A New Intermediate in the Mineralization of 3,4-Dichloroaniline by the White Rot Fungus *Phanerochaete chrysosporium*

HEINRICH SANDERMANN, JR.,1*, WERNER HELLER,1 NORBERT HERTKORN,2 ENAMUL HOQUE,1† DIETMAR PIEPER,1‡ AND REINHARD WINKLER1§

Institut für Biochemische Pflanzenpathologie1 and Institut für Ökologische Chemie,2 GSF—Forschungszentrum für Umwelt und Gesundheit GmbH, D-85764 Oberschleißheim, Germany.

Received 13 March 1998/Accepted 12 June 1998

*Phanerochaete chrysosporium* ATCC 34541 has been reported to be unable to mineralize 3,4-dichloroaniline (DCA). However, high mineralization is now shown to occur when a fermentation temperature of 37°C and gassing with oxygen are used. Mineralization did not correlate with lignin peroxidase activity. The latter was high under C limitation and low under N limitation, whereas the reverse was true for mineralization. The kinetics of DCA metabolism was studied in low-N and low-C and C- and N-rich culture media by metabolite analysis and 14CO2 determination. In all cases, DCA disappeared within 2 days, and a novel highly polar conjugate termed DCAX was accumulated in the growth medium. This metabolite was an end product under C and N enrichment. In oxygenated low-C medium and in much higher yield in oxygenated low-N medium, DCAX was converted to DCA-succinimide and then mineralized. DCAX was purified by high-performance liquid chromatography and identified as N-(3,4-dichlorophenyl)-α-ketoglutaril-b-amide by high-performance liquid chromatography and mass spectrometry and mass spectroscopy, gas chromatography and mass spectroscopy, and nuclear magnetic resonance spectroscopy. The formation of conjugate intermediates is proposed to facilitate mineralization because the sensitive amino group of DCA needs protection so that ring cleavage rather than oligomerization can occur.

In 1985, it was discovered that the white fungus *Phanerochaete chrysosporium* ATCC 24725 has a unique ability to mineralize free and lignin-bound chloroanilines (1). The fungus produced about the same high yield of 14CO2 regardless of whether the 14C label was in the lignin-bound chloroaniline, the natural lignin subunit (coniferyl alcohol), or the free chlorinated anilines. The unique capability of *P. chrysosporium* for mineralization of xenobiotics was also discovered for polychlorinated biphenyls (7) as well as TCDD, DDT, benzo[a]pyrene, and chlorinated biphenyls (6). Numerous reports on the successful mineralization of additional xenobiotics have since appeared (for reviews, see references 4, 12, and 26). The initial report on the mineralization of free and lignin-bound chloroanilines was further confirmed by studies on the mineralization of a chloroaniline-lignin metabolite fraction from wheat (2). A fungal lignin peroxidase preparation was found to react rapidly with chlorinated anilines, but the main reaction was oligomerization rather than ring opening. The highly toxic 3,4,3′,4′-tetrachloroazobenzene and other condensation products were formed (23a, 23a). More complex condensation products of 3,4-dichloroaniline (DCA) have been characterized from an organic collection (3, 23, 23a, 28). Reference substances were generally from our laboratory (1, 20). Reference substances were generally from our laboratory (20). Reference substances were generally from our laboratory (20). Reference substances were generally from our laboratory (20).

The initial purpose of the present work was to define optimal fermentation conditions. In addition, a new intermediate of mineralization was discovered when the kinetics of mineralization were studied. This metabolite, termed DCAX, is now identified as N-(3,4-dichlorophenyl)-α-ketoglutaril-b-amide by isolation from C- and N-rich medium and by standard spectroscopic techniques. A hypothetical mechanism for the formation of this metabolite and its conversion to the succinimide is given. Some of the results have been described briefly (26, 29).

MATERIALS AND METHODS

**Chemicals.** All chemicals used in this study were of analytical grade. The high-performance liquid chromatography (HPLC) solvents used were from Riedel-de-Haën. 3,4-Dichloro[U-14C]aniline was obtained and purified as previously described (1, 20). Reference substances were generally from our laboratory (3, 23, 23a, 28).

