Manganese-Enhanced Biotransformation of Atrazine by the White Rot Fungus *Pleurotus pulmonarius* and Its Correlation with Oxidation Activity

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Received 11 March 1996/Accepted 24 July 1996

Manganese enhanced atrazine transformation by the fungus *Pleurotus pulmonarius* when added to a liquid culture medium at concentrations of up to 300 μ M. Both N-dealkylated and propylhydroxylated metabolites accumulated in the culture medium, with the former accumulating to a greater extent than did the latter. Lipid peroxidation, oxygenase and peroxidase activities, and the cytochrome P-450 concentration increased. In addition, an increase in the spectral interactions between atrazine and components in the cell extract was observed. Antioxidants, mainly nordihydroguaiaretic acid, which inhibits lipoxygenase, peroxidase, and P-450 activities, and piperonyl butoxide, which inhibits P-450 activity, inhibited atrazine transformation by the mycelium. It is suggested that the stimulation of oxidative activity by Mn might be responsible for increasing the biotransformation of atrazine and for nonspecific transformations of other xenobiotic compounds.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), a commonly used herbicide, consists of a triazine ring, with substitutions of Cl^- and ethyl- and isopropyl-amino groups (45). It shows a relatively high persistence in the environment, with a half-life that may reach 300 to 500 days (41). Its maximum concentration in water should not exceed 3 ppb (43), but it has been found in much higher concentrations in the environment. For example, up to 65 $\mu\text{g/liter}$ has been found in groundwater and up to 70 mg/liter has been found in pools and soils in a loading and rinsing area in Iowa (39). When the white rot fungus *Pleurotus pulmonarius* is grown in liquid culture and on straw (its more natural substrate), it has been found to transform atrazine by oxidative N dealkylation to produce deethylatrazine (CAIT) (the molecular structure is shown in Fig. 1 and clarified in Table 1), deisopropylatrazine (CEAT), and deethyl-deisopropylatrazine (25, 27). A fourth propylhydroxylated metabolite (hydroxypropylatrazine [CEIOT]), a product of oxidative hydroxylation, was also produced. N dealkylation, but not propylhydroxylation, of atrazine has also been found in other white rot and soil fungi (9, 19, 29). On straw, the chloro metabolites produced by this fungus were further detoxified through dechlorination by bacteria (25), as in soil (23).

In animals, plants, and various microorganisms, N dealkylation and hydroxylation are known to be catalyzed mainly by phase I reactions, which are generally responsible for the introduction of functional groups, like hydroxyl, carboxyl, and amino, into the xenobiotic molecule in detoxification of the xenobiotic mechanism. The produced metabolites could then undergo the reactions of phase II, which involve coupling of the foreign compound with a readily available endogenous conjugating agent, such as glutathione, glucuronic acid, etc. (1, 10, 33, 37). These reactions are controlled mainly by mixed-function oxidases, of which the dominant enzyme is cytochrome P-450 (37). Evidence for cytochrome P-450 in *P. pulmonarius* was reported by us in a previous study (26), in which it was found in both microsomal and soluble fractions. P-450

was found to be involved in the degradation of atrazine in a *Rhodococcus* sp. which produces the same four metabolites as does *P. pulmonarius* (30). In some cases, however, N dealkylation has been reported to be catalyzed by other hemoproteins, such as peroxidase, and free radicals are assumed to be involved (33). An attempt to find a correlation between the peroxidase activity involved in lignin degradation in the fungus *Phanerochaete chrysosporium* and N dealkylation of atrazine did not show a direct relationship (29). In fungi, no cell-free degradation of atrazine has been reported to date.

Preliminary studies conducted in our laboratory showed that atrazine transformation by *P. pulmonarius* is enhanced by manganese [Mn(II)] in liquid culture (unpublished data). Mn has been reported to regulate enzymes involved in lignin degradation (6). It is a metal ion with prooxidant activity and may enhance oxidation (42). In the present work, the relationship between the enhancement by Mn of atrazine transformation by *P. pulmonarius* and oxygenating activities by whole-cell culture were studied.

