The Oxidative 4-Dechlorination of Polychlorinated Phenols Is Catalyzed by Extracellular Fungal Lignin Peroxidases†

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ABSTRACT: The extracellular lignin peroxidases (ligninases) of *Phanerochaete chrysosporium* catalyzed H_2O_2-dependent spectral changes in several environmentally significant polychlorinated phenols: 2,4-dichloro-, 2,4,5-trichloro-, 2,4,6-trichloro-, and pentachlorophenol. Gas chromatography/mass spectrometry of reduced and acetylated reaction products showed that, in each case, lignin peroxidase catalyzed a 4-dechlorination of the starting phenol to yield a *p*-benzoquinone. The oxidation of 2,4-dichlorophenol also yielded a dechlorinated coupling dimer, tentatively identified as 2-chloro-6-(2,4-dichlorophenoxy)-*p*-benzoquinone. Experiments on the stoichiometry of 2,4,6-trichlorophenol oxidation showed that this substrate was quantitatively dechlorinated to give the quinone and inorganic chloride. $H_2{}^{18}O$-labeling experiments on 2,4,6-trichlorophenol oxidation demonstrated that water was the source of the new 4-oxo substituent in 2,6-dichloro-*p*-benzoquinone. Our results indicate a mechanism whereby lignin peroxidase oxidizes a 4-chlorinated phenol to an electrophilic intermediate, perhaps the 4-chlorocyclohexadienone cation. Nucleophilic attack by water and elimination of HCl then ensue at the 4-position, which produces the quinone. Lignin peroxidases have previously been implicated in the degradation by *Phanerochaete* of several nonphenolic aromatic pollutants [cf. Haemmerli, S. D., Leisola, M. S. A., Sanglard, D., & Fiechter, A. (1986) *J. Biol. Chem. 261*, 6900–6903. Hammel, K. E., Kalyanaraman, B., & Kirk, T. K. (1986) *J. Biol. Chem. 261*, 16948–16952]. It appears likely from our results that these peroxidases could also catalyze the initial dechlorination of certain polychlorinated phenols in vivo.

Little is known about biochemical mechanisms for the degradation of polychlorinated phenols, a major class of environmental pollutants. Some of these phenols are produced for use as broad-spectrum biocides or as precursors for the manufacture of chlorophenoxyacetate herbicides (Alexander, 1974; Reineke, 1984). Others, notably 2,4,6-trichlorophenol, are contaminants of kraft paper mill effluents (Huynh et al., 1985). The microbial catabolism of chlorophenoxyacetates further contributes to the environmental accumulation of certain isomers, especially 2,4-dichlorophenol and 2,4,5-trichlorophenol (Alexander, 1974). In general, the resistance of chloroaromatics to biodegradation increases with the degree of ring halogenation (Reineke, 1984).

Previous work has shown that *Phanerochaete chrysosporium*, a ligninolytic basidiomycete, can degrade a variety of aromatic pollutants, including certain polycyclic aromatic hydrocarbons (Bumpus et al., 1985; Hammel et al.; 1986b, Sanglard et al., 1986) and chlorinated phenols (Huynh et al., 1985). *P. chrysosporium* is notable for its production of extracellular lignin peroxidases (ligninases), normally active in the oxidative depolymerization of lignin, that catalyze sequential one-electron oxidations of numerous aromatic substrates. The reactions catalyzed include the C1–C2 cleavage of lignin model dimers (Gold et al., 1984; Tien & Kirk, 1984; Hammel et al., 1986a; Kirk et al., 1986b; Miki et al., 1986), the oxidation of alkoxylated benzyl alcohols to aldehydes (Gold et al., 1984; Tien & Kirk, 1984), the oxidation of certain alkoxybenzenes to quinones (Kersten et al., 1985b; Paszczyński et al., 1986), and haloperoxidase-type oxidations of bromide and iodide (Renganathan et al., 1987). Recently, both lignin peroxidases and whole cultures of *P. chrysosporium* were shown to oxidize certain polycyclic aromatic hydrocarbons to polycyclic quinones, which indicates a biodegradative role for these enzymes in at least some of the pollutant oxidations accomplished by *Phanerochaete* (Haemmerli et al., 1986; Hammel et al., 1986b). We are interested in the possibility that these peroxidases might be involved in the biodegradation of polychlorinated phenols and now report that they catalyze the oxidative 4-dechlorination of these pollutants.