**Fungal strains and kinetic fermentation studies.** *P. chrysosporium* ATCC 24725 and 34541 were maintained on malt extract agar (1) and grown under the previously described conditions in either static culture (2.0-liter Fernbach flask with 100 ml of medium and no detergent or veratryl alcohol added [1, 11]) or agitated cultures (250-ml Erlenmeyer flask with 0.1% [wt/vol] Tween 80 and 1.5 mM veratryl alcohol added [cf. reference 20]). The composition of the N-limited,
C-limited, and C- and N-rich growth media were as described previously (1, 14). A fungal spore suspension was used for inoculation with 1.3 × 10^6 spores/100 ml of medium. The agitated cultures were shaken (150 rpm, 37°C) for 3 days to allow the fungal mycelium to develop. The culture medium was then reduced from 100 to 40 ml and the agitation speed was set to 60 rpm. The 13C-labelled test chemical was added, and incubation was continued with flushing with pure oxygen twice per week. 13CO₂ development was monitored as described previously (1, 20). All kinetic experiments were performed with 100 µM [13C]DCA (1.5 × 10⁵ dpm). Initially, following flushing with oxygen, 1 ml of growth medium was removed. A sample of 500 µl was directly employed for HPLC analysis of metabolites. Radioactivity (1, 20) and lignin peroxidase activity (20) were also determined. One unit of lignin peroxidase activity is defined as catalyzing the oxidation of 1.0 µmol of veratryl alcohol/min. The growth media were directly extracted with ethyl acetate to obtain DCASI as a major product (cf. reference 3). Prior acidification with KH₂PO₄ also allowed extraction of DCAX with ethyl acetate (28). General fermentation conditions were as previously reported (1, 20). Total radioactivity associated with the mycelium was determined by combustion.

Metabolic isolation. Because of the rapid decline in N-limited cultures (26, 28), C- and N-rich medium (40 ml) was selected for isolation of DCAX. For this purpose, [U-14C]DCA (38.5 kBq, 4.0 µmol) was incubated in N-limiting medium (1, 14). A sample of 500 µl was directly extracted with ethyl acetate to obtain DCASI as a major product (cf. reference 1). One unit of lignin peroxidase activity is defined as catalyzing the oxidation of 1.0 µmol of veratryl alcohol/min. The latter curve was also obtained with heat-inactivated mycelium tested under the conditions used by Hallinger et al. (11) with both strains are also shown (x). The lyophilized culture medium was dissolved in 5 ml of water and passed through an RP18 cartridge (500 mg; Merck no. 1.19849.001). Precipitation of DCAX and DCASA was achieved with argon as a collision gas (1 mTorr) and atmospheric pressure ionization-chemical ionization-mass spectrometry-mass spectrometry (MS-MS) (see Results). Combined HPLC-MS. Combined HPLC-MS was conducted with a single quadrupole mass spectrometer (TSQ 7000; Finnigan MAT, Bremen, Germany). When coupled with HPLC, an acid-free acetonitrile-water gradient on a reversed-phase C18 column (250 by 4.6 mm in diameter [Fisons] and 150 by 4.5 mm in diameter [Finnigan MAT]) was used. The column was connected to the MS system via an electrospray interface. All mass spectra were acquired in the negative ion mode at a typical scan rate of 110 to 167 amu/s. By triple-stage quadrupole MS, the fragmentation of DCAX and DCASA was achieved with argon as a collision gas (1 mTorr) and atmospheric pressure ionization-chemical ionization-mass spectrometry. Ionisation-induced dissociation spectra of DCAX, DCASA, DCAKGA, and DCAX were obtained at energies of 35, 50, and 50 eV, respectively. The atmospheric pressure ionization-chemical ionization mass spectrometry of synthetic DCAKGA was available at 5 eV energy, and the daughter ion (m/z 288) mass spectrum of DCAKGA was available at 18 eV.