MATERIALS AND METHODS

Organism and growth conditions. *P. pulmonarius* was obtained from the Migal collection (no. 100) and maintained at 28°C on potato dextrose agar. The inoculum was prepared as described by Masaphy et al. (27) by subculturing the fungus in 15 g of Difco potato dextrose broth (PDB) per liter and incubating it in a rotary shaker (New Brunswick Scientific, Edison, N.J.) at 28°C and 120 rpm for 6 days, after which the culture was disrupted by a homogenizer (Kinematica, Lucerne, Switzerland) and subcultured again in PDB medium. Homogenized 3-day-old mycelial pellets were used as inocula, and 25-ml aliquots were transferred to 1,500 ml of PDB medium in 3,000-ml flasks. The culture was further incubated in a rotary shaker (28°C, 120 rpm) for 6 days. To study the effects of Mn on enzyme activity and lipid peroxidation, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ solution was added to the culture to give a range of Mn concentrations, and the culture was incubated for a further 10 h before extracting the crude enzyme or determining lipid peroxidation. The Mn concentration in PDB medium without any added Mn was 0.8 μM Mn.

Atrazine transformation. To study atrazine transformation, 20-ml 6-day-old subcultures from the culture mentioned above were transferred to 125-ml Erlenmeyer flasks and 5 mg of atrazine (97%; Riedel de Haen) per liter was added. After 24 h of incubation on a rotary shaker (120 rpm, 28°C), atrazine and its metabolites were extracted with ethylacetate, after adding 2 mg of prometryne (97%; Riedel de Haen) per liter as an internal standard. To study the effects of Mn and antioxidants on atrazine transformation, antioxidants and Mn (as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$) were added at the same time as was atrazine. The effects of MnCl_2 and Na_2SO_4 were also determined as a control. Antioxidant and Mn

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FIG. 1. Structure of atrazine metabolites produced by *P. pulmonarius*. R_1 , R_2 , and R_3 are substitutions on the triazine ring (see Table 1).

TABLE 1. Common names and structure of atrazine metabolites produced by *P. pulmonarius*

Atrazine metabolite	R_1	R_2	R_3
Atrazine	i-C ₅ H ₇	C ₃ H ₅	Cl
CAIT	i-C ₅ H ₇	H	Cl
CEAT	H	C ₃ H ₅	Cl
Deethyl-deisopropylatrazine	H	H	Cl
CEIOT	C ₅ H ₇ O	C ₃ H ₅	Cl

levels were studied in four replicates. All antioxidants were obtained from Sigma Chemical Co., St. Louis, Mo.

Crude enzyme fraction preparation. The crude enzyme preparation was obtained by the method of Ballard et al. (2). After incubation, the fungal biomass was filtered through gauze and squeezed. The crude enzyme was extracted by homogenizing the mycelium in 0.1 M potassium phosphate buffer (pH 7.4), supplemented with glass beads (diameter, 0.5 mm) at a wet biomass/buffer/glass bead ratio of 1:1.5:1 (wt/vol/wt). The biomass was homogenized for 2 min (in cycles of 20 s each) in an ice-cold tube. The homogenate was centrifuged twice for 10 min at 15,000 $\times$ g. The supernatant was kept at -80°C for further analysis or centrifuged again at 100,000 $\times$ g for 60 min. The sediment (microsomal fraction) was resuspended in 0.1 M potassium phosphate buffer containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM reduced glutathione. The 100,000 $\times$ g supernatant (soluble fraction) and resuspended microsomes were kept at -80°C for further analysis. The protein concentration in the enzyme preparation was determined with a Bio-Rad kit for protein determination according to the manufacturer's (Bio-Rad Laboratories GmbH, Munich, Germany) instructions. Bovine serum albumin was used as the standard.

