Ring fission of anthracene by a eukaryote
(white-rot fungi)/Phanerochaete chrysosporium/biodegradation/lignin peroxidase/polycyclic aromatic hydrocarbons

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ABSTRACT Ligninolytic fungi are unique among eukaryotes in their ability to degrade polycyclic aromatic hydrocarbons (PAHs), but the mechanism for this process is unknown. Although certain PAHs are oxidized in vitro by the fungal lignin peroxidases (LiPs) that catalyze ligninolysis, it has never been shown that LiPs initiate PAH degradation in vivo. To address these problems, the metabolism of anthracene (AC) and its in vitro oxidation product, 9,10-anthraquinone (AQ), was examined by chromatographic and isotope dilution techniques in Phanerochaete chrysosporium. The fungal oxidation of AC to AQ was rapid, and both AC and AQ were significantly mineralized. Both compounds were cleaved by the fungus to give the same ring-fission metabolite, phthalic acid, and phthalate production from AQ was shown to occur only under ligninolytic culture conditions. These results show that the major pathway for AC degradation in Phanerochaete proceeds AC → AQ → phthalate + CO₂ and that it is probably mediated by LiPs and other enzymes of ligninolytic metabolism.

Polycyclic aromatic hydrocarbons (PAHs) are major pollutants of both anthropogenic and natural pyrolytic origin, occurring in soils, sediments, and airborne particulates. The crucial step in their biodegradation is oxidative fission of the fused aromatic ring system, an event previously thought unique to certain bacteria (1). Recent evidence necessitates a revision of this view: the lignin-degrading fungi that cause white-rot of wood have also been shown to mineralize a wide variety of aromatic pollutants, including certain PAHs, under culture conditions that promote the expression of ligninolytic metabolism (2–5). A key component of the fungal ligninolytic system is thought to consist of extracellular lignin peroxidases (LiPs), which catalyze the one-electron oxidation of various lignin-related substrates (6–8). LiPs have also been shown to oxidize certain PAHs in vitro, and it has been proposed that they play an important role in the degradation of these pollutants by white-rot fungi (9, 10). However, it has never been demonstrated that LiP-catalyzed oxidation is a significant fate of any PAH in vivo or that the products of such a reaction are subsequently cleaved to smaller, monomeric, compounds. In fact, to our knowledge, no PAH ring-fission metabolite other than CO₂ has ever been identified in any eukaryote. To address these problems, we have examined the fate of anthracene (AC) in cultures of the ligninolytic basidiomycete Phanerochaete chrysosporium, and we now report that ligninolytic metabolism provides a route for the ring fission of this PAH.

MATERIALS AND METHODS
Reagents and Organic Syntheses. [¹⁴C]AC was synthesized from 9,10-anthraquinone (AQ) uniformly labeled with [¹⁴C] in one phenyl moiety. The starting material (Sigma, custom synthesis, 6.1 mCi/mmol⁻¹; 1 Ci = 37 GBq) was diluted with unlabeled AQ to 3.0 mCi mmol⁻¹ and repurified by preparative TLC on silica gel 60 (Merck) in CH₂Cl₂/tetrahydrofuran (99:1) to a radiochemical purity of 98.3%. A portion of this sample (20 mg) was reduced with hydriodic acid (11), and the resulting crude [1-4,4a,9a-¹⁴C]AC, obtained in 90% yield, was purified by preparative TLC on silica gel 60 in benzene to a radiochemical purity of 98.5%. Stock solutions of [¹⁴C]AC and [¹³C]AQ were prepared in toluene or N,N-dimethylformamide and stored at −20°C under argon.

