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The Ligninase of *Phanerochaete chrysosporium* Generates Cation Radicals from Methoxybenzenes*

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Philip J. Karsten‡§, Ming Tien‡, B. Kalyanararnan¶, and T. Kent Kirk‡§

From the ‡Forest Products Laboratory, United States Department of Agriculture-Forest Service, Madison, Wisconsin 53705, the §Department of Bacteriology, *University of Wisconsin, Madison, Wisconsin 53706, and the ¶National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226*

The hemoprotein ligninase of *Phanerochaete chrysosporium* **catalyzes, in the presence of H_2O_2, a variety of seemingly different oxidations in lignin and lignin model compounds. Here we show that the enzyme also catalyzes the oxidation of various methoxybenzenes. ESR spectroscopy shows that the compounds are oxidized to aryl cation radicals. These decompose, evidently by H_2O addition. Thus 1,4-dimethoxybenzene is converted to *p*-benzoquinone and methanol. We propose a unified mechanism, based on formation of aryl cation radicals, to explain the various reactions catalyzed by the ligninase.**

The basidiomycete *Phanerochaete chrysosporium* Burds. produces an extracellular lignin-degrading enzyme (1, 2) which has recently been purified and characterized (3). The hemoprotein catalyzes a variety of oxidations, all of which are dependent on H_2O_2. Among its repertoire is C_α-C_β cleavage of the propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, and phenol oxidation. Molecular oxygen is incorporated into the products of these first two reactions, thus making the enzyme unique as a H_2O_2-requiring oxygenase (3).

Little is known about the mechanism of the enzyme. Ita requirement for H_2O_2, however, resembles that of soybean lipoxygenase, which requires alkyl peroxides for maximal activity (4). Because free radicals have been shown to be involved in lipoxygenase catalysis (5), and because the ligninase catalyzes a number of seemingly different reactions suggestive of radical involvement, we tested the possible formation of radicals by the ligninase. We searched for radical intermediates from simple methoxy-substituted benzenes, because each phenylpropanoid unit in lignin contains one or two aryl methoxyl groups. We found that several of the methoxybenzenes are good substrates for ligninase, and, surprisingly, that some give strong ESR signals at room temperature. The spectra generated are readily interpreted by comparison with published spectra of the corresponding cation radicals generated nonenzymatically.

The simplest methoxybenzene to give an ESR signal is the 1,4-congener. Major products of its oxidation by ligninase/H_2O_2 are methanol and benzoquinone, predictable on the basis of a cation radical mechanism (6). Here we describe the action of the ligninase on the methoxybenzene congeners and propose that formation of cation radicals provides a unified mechanism for the enzyme's action. To our knowledge, this work is the first to describe detection of cation radicals from methoxybenzenes in enzymatic systems.

MATERIALS AND METHODS

Enzyme — The ligninase was produced and purified as previously described (3) using *P. chrysosporium,* strain BKM-F-1767 (ATCC 24725). A millimolar extinction coefficient of 102 at 409 nm was used to determine the concentration of purified ligninase (3).

Chemicals — The mono-, di-, and trimethoxylated benzenes were obtained from commercial sources and their purities were confirmed by thin layer chromatography. 1,2,3,4,-Tetramethoxybenzene was synthesized from 2,3,4-trihydroxyacetophenone by partial methylation to 2-hydroxy-3,4-dimethoxyacetophenone (7), followed by Dakin oxidation and further methylation, m.p. 87 °C (m.p. (literature) 89.5-90°C (8)). 1,2,3,5-Tetramethoxybenybenzene was prepared from 1,2,3,-trimethoxybenzene according to published procedures (7), m.p. 38 °C (m.p. (literature) 47 °C (7)). 1,2,4,5-Tetramethoxybenzene was obtained by methylation of 2,5-dihydroxybenzoquinone with absolute methanol and hydrogen chloride (9) followed by reduction and further methylation with dimethyl sulfate/alkali, m.p. 101-102 °C (m.p. (literature) 102-103 °C (8)). Pentamethoxybenzene was synthesized from 1,2,3,5-tetramethoxybenzene by the method of Baker (7). m.p. 59-60 °C (m.p. (literature) 58–59°C (7)). Hexamethoxybenzene was prepared by reduction of tetrahydroxyquinone (10) followed by exhaustive methylation with dimethyl sulfate/alkali under argon. The product was recrystallized from petroleum ether (b.p. 35-60 °C) and then from water/methanol to give white needles, m.p. 81 °C (m.p. (literature) 81 °C (11)). 1,4-[*methoxy*-^{2}H]Dimethoxybenzene was prepared by methylation of hydroquinone with C^2H_3I (Sigma 99+ atom %) and K_2CO_3. The product was sublimed under vacuum to give white crystals of m.p. 55-56 °C. Mass spectroscopy confirmed the identities of all synthesized compounds.