Lipid peroxidation determination. Thiobarbituric acid (TBA) (Sigma) was used to determine lipid peroxidation by the method of Heath and Parker (16). Dried biomass (0.05 g) was homogenized for 1 min in 2.5 ml of 0.1% (wt/vol) trichloroacetic acid containing 0.01% butylated hydroxytoluene and centrifuged at 10,000 $\times$ g for 5 min. A 1-ml aliquot of supernatant was mixed with 2.5 ml of 0.5% TBA in 20% trichloroacetic acid and incubated in boiling water for 30 min. The specific A_{532} of products was read. The concentration of TBA-reactive matter (TBARM) was calculated by using an extinction coefficient of 155 $\text{mM}^{-1}\text{cm}^{-1}$.

Linoleic acid-dependent oxygenase activity. The consumption of O_2 by the crude enzyme extract with linoleic acid as the substrate (representing mono- or dioxygenase) was determined by measuring the O_2 consumption rate during enzymatic oxidation of linoleic acid with a polarographic O_2 electrode (13). The reaction mixture consisted of 2.9 ml of 8 mM linoleic acid solution in 50 mM phosphate buffer, pH 6.5, and 0.1 ml of fungal crude enzyme from the 100,000 $\times$ g supernatant. The specific unit activity was expressed in micrograms of O_2 per milliliter per milligram of protein consumed per minute.

Peroxidase activity. Peroxidase activity was determined by the method of Kuwahara et al. (22) for phenol red oxidation. The reaction system consisted of 50 μl of crude enzyme and 0.2 ml of phenol red (from a stock of 0.2%) added to 50 μM H_2O_2 in 1 ml of 0.1 M lactate or potassium phosphate buffer, pH 5, for 10 min at 30°C . To stop the reaction, 0.2 ml of 2 M NaOH was added. This activity was expressed as the increase in A_{610} per minute per milligram of protein, and H_2O_2 demand was determined by subtracting the activity without H_2O_2 from the activity with the addition of H_2O_2 .

Determination of P-450. Spectral analysis and quantification of cytochrome P-450 were performed on the crude extract of both microsomal and soluble fractions by the method of Masaphy et al. (26), which is based on the method of Omura and Sato (34). Dithionite was added to the enzyme extract, and the extract was divided into two cuvettes. CO was bubbled into the sample cuvette for 1 min, and the difference spectra for the sample and reference cuvettes were recorded with a Jasco 7800 double-beam scanning spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan) at 390 to 500 nm. The specific concentration of

P-450 was expressed in picomoles of P-450/milligrams of protein $^{-1}$ by using an extinction coefficient of 91 $\text{mM}^{-1}\text{cm}^{-1}$ at 450 nm.

Atrazine binding spectra. The interaction of atrazine with cell extract (soluble or microsomal), representing binding to P-450, was monitored by spectral analysis, as described by Jefcoate (17). Atrazine (0.5 to 5 μl of 5,000-ppm stock solution in dimethyl sulfoxide [DMSO]) was added to 1 ml of the reaction system to a final concentration of 11.6 to 116 μM . The binding spectra were obtained by the following procedure: cell extract (0.1 mg of protein/ml) and DMSO were added to the sample cuvette, and 0.1 M potassium phosphate buffer (pH 7.4) and DMSO were added to the reference cuvette. The difference spectrum of this pair of cuvettes (spectrum 1) was recorded with a Jasco double-beam spectrophotometer at 350 to 500 nm. The cuvettes were replaced by a new pair containing cell extract and atrazine in DMSO in the sample cuvette and buffer and atrazine in DMSO in the reference cuvette. The new difference spectrum was recorded 2 min after atrazine addition (spectrum 2). The interaction between atrazine and P-450 was obtained by subtracting spectrum 1 from spectrum 2.

Analysis of atrazine and metabolite. Atrazine and its metabolites (CAIT, CEAT, and CEIOT) were analyzed by the method of Masaphy et al. (27) with a gas chromatograph (Varian 6000; Varian, Palo Alto, Calif.) equipped with a nitrogen phosphorus detector (Fig. 2). Ethylacetate extract (2 μl) was injected into a 15-m DB17 megabore column (J and W Scientific, Folsom, Calif.) with the temperature programmed at 175°C for 5 min and with subsequent increases of 4°C min^{-1} to 255°C . The injector temperature was 230°C , and the detector temperature was 300°C . Helium was used as the carrier gas.

