Oxidation of Benzo(a)pyrene by Extracellular Ligninases of Phanerochaete chrysosporium

VERATRYL ALCOHOL AND STABILITY OF LIGNINASE*

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Benzo(a)pyrene was oxidized with crude and purified extracellular ligninase preparations from Phanerochaete chrysosporium. Both the crude enzyme and the purified fractions oxidized the substrate to three organic soluble products, namely benzo(a)pyrene 1,6-, 3,6-, and 6,12-quinones. These findings support the recent proposition that lignin-degrading enzymes are peroxidases, mediating oxidation of aromatic compounds by aryl cation radicals. The ligninase which was unstable in the presence of hydrogen peroxide could be stabilized by addition of 3,4-dimethoxy benzyl alcohol to the reaction mixture. The oxidation of benzo(a)pyrene was enhanced in the presence of this alcohol.

Biological degradation of polycyclic aromatic hydrocarbons in the environment is of increasing interest since some of these compounds have been shown to be highly carcinogenic. Benzo(a)pyrene (B(a)P), a particularly hazardous and therefore well investigated representative of this group, is oxidized in mammalian liver microsomes by a mixed function cytochrome P-450 monooxygenase system (1, 2). Among other products B(a)P-dihydrodiol epoxide is formed, which is thought to bind covalently to cellular DNA (1, 2).

Certain bacteria (3) and fungi (4-6) also metabolize B(a)P, and it has been demonstrated (7,8) that the white-rot fungus Phanerochaete chrysosporium effectively degrades B(a)P to CO₂ at rates up to 37 pg/l g fungal dry weight, in a day. The organism produces an extracellular hemoprotein (10, 11), a so-called ligninase that catalyzes different oxidations in lignin-related aromatic molecules (10-16). Multiple molecular forms of this enzyme have been isolated (17-20). Recently ligninase has been proposed to act as a peroxidase by a mechanism involving aryl cation radicals (12, 21-23).

Previously we gave evidence that ligninase is responsible for the initial steps in B(a)P metabolism by P. chrysosporium (8). In this study we have identified the main organic soluble products from the oxidation of B(a)P by different ligninase preparations. We have also shown that the enzyme can be stabilized and the oxidation enhanced by addition of veratryl alcohol (3,4-dimethoxy benzyl alcohol) to the reaction mixture.

MATERIALS AND METHODS

Production of Ligninase

P. chrysosporium (ATCC 24725) was grown in nitrogen or carbon limited cultures. Medium composition was according to Kirk et al. (24). The cultures were inoculated with spore suspension and grown on a rotary shaker (150 rpm, 2.5 cm) in 1000-ml Erlenmeyer flasks (900 ml of medium) for 48 h in air at 37 °C. The pellets were concentrated 4-fold by decanting off excess medium. Veratryl alcohol as an activator of enzyme production was added to a final concentration of 1.5 mM (18); the flasks were then flushed with pure oxygen and put again on the rotary shaker for 24 h at 37 °C (50 rpm for C-limited, 150 rpm for N-limited cultures).

Purification of Ligninases

The extracellular enzyme solution was recovered by filtration through a glass-fiber filter and concentrated 20-fold (N-limited) or 100-fold (C-limited) by ultrafiltration through Amicon PM-10 membrane filters. The concentrate was dialyzed against 0.5 M NaCl in the phosphate buffer. The resulting enzyme concentrate was dialyzed against the K-phosphate buffer and then stored at 4 °C. The crude enzyme from C-limited cultures was further purified by preparative fasted agarose-Sephadex isoelectric focusing (25). Bio-Lyte 3/5 (Bio-Rad) was used in a 1:20 final dilution. The gels were run with 15 watts constant power for 5-8 h. The two major proteins, which had molecular weights of 39,000 and isoelectric points of 4.1 and 4.7, respectively, were eluted from the gel with water. The amphotolites were removed by dialysis against 5 mM K-phosphate buffer, and the enzyme solution was concentrated to 5 mg/ml protein by ultrafiltration. Both purified proteins oxidized veratryl alcohol to veratraldehyde and showed a single protein band in sodium dodecyl sulfate-gel electrophoresis and narrow range isoelectric focusing.

Chemicals

B(a)P was obtained from Sigma and recrystallized from benzene/methanol, 1:1. The B(a)P quinones were purchased from Midwest Research Institute, Kansas City.