Experimental Procedures

Enzyme. Lignin peroxidases were obtained from *trans*-aconitate-buffered agitated cultures of *P. chrysosporium* (ATCC 24725) (Jäger et al., 1985; Kersten & Kirk, 1987). The enzymes were purified by anion-exchange high-performance liquid chromatography (HPLC)[1] on a 0.46 × 25 cm Synchropak Q300 (quaternary aminoethyl) column (Synchrom, Lafayette, IN), using a 60-mL linear gradient of KCl (0–0.5 M, 1.5 mL min^{-1}) in 10 mM potassium phosphate (pH 6.5). When chloride analyses of 2,4,6-trichlorophenol reactions were to be done, the KCl gradient was replaced with a 60-mL gradient of sodium acetate (0.01–1.0 M, 1.5 mL min^{-1}, pH 6.0). These techniques afforded resolution at least as good as that obtained previously on Pharmacia Mono Q (Kirk et al., 1986a).

Chemicals. Polychlorinated phenols were obtained from Ultra Scientific (Hope, RI) and were pure as judged by gas chromatography (GC). Chlorohydroquinone and 2,3,5,6-tetrachloro-*p*-benzoquinone were purchased from Aldrich (St.

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[1] Abbreviations: HPLC, high-performance liquid chromatography; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry.

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Table I: Mass Spectra of *p*-Diacetoxybenzenes Produced by Derivatization of the Quinones Formed during Lignin Peroxidase Catalyzed Polychlorophenol Oxidations[a]

substrate	derivatized product	mass spectrum *m/z* (rel intensity)
2,4-dichlorophenol	2-chloro-*p*-diacetoxybenzene	230 (0.6), 228 (2.3), 188 (5.2), 186 (16.9), 146 (35.9), 144 (100.0)
2,4,5-trichlorophenol	2,5-dichloro-*p*-diacetoxybenzene	264 (0.6), 262 (1.8), 224 (0.8), 222 (7.1), 220 (10.5), 182 (9.2), 180 (60.3), 178 (100.0)
2,4,6-trichlorophenol	2,6-dichloro-*p*-diacetoxybenzene	264 (0.9), 262 (1.3), 224 (1.7), 222 (11.0), 220 (17.1), 182 (10.7), 180 (64.3), 178 (100.0)
pentachlorophenol	2,3,5,6-tetrachloro-*p*-diacetoxybenzene	336 (0.2), 334 (2.1), 332 (5.2), 330 (3.9), 294 (1.1), 292 (4.6), 290 (10.8), 288 (7.9), 254 (0.7), 252 (8.5), 250 (49.7), 248 (100.0), 246 (80.0)

[a] Ions due to ^{13}C are not shown.

Louis, MO). 2,5-Dichloro-*p*-benzoquinone was purchased from Kodak (Rochester, NY) and 2,6-dichloro-*p*-benzoquinone from Pfaltz & Bauer (Waterbury, CT). All other chemicals were of reagent grade.

Reaction Conditions. Typical enzymatic reaction mixtures (1.0–5.0 mL, room temperature) contained a polychlorinated phenol (75–200 μM), *P. chrysosporium* lignin peroxidase H1 or H2 (Kirk et al., 1986a) (0.3–2.0 μM), sodium tartrate (20 mM, pH 3.0), and Tween 80 (0.1% w/v). The reactions were initiated with H_2O_2 (400 μM final concentration). Substrate oxidation was observed by repeated scans on a Varian DMS100 absorption spectrophotometer. When the relative turnover numbers of different lignin peroxidases for 2,4,6-trichlorophenol oxidation were to be determined, the reaction mixtures contained 100 μM 2,4,6-trichlorophenol and 400 μM (saturating) H_2O_2. The reactions were monitored spectrophotometrically at 272 nm (see below). Relative turnover numbers for veratryl alcohol oxidation were obtained from the change in absorbance at 308 nm in reaction mixtures without Tween 80 that contained 1.0 mM veratryl alcohol and 400 μM H_2O_2 (Tien & Kirk, 1984). Lignin peroxidase concentrations were calculated by using an extinction coefficient of 168 mM^{-1} cm^{-1} at 409 nm (Tien et al., 1986).

Product Identifications. The completed enzymatic reactions were purged with argon for several minutes, treated with sodium dithionite, and then extracted three times with 1 volume of CH_2Cl_2. The pooled organic phases were dried over sodium sulfate, concentrated to ca. 10 μL under a stream of dry argon, and acetylated in 20 μL of 1:1 acetic anhydride/pyridine. The reduced and acetylated reaction products were analyzed by gas chromatography/electron impact mass spectrometry (GC/MS) at 70 eV in a Finnegan 4500 instrument fitted with an 8-m nonpolar fused silica capillary column (SPB-1, Supelco). The gas chromatograph was programmed to rise from 50 to 150 °C at 5 °C min^{-1} and then from 150 to 300 °C at 10 °C min^{-1}. Authentic standards of chlorinated *p*-diacetoxybenzenes were prepared from chlorinated quinones or hydroquinones in a scaled-up version of the procedure outlined above. The hydroquinones were recrystallized from glacial acetic acid before acetylation.

