were varied as described in the legend to Fig. 2A.

	K_M μM VA	K_M μM H_2O_2	TN ^a s^{-1}	TN/ $K_M(H_2O_2)$ $M^{-1} \cdot s^{-1}$	TN/ $K_M(VA)$ $M^{-1} \cdot s^{-1}$
I	83	24	2.6	10.8×10^4	3.1×10^4
II	67	21	1.4	6.7×10^4	2.1×10^4

^a TN, turnover number.

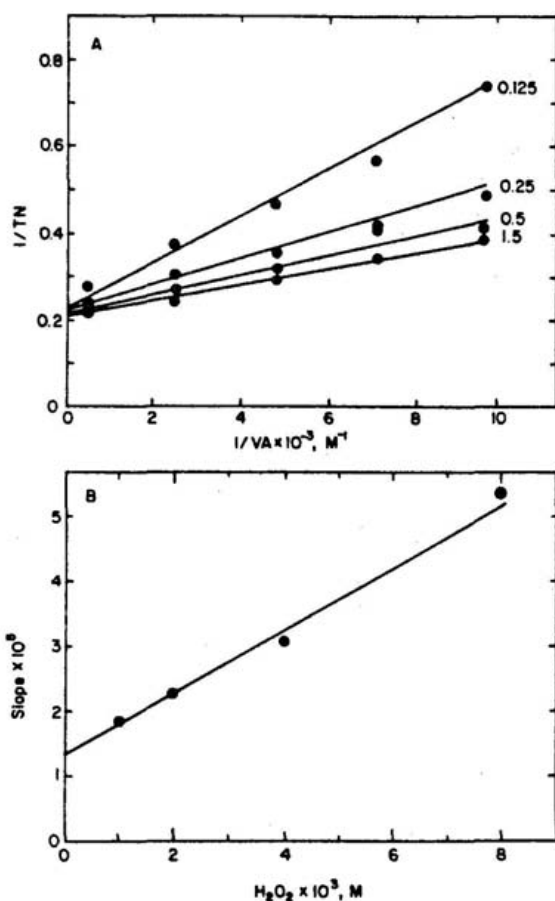


FIG. 3. Evidence for inhibition of ligninase by H_2O_2 . Panel A, double reciprocal plots at concentrations of H_2O_2 approximately 8-fold greater than those used in the experiments of Fig. 2. The H_2O_2 concentrations are as indicated to the right of the lines in reciprocal millimolar units. The reaction mixture contained $0.17 \mu M$ ligninase and the indicated veratryl alcohol (VA) concentration in 50 mM sodium tartrate, pH 3.5. Panel B, plot of the slopes from panel A versus the H_2O_2 concentrations. TN, turnover number.

Transient Kinetics—The reaction of H_2O_2 with resting ligninase was studied using stopped flow and rapid-scan stopped flow techniques in the Soret region of the spectrum. The scans, performed over two different time intervals, show formation of two distinct spectral intermediates (Fig. 4). The first species is formed rapidly (first five scans taken at 5-ms intervals) and is spectrally similar to resting enzyme, except for a decrease in the extinction coefficient, and shows an isobestic point with the resting enzyme at $\sim 430 \text{ nm}$. The second species is formed more slowly (five scans taken at 800-ms intervals). It also has a decreased extinction coefficient, exhibits a red shift in its maximum absorbance to $\sim 416 \text{ nm}$, and has an isobestic point with the oxidized protein at $\sim 420 \text{ nm}$. The latter spectrum is similar to that reported previously