RESULTS

Effects of Mn on atrazine transformation. Manganese enhanced atrazine transformation by the fungus *P. pulmonarius* when added to the culture medium of 6-day-old fungal cultures (Fig. 3). Production of the dealkylated metabolites (CAIT and CEAT) and the propylhydroxylated metabolite (CEIOT) was enhanced. In the range of MnSO_4 concentrations studied, the increase in metabolite accumulation was highest at 300 μM Mn, especially for the N-dealkylated metabolites (265 and 205% of the control for N-dealkylated and propylhydroxylated metabolites, respectively). At higher Mn concentrations (500 and 1,000 μM), the accumulation of metabolites was inhibited. A similar effect was obtained by adding a range of MnCl_2 concentrations, but no increase in metabolite accumulation was observed after the addition of Na_2SO_4 , indicating that Mn was the cause of the enhanced metabolism (Table 2).

At a high Mn concentration, the mycelium became slimy and lost its rigidity, as observed by handling it. In addition, the culture to which Mn was added became brown. This was observed 3 to 5 h after the addition of Mn, depending on the concentration added, reaching a dark intensity when 300 and 500 μM Mn was added, though it was less dark at 1,000 μM (data not shown). This brown color was associated with the fungal pellets, and no significant increase in color was observed

FIG. 2. Gas chromatography analyses of atrazine and metabolites. Peaks: 1, atrazine; 2, CEAT; 3, CAIT; 4, deethyl-deisopropylatrazine; 5, CEIOT; S, prometryne (internal standard).

FIG. 3. Effects of manganese on the accumulation of atrazine metabolites in a 6-day-old culture of *P. pulmonarius*. ■, CAIT + CEAT; ▲, CEIOT. Atrazine (5 mg/liter) was added at the same time as was the Mn solution. The incubation time was 24 h. Data are the means of four replicates. Error bars represent ± 1 standard deviation (SD). The accumulation of metabolites in the control was 0.35 mg/liter for CAIT + CEAT and 0.18 mg/liter for CEIOT.

FIG. 4. Effects of manganese on lipid peroxidation of *P. pulmonarius* mycelium. Lipid peroxidation was determined as the TBARM content of the freeze-dried biomass of a 6-day-old fungus incubated with Mn for 10 h. Error bars represent ± 1 SD.

in the filtered external medium. This browning is probably related to MnO_2 production, as observed by Kerem and Hadar (20) when they studied the effects of Mn on lignin degradation by *P. ostreatus*, and to the black spots found in wood in nature (5).

Effects of Mn on lipid peroxidation. Increasing the concentration of Mn added to the culture medium caused a gradual increase in lipid peroxidation of the mycelium, as determined by the TBARM content (Fig. 4). The TBARM content was not affected by low concentrations of Mn (50 to 100 μM), but higher concentrations gradually increased the TBARM content to a level that was 50% higher in the mycelium incubated for 10 h with 1,000 μM Mn. This increase was also observed when lipid peroxidation was measured by analyzing the fatty acid composition of the mycelium and determining the ratio between unsaturated ($C_{18:2}$ and $C_{18:1}$) and saturated ($C_{16:0}$) fatty acids (data not shown).

Oxygenase and peroxidase activities. The addition of Mn to the culture caused increased O_2 consumption when linoleic acid was added as a substrate (Fig. 5). Fifty percent of the greatest increase in oxygenating activity was already attained at 50 μM Mn. A less steep increase in activity was found at up to

300 μM Mn (increasing by more than three times the result for the control), with a slight decrease at 500 μM Mn.

A similar effect was found in the H_2O_2 -dependent activity (peroxidase); it was rapidly enhanced when Mn was added up to 300 μM . This activity continued to increase more slowly with 500 and 1,000 μM Mn, reaching a level 4.8 times higher than that of the control.