Stoichiometry of 2,4,6-Trichlorophenol Oxidation. The production of 2,6-dichloro-*p*-benzoquinone was monitored at 272 nm. The extinction coefficient of authentic 2,6-dichloro-*p*-benzoquinone in 20 mM sodium tartrate/0.1% (w/v) Tween 80 was determined to be 14.0 mM^{-1} cm^{-1}. The production of Cl^- was determined by a modified mercuric thiocyanate method (Florence & Farrar, 1971) in 0.4-mL samples that were withdrawn periodically from a 3.2-mL reaction. The reaction was stopped in each sample by pipetting it into 0.3 mL of 5.25 M perchloric acid/0.375 M ferric nitrate. Saturated mercuric thiocyanate in ethanol (0.3 mL) was then added, and the absorbance of the sample was measured after 5 min at 460 nm. The chloride concentration in each sample was then determined from a linear calibration plot of chloride standards that had been made up in tartrate/Tween 80 reaction mixture.

Source of the 4-Oxo Substituent in 2,6-Dichloro-p-benzoquinone. $H_2{}^{18}O$-labeling experiments were done in enzymatic reaction mixtures that were enriched to 50 atom % excess $H_2{}^{18}O$ by dilution of 97.4% $H_2{}^{18}O$ (MSD Isotopes). The reactions were terminated with sodium dithionite, and the resulting 2,6-dichlorohydroquinone was extracted (2×) with CH_2Cl_2. The pooled organic extracts were dried over sodium sulfate, concentrated by rotary evaporation, and analyzed by GC/MS as outlined above. The ^{18}O uptake at the 4-position of 2,6-dichloro-*p*-benzoquinone was calculated from the intensities of the β-chlorocyclopentadienone ions detected at *m/z* 114, 116, and 118.

Results and Discussion

Product Identifications. The lignin peroxidases of *P. chrysosporium* catalyzed H_2O_2-dependent spectral changes in all of the 4-chlorinated phenols we examined (Figure 1). With each substrate, these spectral changes were the same for all of the lignin peroxidases tested. For the di- and trichlorophenols, the spectra showed several near isosbestic points, which indicates that these substrates were oxidized to end products with negligible accumulations of intermediate species. The UV absorption spectra of the products from 2,4,5- and 2,4,6-trichlorophenol oxidations (λ_{max} = 272–273 nm) matched those we obtained for standards of 2,5- and 2,6-dichloro-*p*-benzoquinone dissolved in reaction mixture. Similarly, the UV absorption maximum for the products of pentachlorophenol oxidation (290 nm) was the same as the maximum we observed for a standard of tetrachloro-*p*-benzoquinone (*p*-chloranil).

GC/MS of the reduced and acetylated products showed that all of the polychlorophenols we examined had been enzymatically dechlorinated. The only derivatized products detected from oxidations of the trichlorophenols and pentachlorophenol were di- and tetrachlorinated *p*-diacetoxybenzenes, whose GC retention times and mass spectra matched those obtained for authentic standards of the derivatized quinones as shown in Table I. In the case of pentachlorophenol oxidation, the increase in absorbance observed at 250–260 nm (Figure 1C) suggests the formation of additional products, since *p*-chloranil does not exhibit a maximum in this region, but these products were not observed in the GC/MS experiments. The reduced and acetylated products of 2,4-dichlorophenol oxidation included 2-chloro-*p*-diacetoxybenzene (Table I). Control experiments, in which the parent phenols were worked up in the same manner as the reaction mixtures, showed that dechlorination was not an artifact of the derivatization procedure. In some experiments, diazomethane rather than acetic anhydride/pyridine was used for derivatization after dithionite treatment, and this method gave *p*-dimethoxybenzenes that corresponded to the *p*-diacetoxy derivatives shown in Table I (data not shown). We conclude that *P. chrysosporium* lignin peroxidases catalyze the oxidative 4-