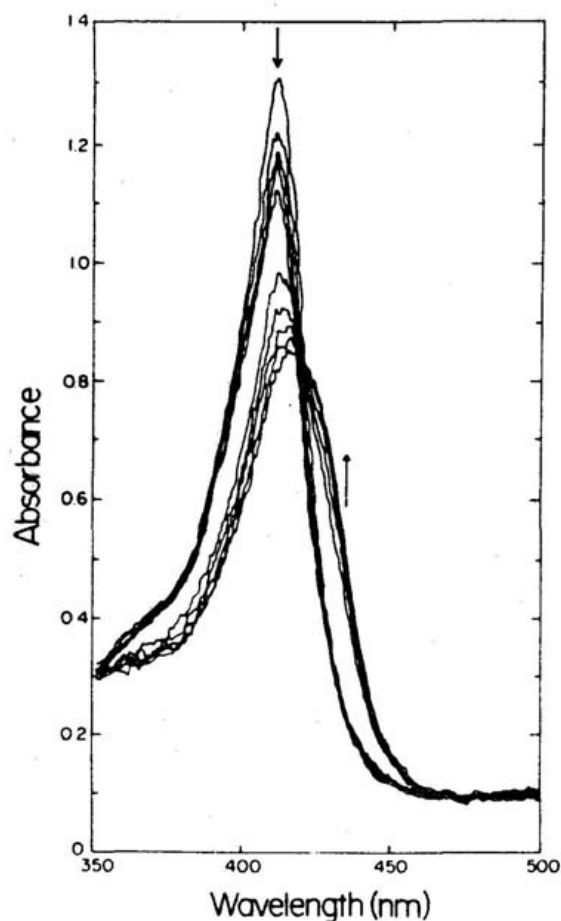


FIG. 4. Rapid-scan spectral analysis of the reaction between H_2O_2 and ligninase. Plot shows changes in absorbance at wavelengths in the Soret region. The topmost scan was taken at time 0, the next five scans were taken at 5-ms intervals, and the last five scans were taken at 800-ms intervals. An individual scan requires 3 ms, therefore, the first few spectra are not fully resolved. Each spectrum is the average of four stopped flow shots. Arrows indicate direction of spectral change. After mixing, the concentration of ligninase was $5.3 \mu M$; the H_2O_2 concentration was $100 \mu M$; and the buffer was $50 \mu M$ sodium tartrate, pH 3.5, at $25^\circ C$.

in which H_2O_2 was added to resting enzyme in a standard optical cuvette (12). No spectral changes were observed upon reaction of ligninase with veratryl alcohol in the absence of H_2O_2 .

The biphasic nature of the reaction of H_2O_2 with ligninase was further examined with single wavelength, stopped flow techniques. Fig. 5A shows the changes in 409 nm absorbance upon reaction of H_2O_2 with nearly equal concentrations of two different preparations of the ligninase. As expected, two different reactions are observed, well separated in time. The first reaction is rapid and complete within 100 ms and is followed by the slower reaction which is complete within $\sim 30 \text{ s}$. In examining the first phase with two different ligninase preparations, we observed a considerable difference in the extent of the initial decrease in absorbance at 409 nm. The total change in absorbance on reaction with H_2O_2 was, however, independent of preparation. The inset in Fig. 5 plots the first order rate constants observed from the reaction of ligninase with H_2O_2 as a function of H_2O_2 concentration. With the fast initial reaction, the line intercepts the origin, so the reaction does not appear reversible. The slope of the line gives a second-order rate constant of $5.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. The slope derived from the slow reaction yields a second-order rate constant of $2.1 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. The single wavelength traces

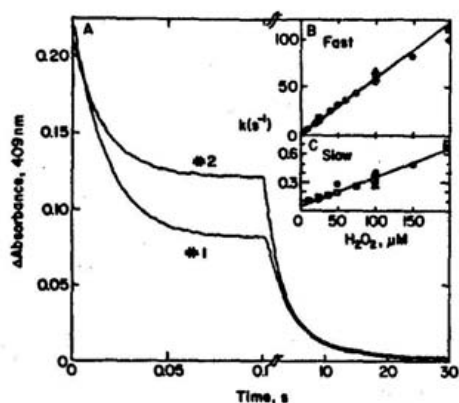


FIG. 5. Panel A, reaction of two ligninase samples with H_2O_2 demonstrating the biphasic bleaching of the Soret band. The data is plotted as the change in 409 nm absorbance ((observed absorbance)-(final absorbance)). After mixing, the concentration of samples 1 and 2 were 0.16 and 0.18 μM ; the concentration of H_2O_2 was 100 μM ; and the reaction was buffered with 50 mM sodium tartrate, pH 3.5. Panels B and C show the dependence of the pseudo-first order rate constants on the concentration of H_2O_2 for the fast and slow reaction, respectively. The results of five different experiments with samples 1 (open triangles) and 2 (closed triangles) are shown. The other symbols indicate different preparations of enzyme.