Effects of Mn on P-450 concentration. The cytochrome P-450-specific concentration in both microsomal and soluble fractions of *P. pulmonarius* mycelium increased gradually with an increasing Mn concentration (Fig. 6). The P-450 concentration in the microsomal fraction was much higher than that in the soluble fraction in all treatments. The gradual increase in microsomal P-450 was higher than that in the soluble fraction, from 15 to 118 pmol/mg of protein in the microsomal fraction versus from 8 to 18 pmol/mg of protein in the soluble fraction.

Atrazine binding spectra. Atrazine interacts with both crude microsomal and soluble fractions (Fig. 7), producing an absorbance peak. The interaction found in the microsomal fraction (indicating microsomal P-450) was very low and difficult to observe in noninduced mycelium (Fig. 7, line A). In the Mn-induced treatments, a spectrum with a peak at approximately 408 to 412 nm and a trough at approximately 385 nm was recorded (Fig. 7, line B). In the soluble fraction, unlike in the microsomal fraction, the peak was at 418 to 422 nm in the noninduced mycelium, with troughs at approximately 385 and 448 to 455 nm (Fig. 7, line C). This spectrum is similar to that representing reverse type I interaction, indicating an interaction between atrazine and oxidized cytochrome P-450 [Fe(III)] (11, 17). Induction by Mn caused an increase in the height of the peak in proportion to the Mn concentration when identical protein concentrations of the crude soluble enzyme for all treatments were used in the assay system (Fig. 7, lines D and E). A shift in the peak position to a lower wavelength (416 nm) and in the first trough position to a higher wavelength (from 385 to approximately 394 to 400 nm) was observed with an increasing Mn concentration (Fig. 7, line E). In a range of atrazine concentrations, a concentration lower than 46 μM (9.9 mg/liter) gave lower peaks, while higher concentrations did not increase them significantly (data not shown), which may indicate saturation.

Effects of antioxidants on atrazine transformation. The concentrations of atrazine metabolites were affected when antioxi-

TABLE 2. Effects of $MnSO_4$, $MnCl_2$, and Na_2SO_4 added to *P. pulmonarius* culture on the accumulation of N-dealkylated and hydroxypropyl metabolites of atrazine^a

Salt	Concn (μM)	Metabolite (% of control) ^b		
		CAIT	CEAT	CEIOT
$MnSO_4$	250	230 (35)	204 (30)	150 (27)
	750	65 (22)	52 (18)	55 (15)
$MnCl_2$	250	205 (23)	198 (26)	130 (20)
	750	49 (17)	50 (14)	45 (15)
Na_2SO_4	250	114 (21)	85 (21)	102 (17)
	750	98 (18)	87 (22)	105 (25)

^a Atrazine and salts were added at the same time to a 6-day-old culture, and metabolite accumulation was determined after a further 24-h incubation.

^b The accumulation of the metabolites in the control (100%) was 0.16, 0.18, and 0.23 mg/liter for CAIT, CEAT, and CEIOT, respectively. Data in parentheses are SDs.

FIG. 5. Effects of manganese added to *P. pulmonarius* culture on oxygenase and peroxidase activities. Mn was added to a 6-day-old culture. The enzyme activities in the $100,000 \times g$ supernatant were measured after 10 h of further incubation. Error bars represent ± 1 SD. ■, oxygenase; ▲, peroxidase. O.D., optical density.

dants were added to the medium concomitantly with atrazine (Table 3). Nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase, peroxidase, and P-450, caused 60% inhibition when added at 0.1 mM and more than 85% inhibition when 0.15 mM was added. Piperonyl butoxide (PBO), a P-450 inhibitor, inhibited the accumulation of all metabolites by more than 85 to 95%. Other antioxidants also had some inhibitory effects, though less than those of NDGA and PBO. Hydroquinone (0.1 mM) inhibited the accumulation of all three metabolites by approximately 20 to 30% each, whereas butylated hydroxytoluene had no effect on CAIT accumulation but caused nearly 20 and 30% inhibition in the accumulation of CEAT and CEIOT, respectively. Butylated hydroxyanisole (0.25 mM) caused 50 to 60% inhibition of the accumulation of each metabolite. Gossypol had an inhibitory effect (20%) only on CEAT, and dithiothreitol had no effect on metabolite accumulation at the concentrations added. NDGA also inhibited atrazine transformation when added together with Mn. The