Oxidation of B(a)P

The oxidations were carried out at room temperature. The reaction mixture contained per ml of medium: dimethylsulfoxide buffer (pH 4.0): 50 nmol of B(a)P, 0.3 milliunit of glucose oxidase (grade 1, Boehringer Mannheim GmbH), 5 nmol of glucose, and 1 unit of ligninase. To some oxidations 120 nmol of veratryl alcohol (Fluka, Switzerland) were added. The reaction was stopped by addition of ethanol and immediate extraction of products as mentioned below. In some experiments, the reaction mixture was flushed with 15 min with nitrogen prior to addition of 250 nmol of H₂O₂ and then continuously flushed with either nitrogen or oxygen. No glucose or glucose oxidase was added in these experiments.

The reaction mixtures were extracted three times with an equal
Oxidation of Benzo(a)pyrene by Ligninases

6901

volume of ethyl acetate, the organic phase washed once with 1 volume of water, dried over Na2SO4, and evaporated in vacuo (15 mm Hg).

Analytical Methods

Ligninase Activity—This was measured according to Tien and Kirk (14). One unit of enzyme oxidized 1 μmol of veratryl alcohol to veratraldehyde in a minute at room temperature.

TLC—Silica Gel 60 F254 TLC plates (Merck, Darmstadt) were developed in benzene/ethyl acetate, 3:1, or in hexane/ethyl acetate, 2:3.

HPLC—Reverse phase HPLC was performed on a Kontron LC-690 system. A 0.46 × 12.5-cm Hyperchrome column filled with Shandon ODS Hypersil (5 μm, RP 18) was used. Samples were injected in methanol, and the system was run at a flow rate of 1.0 ml/min with a linear gradient of methanol/water (20–100% methanol in 25 min, then 100% methanol for 10 min). The absorbance detector was operated at 254 nm.

UV—Spectra were taken in 95% ethanol on a Perkin-Elmer 557 spectrophotometer.

RESULTS

Oxidation of B(a)P—When B(a)P was incubated with crude ligninase (from C-limited cultures) in the presence of an H2O2-producing system, three organic soluble products could be detected by HPLC (Fig. 1). These products accumulated with time. Simultaneously with the appearance of oxidation products, the ligninase was inactivated. In control experiments without ligninase or without an H2O2-producing system, no products were detected. Enzymatic oxidations carried out under pure nitrogen or oxygen resulted in the same products.

Effect of Veratryl Alcohol—Addition of veratryl alcohol to the reaction mixture increased the oxidation rate about 15 times, and the enzyme retained most of its activity during the B(a)P oxidation (Fig. 2). The same three products from B(a)P were detected, but in larger amounts. Veratryl alcohol was oxidized to veratraldehyde in this process. Turnover of B(a)P to oxidized products was always highest in the beginning of the reaction and even when the enzyme remained stable B(a)P was never degraded completely. On the other hand, as soon as veratryl alcohol oxidation by ligninase slowed down, the enzyme was rapidly inactivated (Fig. 2).

To test the influence of H2O2 and veratryl alcohol on enzyme stability, 1 unit of ligninase was incubated with 540 nmol of H2O2 in 3 ml of 20 mM dimethylsuccinate buffer (pH 4.0), and different amounts of veratryl alcohol were added (Fig. 3). A 5-fold excess in the alcohol concentration over peroxide prevented the inactivation almost completely.

Degradation of B(a)P by Different Enzyme Preparations—The purified proteins from C-limited cultures with isoelectric points of 4.1 and 4.7 oxidized B(a)P to the same products as the crude enzyme. Again, addition of veratryl alcohol enhanced product formation considerably.

Crude enzyme preparation from N-limited cultures oxidized B(a)P with similar results. However, the enzyme was more stable than that from C-limited cultures in the absence of veratryl alcohol, and 60% of B(a)P was oxidized. With crude enzyme from C-limited cultures at the most 44% degradation could be observed.

Identification of the Oxidation Products—Compounds I, II, and III as detected by HPLC (Fig. 1) were separated by preparative TLC in the solvent system hexane/ethyl acetate, 2:3 (RF values 0.39, 0.35, and 0.45). To achieve good separation, preparative TLC plates were developed once, dried in air, and developed again in the same solvent system. The products were eluted from the silica gel with ethyl acetate. Subsequent analysis by TLC in hexane/ethyl acetate, 2:3, and benzene/ethyl acetate, 3:1 (RF values 0.59, 0.35, and 0.46) as well as by HPLC confirmed the purity of the isolated substances.