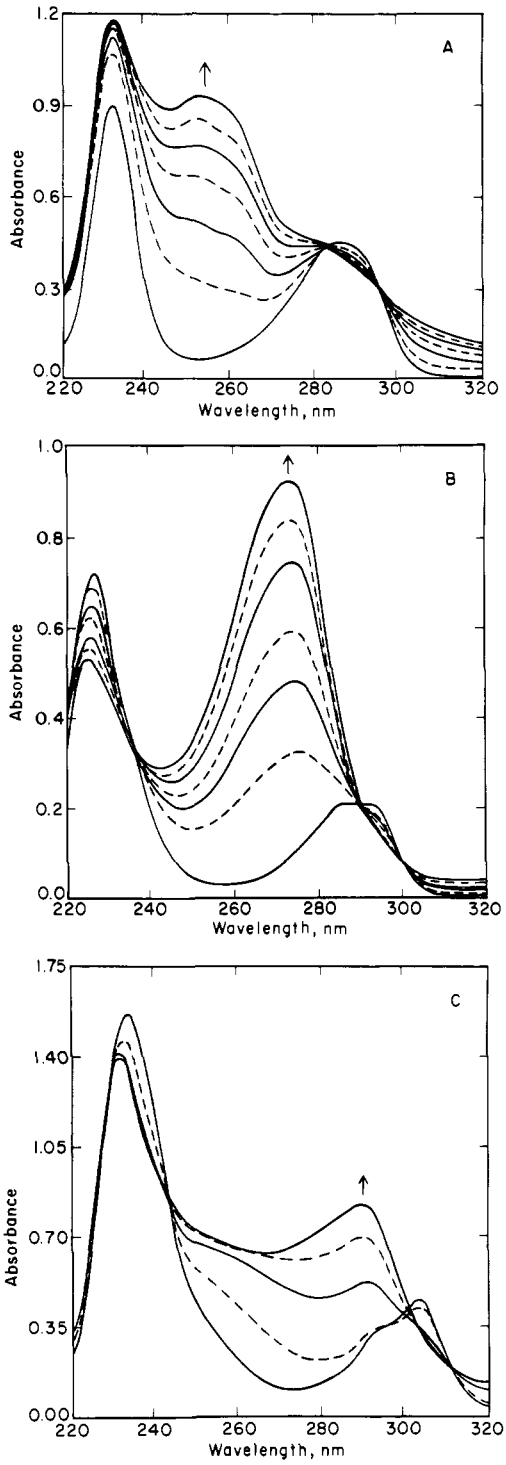

FIGURE 1: Spectral changes in polychlorinated phenols upon incubation with *P. chrysosporium* lignin peroxidase and H_2O_2. The reactions were initiated with H_2O_2 and followed in repetitive scans between 320 and 220 nm, at 100 nm min^{-1}. (A) 2,4-Dichlorophenol (160 μM): The spectrum of the starting material was recorded, and scans were then taken at 0, 1.1, 2.3, 4.4, 7.4, and 14.5 min after initiation. The enzyme concentration was 1.6 μM. (B) 2,4,6-Trichlorophenol (100 μM): The spectrum of the starting material was recorded, and scans were then taken at 0, 1.1, 2.3, 5.4, 8.4, and 15.5 min after initiation. The enzyme concentration was 0.3 μM. (C) Pentachlorophenol (160 μM): The spectrum of the starting material was recorded, and scans were then taken at 0, 1.1, 3.3, 6.4, and 11.6 min after initiation. The enzyme concentration was 0.8 μM. See Experimental Procedures. The spectral changes in 2,4,5-trichlorophenol (not shown) resembled those obtained for 2,4,6-trichlorophenol.

dechlorination of these polychlorophenols to give *p*-benzoquinones.

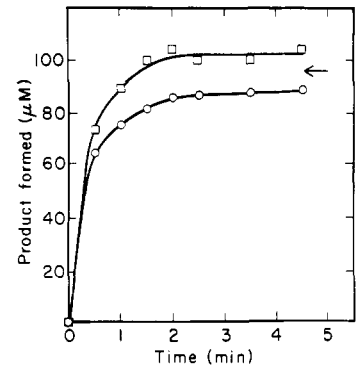

FIGURE 2: Time course of 2,4,6-trichlorophenol oxidation by lignin peroxidase (1.0 μM) and H_2O_2. The open circles indicate production of 2,6-dichloro-*p*-benzoquinone. The open squares indicate production of inorganic chloride. The black arrow indicates the starting concentration of 2,4,6-trichlorophenol (96 μM). See Experimental Procedures.