FIG. 6. Effects of manganese on specific P-450 concentrations in microsomal and soluble fractions of 6-day-old *P. pulmonarius* mycelium. The P-450 concentration was determined 10 h after adding Mn. ▲, microsomal fraction; ■, soluble fraction.

FIG. 7. Atrazine-P-450 binding spectra of manganese-induced and noninduced 6-day-old *P. pulmonarius* mycelia. The treatments for the microsomal fraction (A and B) were 0 (A) and 500 (B) μ M Mn; those for the soluble fraction (C to E) were 0 (C), 250 (D), and 500 (E) μ M Mn. Atrazine was added at a concentration of 46 μ M. O.D., optical density.

addition of 0.2 mM NDGA to the culture with 175 μ M Mn inhibited metabolite accumulation by more than 95%; however, 0.1 mM NDGA added to the culture with 175 μ M Mn had a less inhibitory effect and more than 80% metabolite accumulation was observed.

DISCUSSION

The transformation of atrazine by a whole-cell culture of the lignocellulolytic fungus *P. pulmonarius* was enhanced by Mn (both as MnCl_2 and MnSO_4). The stimulatory effect of Mn on atrazine transformation was observed at concentrations of up to 300 μ M Mn, but a decrease in activity was found at higher concentrations. This phenomenon was observed when the grown fungal pellets either remained in the PDB medium to which atrazine and Mn were added or were transferred to atrazine- and Mn-containing potassium phosphate buffer (0.1

TABLE 3. Effects of antioxidants on atrazine transformation by *P. pulmonarius*^a

Antioxidant	Concn (mM)	Metabolite (% of control)		
		CAIT	CEAT	CEIOT
Control		100 (15)	100 (13)	100 (18)
Butylated hydroxyanisole	0.25	47 (7)	40 (8)	46 (9)
Butylated hydroxytoluene	0.1	100 (17)	81 (15)	70 (18)
Hydroquinone	0.1	79 (13)	80 (7)	75 (15)
Dithiothreitol	0.1	108 (12)	105 (13)	107 (17)
Gossypol	0.15	99 (15)	80 (16)	115 (21)
PBO	0.15	10 (3)	14 (4)	5 (3)
NDGA	0.1	29 (7)	41 (10)	35 (15)
NDGA	0.15	15 (4)	17 (4)	5 (3)
NDGA + Mn (175 μ M)	0.1	80 (15)	67 (17)	95 (23)
NDGA + Mn (175 μ M)	0.2	5 (2)	7 (3)	6 (3)

^a Stock solutions were prepared in methanol and were added to the culture to a concentration of less than 1%. Data are the means of four replicates. Data in parentheses are SDs. The accumulation of the metabolites in the control was 0.14, 0.15, and 0.18 mg/liter for CAIT, CEAT, and CEIOT, respectively.

M, pH 6.8) (data not shown). The enhancement of atrazine transformation was accompanied by increases in the brown color of the mycelium, lipid peroxidation, oxygenating and peroxidizing activities of the mycelium, and interaction between atrazine and cell extract. Mn is a biologically important divalent ion which is found in woody matter at a concentration of several tenths of micromole at certain points in wood tissue (5). It plays a crucial role in lignin degradation by ligninolytic fungi by regulating exocellular enzymes, such as Mn-peroxidase and lignin peroxidase (6), demonstrated also in *P. ostreatus* (20). The present study suggests that Mn enhancement of atrazine transformation was probably the result of two non-specific mechanisms, (i) increasing the permeability of the membrane to nonpolar organic molecules, such as atrazine, due to membrane alteration and (ii) increasing the activities of the enzymes responsible for the oxidation of organic molecules, among which is atrazine.