AC trans-1,2-dihydriodiol was synthesized from 1,2-dihydroxy-AQ (alizarin) as previously described (12, 13) and was recrystallized from acetone. mp 179–180°C (literature mp 177°C); ¹H NMR (CDCl₃, 6 ppm) 4.44 (dd, 1, H₂), 4.81 (dd, 1, H₂), 5.93 (dd, 1, H₂), 6.47 (dd, 1, H₂), 7.28–7.34 (m, 2, aromatic), 7.39 (s, 1, H₉α), 7.61–7.71 (m, 2, aromatic), 7.89 (broad singlet, 1, H₉); J₁₂ = 10.2 Hz, J₂₃ = 2.1 Hz, J₃₄ = 2.4 Hz, J₄₅ = 9.8 Hz, J₅₆ = 1.6 Hz. [ring-¹⁴C]Phthalic acid (12.7 mCi mmol⁻¹, radiochemical purity >98%) was from Sigma. Unlabeled phthalic acid (99%) and AQ (>98%) were from Kodak. All other reagents were of the highest commercially available quality.

To minimize the artifactual oxidation of AC or its metabolites, all syntheses, culture experiments, and workup procedures were performed under dim light, and metabolite samples were stored in the dark under argon at −20°C.

Enzymatic Oxidations. LiP activity in fungal cultures was assayed with 3,4-dimethoxybenzaldehyde as the substrate. One unit of LiP oxidizes 1 μmol of the alcohol to 3,4-dimethoxybenzaldehyde per min in the presence of saturating H₂O₂ (pH 3.0, 22°C) (14). Procedures for the purification of LiP, the in vitro oxidation of AC with LiP, and the analysis of oxidation products by TLC and GC/MS were as described previously (10, 14).

Metabolic Studies. P. chrysosporium (American Type Culture Collection 24725) was maintained on supplemented malt agar slants (14), and precultures for biodegradation experiments were prepared by inoculating conidia from these slants into 125-ml Erlenmeyer flasks that contained 50 ml of basal low trace element medium (15) supplemented with 0.1% Tween 80 (16) and buffered with potassium 2,2-dimethylocitric (10 mM, pH 4.3). The nitrogen source was ammonium tartrate at an initial concentration of 1.1 mM (low-N cultures) or 30 mM (high-N cultures). The precultures were grown at 35°C in a rotary shaker (150 rpm) for 48 hr in air. To prepare each culture for metabolic studies, two precultures were combined into one of the flasks and the volume of extracellular medium was reduced to 25 ml. This procedure resulted in a final biomass concentration of about 2 g of dry weight per liter. ¹⁴C-labeled compounds were then added as <0.1% of the culture volume in N,N-dimethylformamide or toluene, the culture headspaces were flushed with O₂, the flasks were closed with Teflon-coated stoppers (for metabolite workup)

Abbreviations: AC, anthracene; AQ, 9,10-anthraquinone; LiP, lignin peroxidase; PAH, polycyclic aromatic hydrocarbon.

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or gassing manifolds (for mineralization assays), and the cultures were incubated in the rotary shaker (35°C, 150 rpm). The culture headspaces were flushed out daily thereafter with O₂, and in mineralization studies were sparged through an alkaline scintillation cocktail for assay of [²⁴C] (17). No differences in AC metabolism were observed between cultures that received compounds in toluene vs. those that received in N,N-dimethylformamide. The cultures were examined regularly by microscopy for bacterial contamination, with negative results.

**Metabolic Workup.** The extracellular culture fluid from two to six replicate cultures (50–150 ml, containing 0.7–2.0 x 10⁸ dpm) was adjusted to pH 7 with NaOH and extracted (three times) with 2 vol of CHCl₃/acetonitrile (1:1, vol/vol), followed by one extraction with 1 vol of CHCl₃. The combined neutral organic extracts were concentrated to ~50 ml by rotary vacuum evaporation, washed with 10 ml of saturated NaCl, dried over Na₂SO₄, and further concentrated to a few milliliters by sparging with argon. The remaining aqueous phase from the above procedure was acidified to pH 1.5 with HCl and reextracted with CHCl₃/acetonitrile and CHCl₃ as described above. The combined acidic organic extracts were then worked up in the same manner as the neutral extracts, and samples (0.5–2.0 x 10⁸ dpm) were purified from highly polar compounds by chromatography on a 1-ml column of silica gel 60 (Fluka) in benzene/ethyl acetate/formic acid (7:5:1, vol/vol). The eluate, containing an essentially quantitative yield of the applied [²⁴C], was then concentrated by sparging with argon.