Combined GC-MS. The experimental conditions of the combined GC-MS were as follows. GC was done with a 30 m by 0.25 mm MS select (fused silica; J&W, DB-5) column, an injector temperature of 250°C, an initial oven temperature of 80°C, and an initial hold of 3 min, a final temperature of 260°C at an increment of 20°C per minute, and a final hold of 10 min. MS was done with a source temperature of 150°C, a source emission current of 400 mA, 70 eV of electron energy, and CI-reagent gas methane at 10⁻¹⁰ Torr in the source. The mass spectrometer (TSQ 7000; Finnigan MAT) was run in the negative ion mode. 1H-NMR spectra were obtained with a Bruker AC-400 NMR spectrometer (400.13 MHz) at 30°C in acetone-d₆ (δ = 2.04 ppm) and/or
methanol-d$_4$ (δ = 3.30 ppm) at 303 K with a 5-mm inverse geometry probe (90° = 8.5 μs).

**RESULTS**

**Optimal fermentation conditions.** The original work of the efficient mineralization of DCA was carried out with *P. chrysosporium* ATCC 24725 (1). However, *P. chrysosporium* strain ATCC 34541 was reported to be unable to mineralize DCA (11). Hallinger et al. (11) attributed the earlier mineralization results to experimental artifacts. No data that could resolve the controversy have been published since. ATCC strains 24725 and 34541 have now been compared in the two reported fermentation regimes which both use the standard N-limited growth medium (14). The time courses of $^{14}$CO$_2$ development from 3,4-dichloro-[U-1$^{14}$C]aniline are shown in Fig. 1. Both fungal strains were effective mineralizers under the original conditions (1), i.e., 37°C and gassing with pure oxygen in 2.0-liter flasks. It made no difference whether $^{14}$CO$_2$ was collected after passage through a polyurethane plug in 2-aminoethanol (1, 2) or in 1 M NaOH (11). Both strains were inactive under the conditions described by Hallinger et al. (11), i.e., at 27°C and gassing with air in 0.2-liter flasks. When an inactive culture was transferred to 37°C and aerated with oxygen, there was a rapid development of mineralization activity. A similar observation was made with static C-limited cultures that exhibited a low mineralization rate. When C limitation was ended by the addition of 5 mM d-glucose, there was an immediate increase in $^{14}$CO$_2$ development (28). Therefore, inactive fungal cultures remain competent for mineralization when transferred to optimal conditions.

**Lignin peroxidase production.** Lignin peroxidase production in low-N cultures began between fermentation days 2 and 4 (cf. reference 20). Optimal induction in shake cultures has been reported to require C limitation and the addition of veratryl alcohol as well as the detergent Tween 80 (18). This could be confirmed in our study (28). Lignin peroxidase activity (units ·

![FIG. 2. HPLC profiles of aliquots from N-limited growth medium 2 days (upper panel) and 7 days (lower panel) after addition of 100 μM [U-1$^{14}$C]DCA. The column had been calibrated with the authentic reference metabolites (see Materials and Methods). The positions of DCA-X (R$_t$, 11.6 min) and of DCA-SI (R$_t$, 17.6 min) are shown. The initial [U-1$^{14}$C]DCA (R$_t$, 18.3 min) had largely disappeared after 2 days.](http://aem.asm.org/)

![FIG. 3. Kinetics of [U-1$^{14}$C]DCA metabolism. (A) N-limited medium. (B) C-limited medium. (C) C- and N-rich medium. Fermentation was carried out at 37°C, with oxygen gassing every 2 days. The substrate concentration was 100 μM. Aliquots (500 μl each) of the growth medium were directly employed for HPLC analysis (Fig. 2). The distributions of DCA (■-■) and the major metabolites, DCA-X (○-○) and DCA-SI (□-□), are plotted. $^{14}$CO$_2$ (●-●) was trapped and quantitated as described previously (1, 20).](http://aem.asm.org/)
liter$^{-1}$ in agitated cultures) were 40 ± 20 (mean ± standard deviation) under N limitation, 150 ± 30 under C limitation, and <10 in C- and N-rich medium. Total lignin peroxidase activity did not correlate with the extent of mineralization in the three different growth media, since mineralization was high under N limitation and very low under C limitation (see below). These results did not exclude the possibility that mineralization required a low amount of basal lignin peroxidase activity.