shown in Fig. 5 were repeated at several wavelengths. Plotting the change in absorbance observed in both phases of the reaction as a function of wavelength yielded the difference spectra shown in Fig. 6. The difference spectrum shown in the upper panel (fast phase) clearly resembles that of the Compound I of peroxidase (21) having a negative maximum near 410 nm and isobestic points with the oxidized protein at ~ 340 and ~ 435 nm. (The corresponding wavelengths observed for difference spectra of Compound I of horseradish peroxidase (21) are a negative peak at ~ 405 nm and isobestic points near 340 nm and 430 nm.) The difference spectrum of the intermediate formed during the slow phase resembles Compound II of peroxidase (21), showing a trough at ~ 408 nm, a peak at ~ 430 nm, and isobestic wavelengths at ~ 419 and ~ 470 nm (lower panel). (The corresponding wavelengths for the difference spectrum of Compound II of horseradish peroxidase (21) are a trough at ~ 398 nm, a peak at ~ 425 nm, and isobestic points at ~ 420 nm and ~ 465 nm.)

Correlation between Bleaching in the Soret and Turnover Number—Although the difference spectrum obtained from the fast phase clearly resembles Compound I, the extent of the Soret bleaching with ligninase was not as large as that observed with catalase or peroxidase (21). Moreover, as noted above, the extent of rapid bleaching observed with ligninase varied with different preparations, and this was found to correlate with turnover number. The ratio of the rapid decrease in absorbance at 409 nm to the total change in 409 nm absorbance is plotted versus turnover number for several different preparations of enzyme (Fig. 7, A and B). There is clearly a linear correlation between the fraction of the heme able to react rapidly with H_2O_2 and the turnover number of the enzyme preparation. These data suggest that some of the enzyme molecules are modified prior to or during purification such that they react relatively slowly with H_2O_2 and are thus catalytically much less active (or inactive).

These differences in activity could not be attributed to variation in the purity of the protein. As noted, all preparations were found to be of similar purity (see "Experimental Procedures"). Spectroscopic studies of the heme in different preparations also did not reveal any differences. The ESR spectra of four different preparations of ligninase with varying

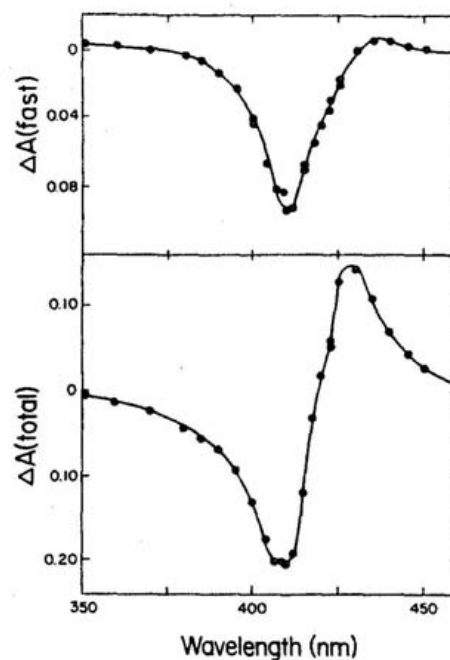


FIG. 6. Difference spectra of the intermediate species. The upper panel corresponds to the difference between the spectra of the rapidly formed species and the resting enzyme. Each data point was taken as the difference between absorbance at approximately 50–100 ms after mixing and the absorbance at zero time. The apparent amplitude of the fast phase was multiplied by 1.13 to account for the dead time of the instrument and plotted accordingly. The lower panel corresponds to the difference between the spectra of the species formed in the slow phase (which accounted for the total absorbance change) and the resting enzyme. Each data point was taken as the difference between the absorbance at approximately 30 s after mixing (total change) and the absorbance at zero time. After mixing, the incubation contained 100 μM H_2O_2 and 1.2 μM ligninase in 50 mM sodium tartrate, pH 3.5. Absorbance values are for a 2-cm light path and the initial absorbance was 0.38 at 409 nm.

values of turnover number indicated the presence of only high-spin ferric heme, and addition of H_2O_2 to each of these preparations diminished the ESR signal intensity by more than 90%; a result that is consistent with formation of either Compound I or II (22). The optical properties of the ferric, the reduced, the reduced-CO complex, the reduced-oxy complex, and the fluoro- and azido-complexed forms of oxidized ligninase are all consistent with the presence of only one heme environment in the different preparations (data not shown).