**Isotope Dilution Experiments.** AQ determinations: For each experiment, a known quantity of unlabeled crystalline AQ (≈500 mg ± 1 mg) was added in 20 ml of CHCl₃ to each of three replicate 25-ml cultures that had been incubated with [¹⁴C]AC (2.0 μM) for the times indicated. Each culture was shaken for 1 hr, the contents of the three flasks were pooled and sonicated, cell debris was removed by centrifugation (16,000 x g, 15 min), and the supernatant liquid was separated from aqueous and CHCl₃ fractions. The CHCl₃ fraction was filtered, dried over Na₂SO₄, and concentrated to dryness by rotary vacuum evaporation. The resulting crude AQ crystals were recrystallized (three or four times) to constant ¹⁴C specific activity, and samples (=20 mg) were weighed and assayed by scintillation counting.

Phthalate determinations: For each experiment, four replicate 25-ml cultures that had been incubated for 7 days with [¹⁴C]AC or [¹⁴C]AQ (2.0 μM) were pooled, and the extracellular medium was separated from the mycelial pellets by centrifugation (16,000 x g, 15 min). A known amount of unlabeled crystalline phthalic acid (3.2–3.3 g ± 1 mg) was added to the extracellular medium, which was then adjusted to pH 8 with NaOH, stirred until the sodium phthalate was completely dissolved, filtered, and extracted exhaustively with CHCl₃ to remove hydrophobic ¹⁴C (mostly [¹⁴C]AQ), from which phthalic acid could not be purified by recrystallization in H₂O. Crystals of phthalic acid were then precipitated from the aqueous phase by adjusting the pH to 0 with HCl, and were recrystallized repeatedly from H₂O. Samples (=200 mg) from each crop of crystals were dried for 8 days over CaSO₄, weighed, and assayed by scintillation counting. Three to six recrystallizations were required to attain constant ¹⁴C specific activity. The mycelial pellets remaining from the above procedure were suspended in 100 ml of H₂O that contained 3.299 g of unlabeled phthalic acid. This suspension was centrifuged (16,000 x g, 15 min), and the supernatant fraction was treated as described above for the culture medium.

**RESULTS AND DISCUSSION**

AC is the simplest PAH to be a LiP substrate, and would be expected, given the high electron density at its 9 and 10 positions, to yield AQ when oxidized by the enzyme in vitro (10). TLC on silica gel and GC/MS of the reaction products from such experiments confirmed that AQ was the only detectable product. Mass spectrum m/z (relative intensity): 208 (M⁺, 100), 180 (– CO, 85), 152 (– 2CO, 35), 151 (15), 76 (15). Since other PAHs that have been examined in detail give mixtures of products when oxidized by LiP (9, 10), we concluded that AC was the substrate most likely to yield diagnostic metabolites in fungal cultures, and we therefore selected it for further studies.

If LiP catalyzes the first step in a pathway for AC degradation by *P. chrysosporium*, and this step is not rate limiting, both AC and its expected oxidation product, AQ, should be mineralized to similar extents in culture. We found this to be the case: When 0.2 μM [¹⁴C]AC was given to 2-day N-limited cultures, 12.9% ± 1.3% of the total was oxidized to ¹⁴CO₂ in 14 days. Likewise, 13.4% ± 3.9% of 0.2 μM [¹⁴C]AQ was mineralized in this time (Fig. 1). When the initial concentration of AC or AQ added was increased to 2.0 μM, the total amount of compound mineralized also increased, but not 10-fold: typically, 2–5% of the total was oxidized to CO₂ under these conditions. The mineralization of both compounds was inhibited by more than 80% when LiP production was suppressed by supplying the cultures with nonlimiting N. All of these findings—the total extents of mineralization obtained, the trend towards saturation of the biodegradative system with increasing concentrations of substrate, and the inhibition of mineralization in N-sufficient cultures—are typical of organopollutant degradation by *P. chrysosporium* (2–5).