The lignin peroxidase catalyzed oxidation of 2,4-dichlorophenol also yielded a dimer that had lost one chlorine. It was tentatively identified after derivatization as reduced and acetylated 2-chloro-6-(2,4-dichlorophenoxy)-*p*-benzoquinone: mass spectrum *m/z* (relative intensity) 388 (M^+, 0.7), 350 (4.8), 348 (19.3), 346 (22.0), 310 (2.2), 308 (30.0), 306 (88.8), 304 (100) (ions due to ^{13}C not shown). The occurrence of this product is consistent with the expected one-electron peroxidative mechanism for phenol oxidation by lignin peroxidase (Tien & Kirk, 1984): The O–C coupling of two 2,4-dichlorophenoxy radicals would give 2,4-dichloro-6-(2,4-dichlorophenoxy)phenol, which itself would be a substrate for oxidative dechlorination by the enzyme to yield the quinone. Although 2,4,5-trichlorophenol could yield an analogous dimer by the same mechanism, since it has a nonchlorinated ortho position, we did not observe this product. The 2,4,5-trichlorophenoxy radical is evidently hindered enough that dechlorination is favored over intermolecular coupling under our reaction conditions (aqueous media, low concentrations of the radicals).

Characteristics of 2,4,6-Trichlorophenol Oxidation. Of the polychlorinated phenols we investigated, 2,4,6-trichlorophenol was oxidized most rapidly by lignin peroxidase. The oxidative dechlorination of this substrate was essentially quantitative: In the presence of excess H_2O_2, 0.93 2,6-dichloro-*p*-benzoquinone and 1.06 Cl^- were produced per 2,4,6-trichlorophenol supplied (Figure 2). The enzyme showed no evidence of saturation by its phenolic substrate at 2,4,6-trichlorophenol concentrations up to 0.5 mM, the highest we could obtain in our reaction mixture. The optimal pH range for 2,4,6-trichlorophenol oxidation by lignin peroxidase was 2.5–3.0, as it is for nonphenolic substrates (Tien et al., 1986).

We consistently observed that the lignin peroxidase isozymes that eluted earlier during anion-exchange HPLC were more active in 2,4,6-trichlorophenol oxidation than the later eluting isozymes were. Purified fractions corresponding to the less acidic isozymes H1 and H2 of Kirk et al. (1986a), which oxidized the standard lignin peroxidase substrate veratryl alcohol (1.0 mM) with turnover numbers of 16–20 s^{-1} under our conditions, oxidized 2,4,6-trichlorophenol (0.1 mM) at rates of 3–5 s^{-1}. By contrast, purified acidic isozymes that corresponded to lignin peroxidases H6–H10 oxidized veratryl alcohol with comparable turnover numbers (14–26 s^{-1}), yet oxidized the trichlorophenol more slowly (ca. 1 s^{-1}). The capacity to oxidize 2,4,6-trichlorophenol was not limited only to those peroxidases with lignin peroxidase activity. Other extracellular fungal peroxidases, i.e., the *P. chrysosporium*

H3–H5 fractions (Kirk et al., 1986a) and an enzyme from *Geotrichum candidum* (ATCC 26195; Bordeleau & Bartha, 1972), as well as a plant (horseradish) peroxidase, all oxidized this substrate to 2,6-dichloro-*p*-benzoquinone.

Source of the 4-Oxo Substituent in 2,6-Dichloro-p-benzoquinone. The reduced but underivatized product of 2,4,6-trichlorophenol oxidation, 2,6-dichlorohydroquinone, chromatographed satisfactorily by GC, and we exploited this finding in $H_2{}^{18}O$-labeling studies of the dechlorination reaction. Figure 3A shows the results obtained when a lignin peroxidase catalyzed trichlorophenol oxidation was done in natural abundance reaction mixture. The products were treated with dithionite and extracted, and the resulting hydroquinone was analyzed by GC/MS. The ions observed at m/z 142 and 144 indicate the loss of HCl from the molecular ions, whereas the ions at m/z 114 and 116 show the further loss of CO to yield, presumably, monochlorinated cyclopentadienone radical cations.