Enzyme-monitored Turnover—As shown in Fig. 5A, mixing H_2O_2 with ligninase caused a decreased absorbance at 409 nm. This is the result of two second order processes differing in rates by a ratio of ~ 280 . We examined the effect of including a constant amount of veratryl alcohol as the concentration of H_2O_2 was increased on these processes. The absorbance changes that occur on mixing the enzyme with both substrates are shown in Fig. 8. When the H_2O_2 concentration is less than the veratryl alcohol concentration, both the fast and slow phases are diminished in amplitude, and the final absorbance returns to that of ferric enzyme (curves 1–3). For comparison, curve 4 shows the results of an experiment in which veratryl alcohol was omitted. If the fast phase of Fig. 8 is representative of the overall enzyme activity, then one expects that its amplitude should vary with H_2O_2 concentration in a manner such that half-maximal amplitude occurs at the H_2O_2 concentration corresponding to the K_M for H_2O_2 ; this is roughly what is observed. Thus, for curve 2, the H_2O_2 concentration is 25 μM ($K_M = 29 \mu\text{M}$) and approximately half-maximal amplitude is observed. This strongly suggests that the fast phase ob-

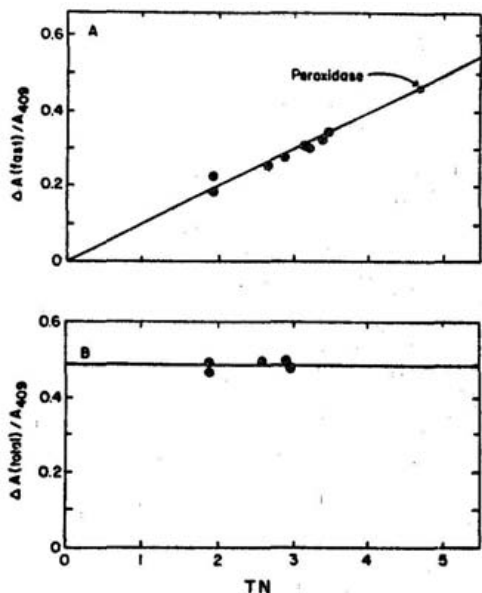


FIG. 7. Correlation of specific enzymatic activity with the amplitude of the fast (A) and slow (B) reaction with H_2O_2 . Amplitude of the fast phase and slow phase (total change) were measured as described in the legend of Fig. 5 for five different preparations of enzyme. The turnover number (TN) was determined from the standard assay mixture: 50 mM sodium tartrate, pH 3.5, 0.4 mM H_2O_2 , 2 mM veratryl alcohol, and six enzyme concentrations between 0.5 and 1 μM .

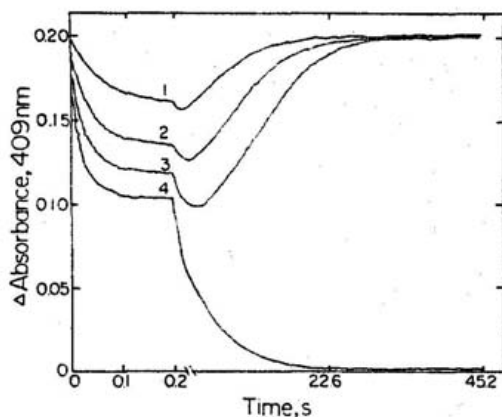


FIG. 8. Enzyme-monitored turnover studies of ligninase. Plot shows the change in absorbance versus time similar to those described in the legend of Fig. 5 except that varying amounts of H_2O_2 and veratryl alcohol were included in the reaction mixture. The fast phase was monitored over a period of 0.2 s and the slow phase over a period of 45 s. After mixing, the reaction mixtures contained 1.26 μM ligninase in 50 mM sodium tartrate, pH 3.5, and the following additives: 2 mM veratryl alcohol, 12.5 μM H_2O_2 (curve 1); 2 mM veratryl alcohol, 25 μM H_2O_2 (curve 2); 2 mM veratryl alcohol, 50 μM H_2O_2 (curve 3); 50 mM H_2O_2 (curve 4).