The observation that AC and AQ were mineralized to comparable extents by *P. chrysosporium* was consistent with a role for LiP in AC degradation, but it was necessary to ascertain in addition whether AC was actually oxidized to AQ by the fungus. The cultures employed in these experiments exhibited high levels of LiP activity: typically 20 units (U) liter⁻¹ on day 1, 100 U liter⁻¹ on day 2, 10 U liter⁻¹ on day 3, and 5 U liter⁻¹ on day 4. When the neutral organic-soluble metabolites formed from [¹⁴C]AC in culture were extracted and analyzed by reversed-phase HPLC and silica gel TLC, it was evident that AC was rapidly oxidized to AQ in vivo (Fig. 2). AC trans-1,2-dihydrodiol, a major AC metabolite formed by the action of cytochrome P-450 and epoxide hydrolase in one or more of the cultures with AC, was also the major AQ metabolite formed by the fungus when AQ was added to the cultures.
other eukaryotes, including previously examined fungi (18, 19), was not formed in detectable amounts by P. chrysosporium. When \[^{14}C\]AQ was supplied to the cultures, no prominent neutral compound other than the starting material was found.

The identification and quantitative significance of AQ as an intermediate in AC metabolism were confirmed in an isotope dilution experiment, which showed that a large fraction of initially added AC was converted in culture to a product that recrystallized repeatedly with authentic AQ. The oxidation of AC to AQ preceded the onset of significant mineralization, and in low-N cultures accounted for about one-third of originally added AC after 24 hr. Oxidation to the quinone was significantly slower in high-N cultures and was negligible in uninoculated cultures (Table 1).

It is clear from these results that the oxidation of AC to AQ is rapid and quantitatively important in N-limited P. chrysosporium cultures. Since the rates and extents of mineralization obtained for AC and AQ were similar, we can further conclude that the pathway AC $\rightarrow$ AQ $\rightarrow$ CO$_2$ is a major one for AC degradation by the fungus. The simplest explanation for the data, in accord with the high LiP activity exhibited by these cultures and the suppression of AQ formation in high-N medium, is that LiP catalyzes the first step in AC degradation.

A crucial test of LiP involvement in AC degradation was to identify a diagnostic ring fission product from AQ in fungal cultures and then determine whether this metabolite was also produced from AC. Polar metabolites were formed in significant amounts from both compounds: typically, about 20% of \[^{14}C\]AC and 10% of \[^{14}C\]AQ were recovered from the extracellular medium as H$_2$O-soluble or acidic organic-soluble \(^{14}C\) after 7 days in culture. The acidic organic-soluble fractions from both AC and AQ experiments contained a metabolite that was indistinguishable from phthalic acid by ion exclusion HPLC (Fig. 3A). The same fractions, after methylation with diazomethane, both gave a product that chromatographed identically with authentic dimethyl phthalate by silica gel TLC (Fig. 3B). Phthalate was the only aromatic acid to accumulate from AQ, whereas as a second, more polar metabolite was also formed from AC, and was presumably the product of an oxidative pathway that is not initiated by LiP. This product appeared to be present in the extracellular medium at roughly the same level as phthalic acid, but large workup losses due to sample volatility make comparison difficult. Attempts to identify the more polar metabolite were unsuccessful; it is unclear whether it is a ring-fission product or a conjugate between AC and some acidic species.

An isotope dilution experiment confirmed the identification of phthalic acid and established its quantitative importance in AC degradation. Both AC and AQ gave phthalic acid in 12–13% yield after 7 days (Table 2), which is consistent with the pathway AC $\rightarrow$ AQ $\rightarrow$ phthalate. It is particularly significant that the cleavage of AQ to phthalate was inhibited by 94% under high-N conditions; this result shows that AQ ring fission in Phanerochaete is expressed coordinately with ligninolytic metabolism. The mechanism for this reaction remains to be determined. One obvious possibility is that the quinone might be directly cleaved to phthalic acid via oxygen insertions adjacent to C9 and C10, in a monoxygenase-catalyzed equivalent of the Baeyer–Villiger oxidation (20), but the low electron density on the end rings of AQ makes them poor candidates for the required migration to electron-deficient oxygen. A more plausible, but still unsubstantiated, mechanism would entail the hydroxylation of AQ before ring fission. In any event, it is clear that AC degradation in

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>14C present as AQ after given time in culture, % of total 14C added</th>
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</thead>
<tbody>
<tr>
<td>Low-N</td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Uninoculated (low-N)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>High-N</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>8.3</td>
</tr>
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ND, not determined.
Phanerochaete differs fundamentally from the process in bacteria, which proceeds via AC cis-1,2-dihydrodiol rather than AQ and has been proposed to yield salicylate rather than phthalate as a monocyclic cleavage product (21–23). The formation of a quinone to prepare the aromatic ring for cleavage is an unusual biodegradation strategy, and it appears to be of general importance in *P. chrysosporium*: LiPs have also been implicated in the degradation of polychlorinated phenols by this organism (24, 25).