ESR Measurements — ESR measurements were performed on Varian E-9 and E-109 spectrometers operating at 9.5 GHz and employing 100 kHz field modulation. Magnetic field measurements were made with a Radiopan MJ-110 gaussmeter. For microwave frequency measurements an EiP 200 counter was used. Hyperfine splittings were measured (to 0.05 G) either directly from magnetic field separations or from computer simulations of spectra. The *g* values were obtained from measurements of magnetic field and microwave frequency, after correcting for the position of the gaussmeter probe. Radical concentrations were measured relative to a standard solution of the stable nitroxide radical 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (Aldrich), taking into account differences in line width and hyperfine multiplicity between the radical cation and the nitroxide spectra.

The reaction mixtures for ESR spectroscopy contained, in a final volume of 2 ml, 0.5 μM ligninase, 0.32 mM H_2O_2, 0.1 M sodium tartrate, pH 2.5, and 5 mM of the specified substrate as indicated in the figure legends. The reactions, at room temperature, were initiated by addition of enzyme and were scanned within 5 min. No ESR signal was observed in the absence of enzyme, H_2O_2, or substrate. With 1,2,4,5-tetramethoxybenzene, radical concentrations up to 200 μM were observed.

Product Identification from 1,4-Dimethoxybenzene—Incubations were performed to identify the products (methanol and benzoquinone)

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from 1,4-dimethoxybenzene. Reaction mixtures for methanol identification contained 1 mM 1.4-[methoxy-^{2}H]dimethoxybenzene, 2 mM H_2O_2, 1.2 μM ligninase, and 0.1 M sodium tartrate, pH 3.0, in a final volume of 5 ml. Reactions were at room temperature and were initiated by H_2O_2 addition. After 1 h, the [^{2}H]methanol was derivatized to its 3,5-dinitrobenzoate, which was analyzed by gas chromatography/mass spectrometry (12). Reaction mixtures for benzoquinone identification contained 1 mM 1,4-dimethoxybenzene, 1 mM H_2O_2, 1 μM ligninase, and 0.1 M sodium tartrate, pH 3.0, in a total volume of 5 ml. The reaction was initiated with H_2O_2 addition and performed at room temperature. After 45 min, the reaction mixture was extracted with ethyl ether, solvent was removed by vacuum distillation, and the residue was treated with excess sodium hydrosulfite (dithionite) in 1 ml of H_2O. Re-extraction with ether and solvent evaporation yielded a residue which was acetylated with acetic anhydride/pyridine. The residue remaining after removing liquids by vacuum evaporation was examined by gas chromatography/mass spectrometry.

For both analyses, a Finnigan MAT (San Jose, CA) 4510 gas chromatograph/mass spectrometer was used with a 30-m, 0.25-μm film thickness DB-1 (bonded methyl silicone) fused silica capillary column (J & W Scientific, Rancho Cordova, CA). Electron impact mass spectra were obtained at 70 eV.

RESULTS

Radical Identification—The enzyme ligninase functions efficiently at low pH (3). This, together with the fact that radical cations are remarkably stable at low pH, enabled us to detect such radicals by direct ESR. The spectra of the radicals generated from 1,4-di-, 1,2,3,4-tetra-, and 1,2,4,5-tetramethoxybenzenes are shown in Fig. 1. Hexamethoxybenzene also gave a good spectrum (not shown). The magnetic parameters (Table I) of these radicals agree well with the literature data reported for the corresponding cation radicals generated chemically (13).

Enzyme Dependency—The steady-state concentration of radical generated from the enzymatically treated 1,2,4,5-tetramethoxybenzene remained constant over a long period of time. The steady-state concentration of radical varies linearly with the enzyme concentration (Fig. 2). Because a true steady state is obtained, this means that the cation radical decays via a first order reaction. The cation radical probably decays by reacting with the solvent water, as described below for 1,4-dimethoxybenzene.