Figure 3B shows the results obtained in a control experiment, in which an authentic standard of 2,6-dichloro-*p*-benzoquinone was incubated for 4.0 min in reaction mixture that had been made up in 50 atom % excess $H_2{}^{18}O$. The hydroquinone was then obtained and subjected to GC/MS as outlined above. The relative intensities of the ions at m/z 114, 116, and 118 demonstrate that one of the two dichloroquinone oxygens, i.e., the oxygen that was not subsequently lost as CO from the hydroquinone, did not exchange rapidly with solvent water. The calculated amount of exchange under these conditions was 20%. By contrast, the other carbonyl oxygen, i.e., the one to be lost from the molecular ion of the hydroquinone as CO, exchanged completely with water during the same time, as can be seen from the relative intensities of the molecular ions at m/z 178, 180, 182, and 184. Prominent ions indicating the loss of HCl and CO in the mass spectrometer are characteristic of a phenol with two ortho chlorines, but not of one with two meta chlorines (Heller & Milne, 1978), which leads us to the conclusion that the oxygen at C1 of 2,6-dichlorohydroquinone is the first to be lost upon ionization. Moreover, exchange of the oxo substituent at C1 of the quinone is expected to be more rapid than at C4, because the inductive effect of two ortho chlorines would favor nucleophilic attack by water. These points accord with our observation that the first oxygen to be lost from the hydroquinone molecular ion in the mass spectrometer is the same oxygen that exchanges rapidly with water in the quinone. Therefore, we conclude that the peaks at m/z 114, 116, and 118 represent β-chlorocyclopentadienone ions that still contain the 4-oxo substituent of the original quinone.

This 4-oxo substituent is the one introduced during oxidative 4-dechlorination of 2,4,6-trichlorophenol by lignin peroxidase. We therefore asked whether the enzymatic oxidation of this phenol in 50 atom % excess $H_2{}^{18}O$ would lead to significant labeling of the cyclopentadienone ions seen at m/z 114, 116, and 118. This experiment, done simultaneously with the above-mentioned control (total reaction time 4.0 min), showed that 100% of the newly introduced quinone oxygens come from water (Figure 3C).

Mechanism of Oxidative Dechlorination. Our $H_2{}^{18}O$-labeling experiments indicate that this reaction involves attack by water on an electrophilic intermediate, perhaps the 4-chlorocyclohexadienone cation. The resulting 4-chloro-4-hydroxycyclohexadienone would eliminate HCl, in agreement with our observed stoichiometry, to give the final *p*-benzoquinone (Figure 4). A chlorinated phenoxy radical is shown as an intermediate in Figure 4 because phenoxy radicals are