served in these experiments is in the dominant catalytic cycle. The rate of return to resting enzyme was unchanged when the concentration of veratryl alcohol was decreased to 0.14 mM (data not shown), suggesting that this process is nominally first order and allows one to estimate a rate constant of $\sim 0.1 \text{ s}^{-1}$. Compared to turnover number, this process is much too slow to be involved in catalysis.

Spin-trapping Experiments—Previous studies have shown that free radicals can be formed during ligninase-catalyzed reactions, although this seems to be substrate dependent. Thus in the cases of methoxybenzenes, Kersten et al. (14) demonstrated with ESR and optical spectroscopy the cation free radicals are products of enzymatic catalysis. Harvey et

al. (23) have demonstrated that the one-electron oxidant Fe(III) (orthophenanthroline)₃ is able to promote C_1 – C_2 cleavage of 1,2-di(3,4-dimethoxyphenyl)-1,3-propanediol to the same products as ligninase and have suggested that an “oxyferryl” heme complex of the enzyme acts to produce a cation radical in the substrate. Shoemaker *et al.* (24) have speculated on a more general one-electron mechanism of lignin degradation. More recently, Hammel *et al.* (15) have used spin-trapping agents to demonstrate free radical formation during C_1 – C_2 cleavage. We have attempted to detect free radicals by the use of the spin-trap 5,5'-dimethyl-1-pyrroline-*N*-oxide during the action of ligninase on veratryl alcohol (see “Experimental Procedures”); none was observed.

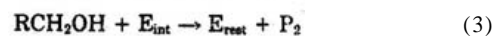
DISCUSSION

The above results provide an experimental base for formulating a mechanism of ligninase catalysis. The following observations must be considered: (a) there exists a 1:1 stoichiometry in the oxidation of veratryl alcohol by H_2O_2 ; (b) the enzyme operates through a ping-pong mechanism during steady-state catalysis; (c) peroxidase-like Compounds I and II are observed in transient kinetic experiments; (d) free radicals are formed with some substrates but apparently not with others, in particular not with veratryl alcohol; and (e) an unidentified residue at the active site must be protonated in order for the enzyme to be active. In addition, there are two complexities which must be of concern: (a) the enzyme is inhibited by modest concentrations of H_2O_2 , and (b) there is a dependence of enzyme activity on the history of the protein sample.

The stoichiometry of the reaction is consistent with the overall process



in which RCH(OH)_2 corresponds to the hydrated aldehyde which will be in equilibrium with the non-hydrated form. This is independent of the presence of dioxygen as shown by the results of Table I. A ping-pong mechanism requires that the enzyme operate by first forming an intermediate with one of the substrates followed by a reaction with the second substrate to return it to its initial or resting state. It is reasonable to formulate this as follows

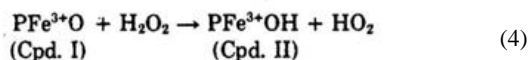


Where the nature of the products and the intermediates remains undefined.

The observation of intermediates having spectral properties similar to Compounds I and II suggests that these compounds may be intermediates in the reaction scheme. The second order reaction of H_2O_2 with active, resting ligninase to form Compound I has a rate constant of $5.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at pH 3.5, and this is comparable to turnover number/ K_M $1.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for the overall reaction under turnover conditions. The latter value must be considered a minimum because it does not include a correction for the amount of active enzyme contributing to activity. The observations of Fig. 8 show that the formation of Compound I is suppressed when veratryl alcohol is present in the reaction mixture. This suggests that the alcohol may react with Compound I either directly to form resting enzyme and aldehyde, an apparent oxygenation reaction, or to form Compound II and a cation-free radical. The transient kinetic observations give no evidence that Compound II formation is promoted in the presence of the alcohol, and attempts to detect intermediate-free radicals by