Product Identification from 1,4-Dimethoxybenzene—The UV spectral changes with 1,4-dimethoxybenzene (maximum absorbance at 286 nm) upon incubation with ligninase and H_2O_2 are shown in Fig. 3. Concomitant with the decrease in absorbance at 286 nm is an increase in absorbance at 245 nm. This pattern is consistent with the conversion of substrate to *p*-benzoquinone. Benzoquinone, as hydroquinone diacetate, was identified as a product by gas chromatography/mass spectrometry. Mass spectrum: *m/z* (relative intensity): 194 (M+, 4), 152 (18), 110 (100), 81 (2). The spectrum was identical to that of an authentic sample prepared by acetylating hydroquinone. Analysis by high performance liquid chromatography of underivatized products confirmed *p*-benzoquinone as product; hydroquinone was not detected.

Methanol was also identified as a major product from 1,4-dimethoxybenzene by using the deuterated analog (1,4-[*methoxy*-^{2}H]dimethoxybenzene) as the substrate and identifying deuterated methanol as its 3,5-dinitrobenzoate. Mass spectrum: *m/z* (relative intensity): 229 (M+, 11), 197 (33), 195 (100), 183 (2), 149 (14), 75 (38), 74 (10). The *m/z* 195 base peak fragment corresponds to the acylium ion resulting from loss of OC^2H_3 and is diagnostic of the alcohol derivative (12). In a parallel reaction, 2 mM (stoichiometric with substrate methoxyls) natural abundance methanol was added prior to derivatization. In this case, the added methanol gave a *m/z* 226 parent ion of comparable intensity to the *m/z* 229 peak arising from the deuterated derivative, confirming that methanol is a major product of the reaction. No products were detected in reactions lacking either enzyme or H_2O_2.

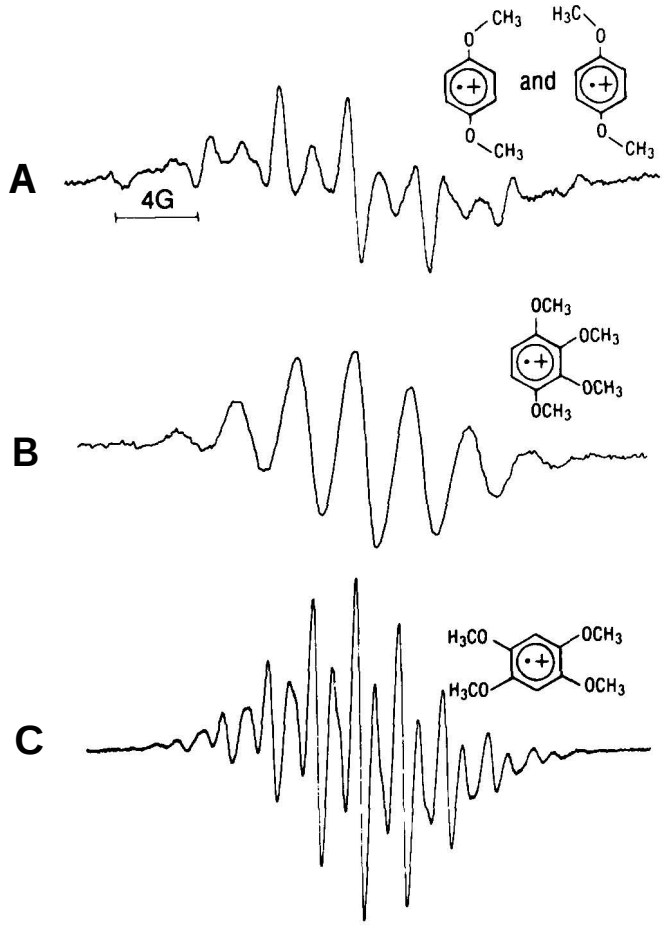

FIG. 1. ESR spectra obtained from reaction mixtures containing methoxybenzenes, ligninase, and H_2O_2, as described under "Materials and Methods." *A,* 1,4-dimethoxybenzene; the spectrum is a composite of the cis and trans isomers. *B,* 1,2,3,4-tetramethoxybenzene. *C,* 1,2,4,5-tetramethoxybenzene

TABLE I

Magnetic parameters for radical cations formed by the action of ligninase

Parent compound		Hyperfine splitting[a]		
		$a^H_{OCH_3}$	a^H_{ring}	g[b]
		G		
1,4-Dimethoxybenzene	*cis*	3.2 (6)	1.8 (2), 2.6 (2)	2.0040
	trans	3.4 (6)	1.5 (2), 2.9 (2)	2.0040
1,2,3,4-Tetramethoxybenzene		2.8 (6)	2.8 (2)	2.0040
1,2,4,5-Tetramethoxybenzene		2.15 (12)	0.88 (2)	2.0040
Hexamethoxybenzene		1.33 (18)		2.0040

[a] Parameters were obtained from computer simulation. Values are ±0.05; number in parentheses denotes number of protons contributing to signal.