**Intermediates of mineralization.** Incubation of an N-limited static culture with [U-14C]DCA led upon ethyl acetate extraction to the succinimide conjugate as the main soluble metabolite (3). A systematic comparison of N-limited, C-limited, and C- and N-rich growth media has now been performed with HPLC metabolite analysis as shown in Fig. 2. In all three growth media, DCA disappeared within 2 days of application, with the concomitant appearance of a highly polar metabolite that was termed DCAX ($R_t$, 11.6 min). The time courses determined in the three growth media are shown in Fig. 3. The HPLC profiles obtained with N-limited growth medium showed that DCAX was high after 2 days (Fig. 2). This was followed by a rapid decline (Fig. 3) with a concomitant rise of DCASI, which reached a maximum at day 10. DCASI then declined in favor of CO$_2$ development. In C-limited and even more in C- and N-rich medium, DCAX remained high as a dead-end metabolite up to day 21. There were much lower amounts of DCASI, and CO$_2$ formation was very low (C limitation) or not detectable (C- and N-rich medium). Smaller amounts (<20%) of additional metabolites were detected (unidentified products as well as N-glucosyl-DCA [cf. reference 30] and DCASA [cf. reference 3]). These minor metabolites could not be correlated with 14CO$_2$ development and are not documented here. Mean total recoveries of 14C over the nine time points per kinetic experiment were 99.9% ± 11.3% (N limitation), 98.6% ± 8% (C limitation), and 100.0% ± 5.1% (C- and N-rich medium). The representative test series shown in Fig. 3 was reproduced three times under slightly different conditions.
experimental conditions. Treatment of incubation medium with ethyl acetate led to the selective extraction of DCASI (cf. reference 3). After acidification with KH₂PO₄, DCAX could also be extracted with ethyl acetate, but it decomposed slowly to material chromatographing near the DCASI standard (Rᶠ, 0.7; TLC analysis [28]). Fungal mycelium never contained more than 2 to 5% of applied radioactivity. In the case of N limitation, DCASI was the main mycelial component (TLC analysis [28]).

Isolation of DCAX. The new intermediate DCAX was labile upon solvent extraction, even though it was quite stable in C- and N-rich growth medium at pH 4 to 5 (26, 28). Therefore, no solvent extraction of DCAX from the C- and N-rich culture medium with organic solvents was performed. Instead, DCAX from the C- and N-rich cultures was purified by lyophilization and HPLC with a step-wise gradient from water to organic solvent. The collected fraction (Rₚ, 12.1 min) was dried under a stream of N₂ and could be stored under N₂ at -18°C for several weeks without significant decomposition.

Chemical structure of DCAX. DCAX and synthetic DCAKGA had the same Rₚs of 12.1 and 13.3 min upon C₆ and C₁₈ reversed-phase HPLC, respectively. The purified DCAX fraction was analyzed by combined HPLC-MS after being dissolved in CH₃CN-MeOH (1:1). A main product at an Rₚ of 10.9 min and a by-product at an Rₚ of 17.45 min appeared upon combined HPLC-MS (electrospray ionization, negative mode). The UV analog signals (at 250 nm) agreed with the mass scan maxima at the calculated values of m/z 288 ([M-H]⁻; DCAX); Rₚ, 10.9 min) and m/z 260 ([M-H]⁻; DCASA; Rₚ, 17.45 min). Both peaks showed the isotope cluster for two chlorine atoms. This result was confirmed by three independent HPLC-MS systems (data not shown). The mass spectra obtained with the [¹⁴C]DCAX sample and with synthetic DCAKGA are shown in Fig. 4. The proposed fragmentation pattern is indicated. The fragment with an m/z of 288 led to major daughter ions at m/z of 160, 216 and 244. The latter fragments corresponded to the 3,4-dichloroaniline fragment and to fragments due to the loss of the -COOH and -CO-COOH groups. Incubation of the fungus with DCASA (instead of DCA) in C- and N-rich medium also led to the formation of DCAX, which was identified by direct HPLC-MS analysis showing a mole peak at m/z 288 ([M-H]⁻) with an isotope cluster for two chlorine atoms. Fungal mycelium was found to contain aryldiamidase activity for DCASA (0.73 pkat/mg of soluble protein) (data not shown). The presence of a COOH group in DCAX was further demonstrated by methylation and HPLC on the reversed-phase C₆ column (Rₚ, 11.2 min). The mass spectra of methylated DCAX and DCAKGA were identical and indicated that the -NH₂ and the -CO₂H groups had both been methylated (Fig. 5). Synthetic DCAKGA was further characterized by its ¹H-NMR spectrum (Fig. 6 and Table 1). The spectrum showed an intact 3,4-dichloroaniline ring system as well as the expected side-chain protons.