The phthalic acid that is produced from AC is degraded further to CO₂, although less readily than AC and AQ are (Fig. 1). The relative persistence of phthalic acid in the cultures probably explains our success in identifying it as an intermediary metabolite, and is not surprising. Since phthalate is presumably derived from one phenyl ring of AQ that carries both of the original carbonyl groups (C-9 and C-10), the remainder of the cleaved AQ molecule must appear either as a catechol or an o-quinone (if AQ is oxidatively cleaved adjacent to the carbonyl groups), or as aliphatic fragments (if an end ring of AQ is cleaved first). All such products are expected to be more labile in culture than phthalic acid is, and probably account for the bulk of AQ mineralization.

We are indebted to P. J. Caluwe for illuminating discussions on AQ chemistry, to P. J. Tardone for the synthesis of [¹⁴C]AC, and to D. J. Kiemle for the mass spectral and NMR analyses. This work was supported by U.S. Environmental Protection Agency Cooperative Research Agreement 813530 (J. A. Glaser, project officer) and a grant from the New York State Center for Hazardous Waste Management.

![Fig. 3. Chromatographic analysis of acidic organic-soluble metabolites formed from [¹⁴C]AC and [¹⁴C]AQ (2.0 μM initial concentration) by *P. chrysosporium*. (A) HPLC of metabolites after 7 days in culture. Samples of acidic organic extracts (0.25–0.40 ml, 8.0 x 10⁶ dpm) were combined with 0.10 ml of distilled H₂O, and the organic solvents were evaporated by sparging with argon. Approximately 20% of the ¹⁴C was lost during this procedure. A sample of 0.05 ml of the resulting aqueous solution was then subjected to ion-exclusion HPLC (Interaction Chemicals ARH-601 column, 6.5 x 100 mm) with 0.05 M H₂SO₄ as the eluant at 0.60 ml/min⁻¹ temperature, 45°C; fraction size, 0.30 ml. The collected fractions were analyzed for ¹⁴C by scintillation counting. The phthalic acid standard (0.5 μmol in 0.05 ml) was detected by its absorbance at 280 nm. (B) TLC autoradiogram of methylated acidic metabolites. Samples of acidic organic extracts that had been purified on silica gel (0.2–0.7 ml, 6–7 x 10⁶ dpm) were treated with an excess of diazomethane in diethyl ether at 0°C for 5 min. The ether solution was then concentrated by sparging with argon to a volume of ~0.2 ml, whereupon 0.1 ml of benzene was added. The remaining ether was then removed by further sparging (losses of ¹⁴C during workup of the methylated sample were 30–40%). The methylated sample was combined with a standard of dimethyl phthalate, applied to the preconcentration zone of a silica gel 60 F₂₅₄ TLC plate (Merck; 20 cm long, 0.25 mm thick), and developed in hexanes/ethyl acetate (3:1, vol/vol). Standards were viewed under UV illumination.](image)

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### Table 2. Determination by isotope dilution of the [¹⁴C]phthalic acid produced by *P. chrysosporium* from [¹⁴C]AC or [¹⁴C]AQ

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Extracellular medium</th>
<th>Mycelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁴C]AC added</td>
<td>Low-N</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>High-N</td>
<td>0.1</td>
</tr>
<tr>
<td>[¹⁴C]AQ added</td>
<td>Low-N</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>High-N</td>
<td>0.1</td>
</tr>
<tr>
<td>Un inoculated</td>
<td>(low-N)</td>
<td>—</td>
</tr>
</tbody>
</table>