[b] ±0.0002.

Other Methoxybenzenes—1,2-Dimethoxybenzene did not give a detectable ESR signal, although UV spectrophotometric results (not shown) clearly show that it is a substrate; furthermore, methanol was identified as a product using the above derivatization procedure. Radicals from penta-, 1,2,3,5-tetra-, and trimethoxybenzenes were not detected. Anisole

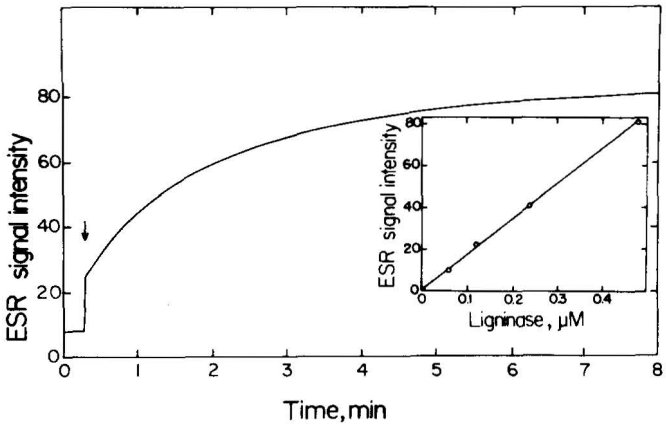

FIG. 2. Time course of cation radical formation from 1,2,4,5-tetramethoxybenzene. The reaction mixture was as described under "Materials and Methods," except for varying enzyme concentration. The signal intensity was monitored by turning off the magnetic field, and with the following ESR spectrometer conditions: gain, 2.0×10^4; modulation amplitude, 5 G time constant, 0.064 s; scan time, 8 min; microwave power, 20 milliwatts. *Inset* shows the relationship between enzyme concentration and the steady-state radical concentration. Arrow indicates entry of sample into ESR cell.

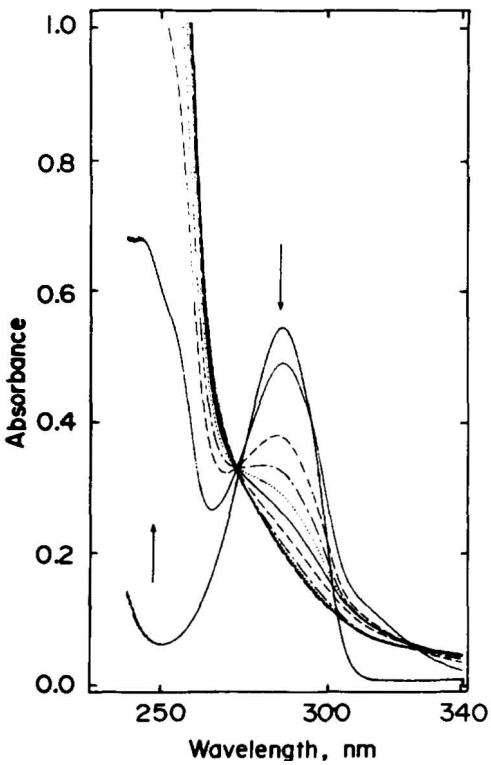

FIG. 3. UV spectral changes in 1,4-dimethoxybenzene on incubation with ligninase and H_2O_2. The reaction with 2×10^{-4} M 1,4-dimethoxybenzene, 0.5×10^{-6} M ligninase, and 8×10^{-4} M H_2O_2 in 1 ml of 0.1 M tartrate (pH 3) was followed in repetitive scans between 240 and 340 nm with a cycle time of 2 min (Perkin-Elmer Lambda 7 UV/visible spectrophotometer). The reaction was initiated with H_2O_2. The starting material exhibits λ_{max} 286 nm.

and 1,3-dimethoxybenzene are not substrates as determined by lack of both UV spectral changes and ESR signals.

DISCUSSION

Detection of aryl cation radicals from methoxybenzenes in a biological reaction is apparently unprecedented. Previously, they had only been chemically generated and detected in solvents that extend their half-lives (14). This research has demonstrated that the ligninase of *P. chrysosporium* generates cation radicals from methoxybenzenes. Evidence given here indicates that these are formed directly by one-electron oxidation by the ligninase, rather than via an initial two-electron oxidation followed by a reverse disproportionation reaction. Normally, one would obtain a square root relationship between the enzyme concentration and the steady-state radical concentration for a second order dismutation reaction. A pseudo-first order reaction usually is observed if the radical reacts with the solvent, as presumably occurs here. Although the linear relationship between enzyme concentration and radical concentration can also be found in the case of a reverse dismutation, this possibility is excluded by the fact that a true steady state is observed. A one-electron mechanism is also indicated by our unpublished spectroscopic studies of the heme (Soret band) during turnover; spectral transients that resemble compounds I and II of horseradish peroxidase are observed during turnover studies.