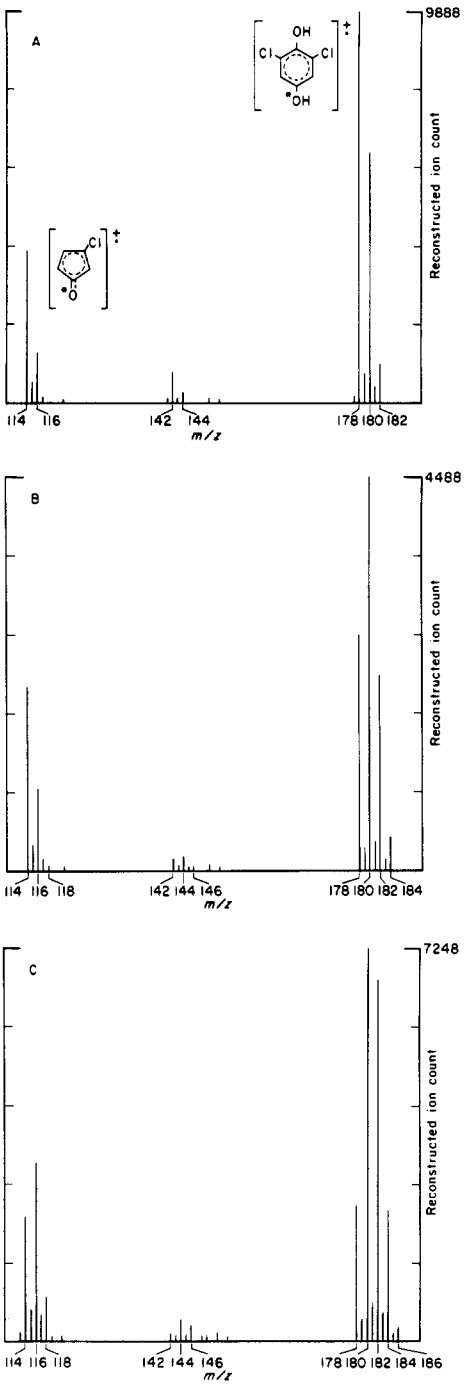

FIGURE 3: Mass spectrum of 2,6-dichlorohydroquinone obtained after reduction of the quinone. (A) The quinone was obtained by lignin peroxidase catalyzed oxidation of 2,4,6-trichlorophenol in natural abundance reaction mixture and then reduced and extracted. The ratio of the peaks at m/z 114 and 116, both theoretical and found, is 1.00:0.33. The asterisks on the chemical structures depicted show the oxygen atom that is introduced during the lignin peroxidase catalyzed oxidation of 2,4,6-trichlorophenol to 2,6-dichloro-*p*-benzoquinone. (B) An authentic standard of the quinone was incubated in 50 atom % excess $H_2{}^{18}O$-containing reaction mixture for 4.0 min and then reduced and extracted. Given 20% exchange of the quinone's 4-oxo substituent with 50 atom % $H_2{}^{18}O$, the theoretical ratio of the peaks at m/z 114, 116, and 118 is 1.00:0.44:0.03. The ratio found was 1.00:0.44:0.02. (C) The quinone was obtained by lignin peroxidase catalyzed oxidation of 2,4,6-trichlorophenol in 50 atom % excess $H_2{}^{18}O$-containing reaction mixture (total reaction time 4.0 min) and then reduced and extracted. Given quantitative incorporation of 50 atom % $H_2{}^{18}O$ into the new 4-oxo substituent, the theoretical ratio of the peaks at m/z 114, 116, and 118 is 0.76:1.00:0.24. The ratio found was 0.70:1.00:0.25. See Experimental Procedures.

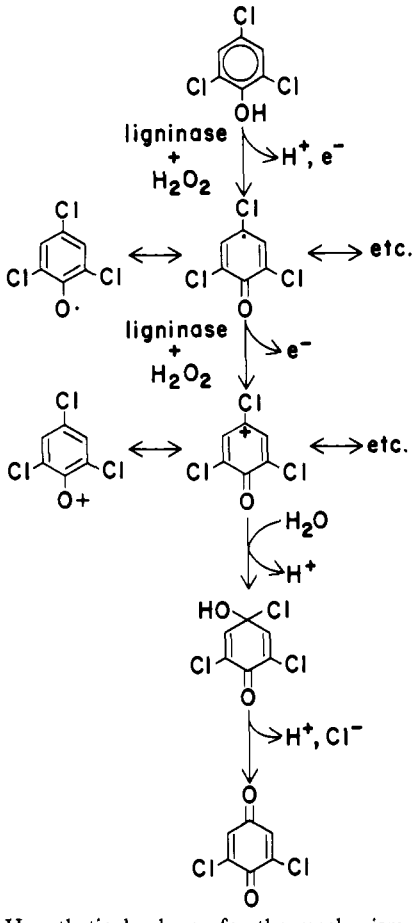

FIGURE 4: Hypothetical scheme for the mechanism of 2,4,6-trichlorophenol oxidation by lignin peroxidase and H_2O_2.

known to be intermediates in other peroxidase-catalyzed phenol oxidations, and the occurrence of a dimeric product from the lignin peroxidase catalyzed oxidation of 2,4-dichlorophenol does indirectly indicate the production of phenoxy radicals. However, our data do not establish that the dechlorination pathway that yields *p*-benzoquinones must occur in one-electron rather than two-electron steps, and we have not so far been able to detect any phenoxy radicals by ESR techniques (B. Kalyanaraman and K. Hammel, unpublished observations). It was recently suggested that phenoxy radical intermediates from peroxidase-catalyzed phenol oxidations might react at their ortho or para positions with molecular oxygen to yield peroxy radicals, which would then react further to give catechols, hydroquinones, or quinones (Ortiz de Montellano & Grab, 1987). Although phenoxy radicals are likely intermediates in the reactions we have studied, our $H_2{}^{18}O$-labeling experiments do not support a role for dioxygen in the lignin peroxidase catalyzed oxidation of 4-chlorinated phenols.

Conclusions

The lignin peroxidase catalyzed 4-dechlorination of polychlorophenols bears some resemblance to the reaction of these compounds with certain inorganic oxidants, e.g., lead dioxide (Hunter & Morse, 1926). The possibility that peroxidases might catalyze such dechlorinations was noted by Saunders and Stark (1967), who were, however, unable to detect 2,6-dichloro-*p*-benzoquinone as a product when 2,4,6-trichlorophenol was incubated for three days with turnip peroxidase and H_2O_2. Chloroquinones have been reported to be unstable in water (Hancock et al., 1962; Saunders & Stark, 1967), and we also have observed that 0.1 mM aqueous solutions of 2,6-dichloro-*p*-benzoquinone and 2,3,5,6-tetrachloro-*p*-benzoquinone develop a purple color with concomitant disappearance of the quinones (as determined by HPLC, data not shown) within ca. 1 h. Our success in identifying the chloroquinones as products is probably attributable to short reaction times (<20 min) and immediate derivatization. We have not been able to determine whether chloroquinones actually occur in whole fungal cultures that have been given chlorophenols because the quinones are not stable throughout the long incubation times necessary in these experiments.