Decomposition products of DCAKGA. During purification by HPLC, DCAX as well as DCAKGA partially decomposed into DCASA and smaller amounts of DCASI. DCASA and DCASI were identified by HPLC retention times on C₆ and C₁₈ columns and by ¹H-NMR spectroscopy (Table 1). When DCAX was transferred from a pH of 4 to 5 to a pH of 5.5, 6.0, or 6.5, complete conversion to a distinctly more polar, unidentified product occurred. It seems likely that the known revers-
The present metabolite structure can explain the formation of DCASI by the reaction sequence shown in Fig. 7. Cleavage of DCAGKGA by an α-ketoglutaryl-dehydrogenase-type reaction leads to the formation of the activated coenzyme A ester of DCASA, which can then spontaneously cyclize to yield DCASI. In model reactions, chemical activation of DCASA to

**DISCUSSION**

**Optimal fermentation conditions.** The previous failure to obtain mineralization of DCA with *P. chrysosporium* strain 34541 can now be explained by the inadequate fermentation conditions used. The use of smaller flask and gassing with air rather than oxygen both reduced the oxygen partial pressure. The latter is well known to be crucial for mineralization of lignin (13, 17). In addition, room temperature was used instead of the optimal growth temperature of 37°C. Inactive fungal cultures are shown here to lead within 2 days to a yield of up to 90% of a novel, highly polar intermediate termed DCAX. In agreement with the previous comparison of N-limited, C-limited, and C- and N-rich growth media (1), significant mineralization occurred only under N-limited growth conditions. These conditions also led to a near-stoichiometric conversion of DCAX to DCASI (Fig. 2 and 3). Extraction of DCAX with ethyl acetate required prior acidification. This observation and the low *Rf* value upon TLC led to the proposal of DCAX being the anilide of a dicarboxylic acid (28). The chemical lability of DCAX was consistent with a β-keto-acyl structure. β-Ketoadipic acid is a known intermediate of aromatic ring cleavage in *P. chrysosporium* (cf. reference 25), and its terminal anilide could conceivably rearrange to yield DCASI. However, the above-mentioned detailed structural studies have shown that DCAX is the δ-anilide of α-ketoglutaric acid.

The various conjugates were isolated to >90% from the growth medium, always with less than 5% of the applied radioactivity associated with the fungal mycelium (1, 2, 28). Coenzyme A transferases and the ring cleavage and mineralization systems are most likely intracellular, so there may be multiple hydrolase and efflux as well as cellular uptake and activation steps. The substrates may be circulating with an apparent waste of chemical energy. Interestingly, about the same high-percentage yield of 14CO2 was found when 4-chloro-roaniline and DCA were increased from 1 to 10 ppm (3). This lack of system saturation is consistent with an unusually high overall *Km* value or with a nonenzymatic step being rate limiting for mineralization.

**Structure of the new intermediate.** The comparison between DCAX and synthetic DCAGKGA by HPLC retention times, HPLC-MS, and GC-MS gave unequivocal evidence for the identity of both compounds. Analogous to the spontaneous formation of DCASA from DCA and succinyl-S-coenzyme A (3), one would expect the new compound to form from DCA and α-ketoglutaryl-δ-S-coenzyme A. The latter could be formed by a broad-specificity coenzyme A ligase. The derived structure of DCAX contrasts with that of α-hydroxy-glutaryl-S-coenzyme A, which is activated at the vicinal rather than the distal carboxyl group (22). Hallinger et al. (11) also placed DCA at the A, which is activated at the vicinal rather than the distal carboxyl group (22). Hallinger et al. (11) also placed DCA at the
spontaneous cyclization to give DCASI, which is the closest intermediate of DCASA by a new intermediate DCAKGA is converted to the coenzyme A derivative of a-t-ketoglutarate dehydrogenase-type reaction. This is followed by oxidative ring cleavage reactions and mineralization of xenobiotics as well as lignins (26). Such a role has also been proposed for xylosyl conjugation (15).

REFERENCES