The detection of cation radicals in the enzymatic system from methoxybenzenes permitted predictions of the products formed from ligninase-catalyzed oxidation. The cation radical produced by voltametric oxidation of 1,4-dimethoxy-2,3,5,6-tetramethylbenzene decomposes to give 2,3,5,6-tetramethyl-*p*-benzoquinone (6). We therefore predicted, and observed, *p*-benzoquinone (and methanol) as major products from 1,4-dimethoxybenzene. The mechanism of demethoxylation presumably involves one-electron oxidation of the methoxybenzene by the oxidized enzyme and subsequent addition of H_2O to the cation radical, followed by or simultaneous with methoxyl elimination as methanol. Nucleophilic attack by water on aromatic cation radicals has been noted in other (chemical) systems (15). Fig. 4 shows a hypothetical scheme which is consistent with the data presented here.

Biological systems can cleave methyl ethers by hydroxylation to form hemiacetals which decompose to release formaldehyde (16). Alternatively, the ether linkage may be converted via a series of reactions to an ester which is hydrolyzed to release methanol (12, 17). The ligninase reaction is a new biological mechanism for ether cleavage. Methoxyl loss has long been associated with lignin biodegradation by white-rot fungi (18, 19). Further research will assess the role of ligninase in that loss.

The ability of ligninase to generate cation radicals is not

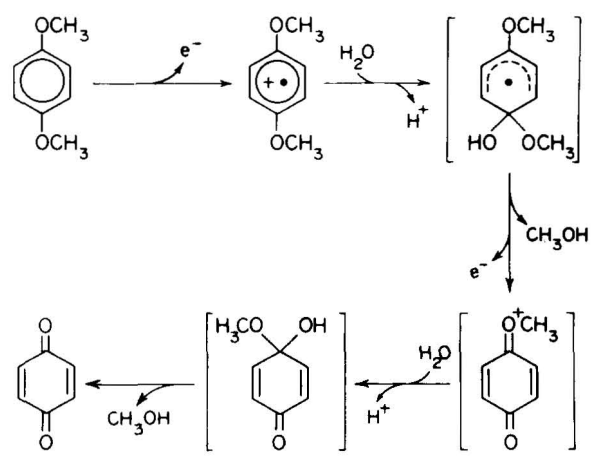

FIG. 4. Hypothetical scheme showing two sequential one-electron oxidations of 1,4-dimethoxybenzene and addition of water with loss of methanol. The intermediate cation radical and products *p*-benzoquinone and methanol have been identified.

restricted to methoxylated benzenes. 1,4-Diethoxybenzene also proved to be a good substrate, showing a strong ESR signal and UV spectral changes (not shown). As predicted from the results with the methoxybenzenes, ethanol and benzoquinone are the products. This finding, together with our most recent results, indicates that the demethoxylase activity is more appropriately termed a "dealkoxylase" activity. Research in progress has shown that more complex ether substituents are also removed by ligninase. These include the ß-glyceryloxy substituent, which represents the most abundant intermonomer linkage in the lignin polymer. Thus the activity described here may be important in cleaving interunit glycerol ether linkages, and thereby in depolymerizing lignin.

Formation of aryl cation radicals may lead to the side-chain modifying reactions catalyzed by ligninase in lignin model compounds (2, 3). Work presented in a companion paper[1] shows that side-chain cleavage in a lignin model involves radical intermediates. Such side-chain cleavages, as well as the benzyl carbon oxidation reactions catalyzed by ligninase (3), resemble the reactions found in oxidation of 1-phenylalkanols by sulfate anion radical, in which aryl cation radicals are postulated intermediates (20).

In conclusion, we are led to propose that the various reactions catalyzed by ligninase involve a mechanism based on aryl cation radical formation. This does not preclude possibilities of two-electron oxidations or hydroxylation by the enzyme with other substrates. However, the radical mechanism is consistent with the nonstereoselectivity of the enzyme (3).

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[1] K. Hammel, M. Tien, B. Kalyanaraman, and T. K. Kirk, manuscript submitted for publication.

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