Some other phenol-oxidizing enzymes have also been examined for their capacity to oxidize polychlorophenols. A fungal laccase was reported to catalyze the coupling of 2,4-dichlorophenol to give a tetrachlorinated dimer of fw 322, and it was proposed that such radical coupling reactions may operate to immobilize phenolic pollutants in soils (Bollag et al., 1977). We did not observe this product in lignin peroxidase catalyzed reactions with 2,4-dichlorophenol but note that it would probably be a substrate for the enzyme. The trichlorinated 6-phenoxy-*p*-benzoquinone (inferred fw 302), whose reduced and acetylated derivative we did observe, probably results from the oxidative dechlorination of a tetrachlorinated diphenyl ether of fw 322. Lactoperoxidase has also been reported to catalyze transformations of polychlorophenols, but the products were not identified (Öberg & Paul, 1985).

The lignin peroxidase catalyzed oxidation of 4-chlorinated phenols is particularly interesting because it accomplishes an oxidative dechlorination of the intact ring in one enzymatic step. It contrasts with currently well-understood oxidative pathways for the biochemical dechlorination of aromatics, which involve prior hydroxylation and ring cleavage. For example, the ortho cleavage of certain halocatechols gives chloromuconates, which then lactonize and eliminate halide to yield maleyl acetates (Evans et al., 1971; Schmidt et al., 1980; Schmidt & Knackmuss, 1980), and halide is also eliminated during the meta fission of 5-halogenated protocatechuates by protocatechuate 4,5-dioxygenase (Kersten et al., 1985a). Certain monooxygenases, e.g., phenylalanine hydroxylase (Kaufman, 1961) and *p*-hydroxybenzoate hydroxylase (Husain et al., 1980), can catalyze, without ring cleavage, the defluorination of fluoroaromatics that resemble their natural substrates, but these reactions, which require O_2 and NADPH as cosubstrates, clearly differ from the peroxidative dechlorinations we have described. Mechanisms for dechlorination that do involve ring cleavage have been proposed to operate when mono- and dichlorophenols are degraded by *Pseudomonas* sp. B13 or *Alcaligenes eutrophus* (Knackmuss & Hellwig, 1978) but evidently have not been demonstrated for more highly chlorinated isomers. We envisage that a number of microbial peroxidases, of which the lignin peroxidases are simply a particular class, may accomplish the initial 4-dechlorination of these more recalcitrant phenols in vivo.

Acknowledgments

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Registry No. 2,4,6-Trichlorophenol, 88-06-2; 2,4-dichlorophenol, 120-83-2; 2,4,5-trichlorophenol, 95-95-4; lignin peroxidase, 42613-30-9; *p*-chloranil, 118-75-2; 2,5-dichloro-*p*-benzoquinone, 615-93-0; 2,6-dichloro-*p*-benzoquinone, 697-91-6; pentachlorophenol, 87-86-5; 2-

chloro-6-(2,4-dichlorophenoxy)-*p*-benzoquinone, 76540-52-8; 2-chloro-*p*-benzoquinone, 695-99-8.

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Inhibition of Cathepsin B by Peptidyl Aldehydes and Ketones: Slow-Binding Behavior of a Trifluoromethyl Ketone†

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ABSTRACT: Inhibition of the cysteine proteinase cathepsin B by a series of *N*-benzyloxycarbonyl-L-phenylalanyl-L-alanine ketones and the analogous aldehyde has been investigated. Surprisingly, whereas the aldehyde was found to be almost as potent a competitive reversible inhibitor as the natural peptidyl aldehyde, leupeptin, the corresponding trifluoromethyl ketone showed comparatively weak (and slow-binding) reversible inhibition. Evaluation of competitive hydration and hemithioketal formation in a model system led to a structure–activity correlation spanning several orders of magnitude in both cathepsin B inhibition constants (K_i) and model system equilibrium data ($K_{RSH,apparent}$).

It has been known for some time that peptidyl aldehydes are effective, reversible inhibitors of serine (Thompson, 1973; Thompson & Bauer, 1979; Dutta et al., 1984; Stein & Strimpler, 1987) and cysteine (Aoyagi et al., 1969; Westerik & Wolfenden, 1972; Mattis et al., 1977; Knight, 1980; Baici & Gyger-Marazzi, 1982; Ogura et al., 1985; Mackenzie et al., 1986) proteinases by forming hemiacetals and hemithioacetals at the active site (Scheme I). In recent years interest has focused on the potential of trifluoromethyl ketones to inhibit proteinases in a similar fashion, by the formation of

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