The UV absorption spectra of compounds I, II, and III were identical with those obtained by B(a)P 1,6-, 3,6-, and 6,12-quinones, respectively (Table I). Further evidence of their identity was provided by HPLC. The retention times for the

FIG. 1. Reversed phase HPLC of the products from oxidation of B(a)P by P. chrysosporium ligninase with added veratryl alcohol. Conditions were as described under "Materials and Methods.

FIG. 2. Oxidation of B(a)P (O, O) by ligninase from P. chrysosporium to organic soluble products (C, □) as a function of time, with (open symbols) and without (closed symbols) addition of veratryl alcohol. △, ligninase activity; +, concentration of veratryl alcohol in per cent of original concentration.

FIG. 3. Activity of ligninase from P. chrysosporium in 180 μl H2O2 in the presence of no (O, O), 0.2 (□), and 1.0 (■) mM veratryl alcohol. *, activity of ligninase when neither H2O2 nor veratryl alcohol were added.
of standard compounds and the products of the enzymatic reaction are identical (Fig. 1).

The isomer ratio determined by HPLC (corrected for absorbance at 254 nm) was 52% 1,6-, 25% 3,6-, and 23% 6,12-quinone.

DISCUSSION

In this study we have shown that the extracellular ligninase of P. chrysosporium oxidized B(a)P mainly to 1,6-, 3,6-, and 6,12-quinones. The use of crude enzyme or purified enzyme fractions yielded the same results. Addition of veratryl alcohol to incubations enhanced the oxidation of B(a)P.

Chemical and electrochemical oxidation of B(a)P results in a mixture of the same quinones as obtained by the enzymatic reaction (26, 27). An isomer ratio of 46.7% 1,6-, 33.3% 3,6-, and 20% 6,12-B(a)P quinone has been reported for the chemical oxidation (27). This is similar to the ratio found in this study. The electrochemical oxidation of B(a)P has been shown to lead through a 1-electron transfer to a cation radical which undergoes several hydrolysis and oxidation reactions resulting in quinone end products (27). Ligninase attacks different types of aromatic compounds (10–16) creating cation radicals. We, therefore, propose for the oxidation of B(a)P catalyzed by this enzyme a reaction pathway (Fig. 4) where ligninase is only responsible for the initial steps and subsequent reactions occur spontaneously. We were not able to detect 6-hydroxy-B(a)P in the reaction mixtures although this compound would be stable enough to allow determination by HPLC (28). Probably 6-hydroxy-B(a)P is instantaneously oxidized further by ligninase. A similar mechanism has been proposed for the formation of these quinones in rat liver homogenate (29). Nucleophilic interactions with water are indeed expected to occur at the 6-position, since this is the most reactive site in the B(a)P cation radical according to Hückel molecular orbital calculations (27). The suggested reaction mechanism corresponds with the view that lignin peroxidase plus H2O2 can act as a fairly nonspecific oxidation reagent for aromatic compounds (21).

Equal products were obtained in B(a)P oxidation by crude enzyme from C- and N-limited cultures and by purified enzyme fractions. Similar results with other substrates have been reported by Renganathan et al. (19). This points to a certain similarity among the many extracellular ligninases of P. chrysosporium, although they have different isoelectric points or molecular weights. Possibly they differ mainly in their glycosidic parts. Considering the different availabilities of carbohydrates in C- and N-limited cultures, one could expect to find a difference in the glycosylation pattern of proteins obtained from these cultures.

An explanation has been given (19, 30) on how veratryl alcohol promotes product formation. Harvey et al. (30) have suggested that veratryl alcohol has a mediator role in oxidation reactions catalyzed by ligninase. In their scheme, veratryl alcohol cation radicals function as 1-electron oxidants. This would mean that cation radicals of veratryl alcohol created by ligninase react with B(a)P, resulting in the B(a)P cation radical and original veratryl alcohol. We further suggest that at the same time the oxidation of veratryl alcohol prevents inactivation of the enzyme. The ligninase, oxidized by hydrogen peroxide, is rapidly reduced again in the presence of veratryl alcohol, and through this cycle excess H2O2 is removed from the reaction mixture. High peroxide concentrations destroy the ligninase (Fig. 3), probably because either the enzyme can be overoxidized by H2O2 or the peroxo form is especially unstable. The stabilizing effect by veratryl alcohol possibly also by other compounds is important when one considers practical applications where ligninases are used to oxidize water-insoluble organic substrates.

Additional studies are planned to clarify the degradation pathway of B(a)P by the intact fungus.

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Oxidation of Benzo(a)pyrene by Ligninases

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