spin-trapping were unsuccessful. These results favor the possibility that the alcohol is converted directly to product at the active site either by oxygenation or by two rapid and consecutive one-electron oxidations. Our data do not allow us to distinguish the latter two processes; however, with other substrates such as methoxybenzenes, solid evidence exists that free radicals are formed as products (14). We predict that Compound II formation will be enhanced by these substrates? Finally, the data require that an unidentified ionization occur at the active site having an apparent $pK_a \sim 3.1$. Preliminary attempts to detect an ionization in the oxidized protein which affects the spectral properties of the heme were unsuccessful. Moreover, the rate of reaction of H_2O_2 with active enzyme is essentially independent of pH, suggesting that, if this ionization occurs in the oxidized protein, it does not involve large changes in the heme environment. It is, thus, reasonable to speculate that the ionization affecting activity is actually occurring in one of the intermediate forms of the enzyme. The above formulation accounts for all available data,³ and suggests that ligninase acts in a manner entirely analogous to other peroxidases (28).

H_2O_2 is both a substrate and a competitive inhibitor of catalysis.⁴ The data of Fig. 5 offer a possible explanation for this behavior. The reaction of oxidized ligninase with H_2O_2 occurs in two phases. The rapid phase results in the formation of Compound I as shown by the kinetic difference spectra of Fig. 6; the subsequent slower reaction is consistent with the formation of Compound II. Both are first order reactions with respect to H_2O_2 . Conversion of Compound I (Cpd I) to II (Cpd II) can be explained if Compound I oxidizes H_2O_2 to the perhydroxyl radical HO_2



The perhydroxyl radical rapidly dismutates to dioxygen and H_2O_2 (29). Is reaction 4 sufficiently fast to account for inhibition of catalysis? Whereas the specific rate of reaction 4 is relatively small ($\sim 2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$), the velocity of reaction 4 is $\sim V_{\text{max}}$ when the H_2O_2 concentration is 2.8 mM ($\sim K_1$). This would suggest that half of the active enzyme is in the form of Compound II under these conditions. It is also necessary to postulate that the resulting Compound II is unable to convert substrate to product at a catalytically competent rate. This postulate is supported by observations (Fig. 8) showing the specific rate of conversion of Compound II to resting enzyme is ~ 30 times smaller than turnover number.

² Gold *et al.* (12) have demonstrated ethylene formation from H_2O_2 -dependent oxidation of α -keto- γ -methiolbutyric acid catalyzed by the ligninase in the presence of veratryl alcohol. Ethylene formation arises from a one-electron oxidation of α -keto- γ -methiolbutyric acid (25). Because ethylene formation arises from a one-electron oxidation, we suspect that it may be reacting with intermediates formed during veratryl alcohol oxidation.

³ The reader is referred to Refs. 26 and 27 for data concerning the physical properties of ligninase (ESR and resonance Raman of the heme active site).

⁴ To distinguish between competitive and noncompetitive inhibition, the results were submitted to computer analysis using the MLAB program available at NIH. Although the data were best fit to noncompetitive inhibition, the intercept effect was negligible. Thus the inhibition by high H_2O_2 is best described as competitive with veratryl alcohol.

The results present here do not provide a mechanism for the conversion of Compound II back to resting enzyme. This one-electron reduction occurs in the presence of veratryl alcohol, which leads us to speculate that veratryl alcohol serves as the reductant. If such is the case, it is uncertain whether this reaction leads to veratraldehyde formation. Because the low rate of this conversion and its lack of importance in turnover number, further details of this reaction were not pursued.

There remains the question of the preparation dependence of ligninase activity. We presently have no insight into this problem but our data exclude the idea that it is caused by a structural modification which affects the electronic properties of the heme in its oxidized form. However, whatever structural change that has occurred certainly affects the chemical behavior of the oxidized form of the protein because it greatly reduces the rate at which the enzyme reacts with H_2O_2 . It is, thus, likely that a structural change has occurred near the iron, restricting the approach of H_2O_2 to the iron. The presence of inactive enzyme has greatly limited our ability to formulate a more definite mechanism for the enzyme, surely interpretation of future kinetic studies will require a solution to this problem.

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