Atrazine was transformed by oxidative N dealkylation and propylhydroxylation to CAIT, CEAT, and CEIOT, and the accumulation of both N-dealkylated and propylhydroxylated atrazine metabolites was enhanced by Mn. In a previous study, we found that these metabolites contained more than 75% of the atrazine which had disappeared (27). At high Mn concentrations (500 to 1,000 μ M), a decrease in the accumulation of the metabolites, as well as a decrease in the intensity of the brown color and an increase in mycelial sliminess probably due to increased Mn toxicity, was observed. Neilson et al. (32) suggested that the sedimentation of MnO₂, the cause of this mycelium browning, is a biological mechanism aimed at reducing the toxicity of Mn and is performed by various microorganisms.

The increased sliminess could be explained by the increase in lipid peroxidation found with an increasing Mn concentration. The enhanced lipid peroxidation caused by the addition of Mn could be caused either directly by Mn (by producing oxygen-reactive species, such as singlet-oxygen via oxidation of polyunsaturated lipids [42]) or by enhancing the enzymatic activities of oxygenase and peroxidase, which involves the production of free radicals (12). Lipid peroxidation could cause changes in the membrane, as reported for wheat thylakoids (36). This alteration could increase membrane permeability to lipid-soluble organic molecules, such as atrazine, and hence increase its interactions with endocellular enzymes. This might be the reason for increases in both N dealkylation and hydroxy-

lation. At a high Mn concentration, the damage in the membrane is probably too high, causing damage to all the vital activities of the fungus. Further work is needed to ascertain the role of increasing membrane permeability on the biodegradation of atrazine and other xenobiotics.

The second mechanism consists of enhanced enzymatic activities. N dealkylation and oxidative hydroxylation are catalyzed by P-450 in higher organisms (1, 10, 37). N dealkylation could also be catalyzed by hemoproteins other than P-450, including peroxidase (33). In the present study, Mn enhanced the activities of linoleic-dependent oxygenases (mono- or dioxygenases), which could represent lipoxygenase, eight dioxygenases, and P-450 (4, 15), and the activity of peroxidase in the mycelium of *P. pulmonarius*. Those oxidizing enzymes are involved in the transformation of xenobiotic compounds. Various organic molecules were reported to be cooxidized by the linoleic-dependent activity of oxygenase (such as lipoxygenase) and peroxidase and non-linoleic-dependent peroxidase. Lipoxygenase oxidizes xenobiotics, such as parathion, to paraoxon (31) and causes epoxidation of benzo(a)pyrene (7). In *P. pulmonarius*, this enzyme was found to be involved in the production of 1-octen-3-ol from linoleic acid in *P. pulmonarius* (4). Mn-peroxidase was also found to oxidize xenobiotic compounds, such as phenanthrene and nonphenolic lignin, in the presence of unsaturated fatty acids in the white rot fungus *P. chrysosporium* (3, 28).

Cytochrome P-450 was determined specifically and found to increase in both microsomal and soluble fractions after Mn was added to the culture. This is the most important enzyme responsible for the detoxification and toxification of various organic compounds (37). So far, stimulation of P-450 by Mn has been found only in Jerusalem artichoke (38), and no report on Mn influence on fungal P-450 is found in the literature. The concentration of P-450 was much higher in the microsomal fraction, as expected, but the interaction between atrazine and P-450 (represented by the binding spectra) was higher in the soluble fraction of the Mn-treated mycelium (Fig. 6). In a previous study, we found that benzo(a)pyrene hydroxylation was mediated by P-450 in both soluble and microsomal fractions (26) of *P. pulmonarius*. This indicates that the P-450 found in the soluble fraction is also active. Evidence of soluble P-450 in *Fusarium oxysporum* was also demonstrated by Shoun et al. (40). The binding of a substrate to the microsomal fraction has been reported to indicate binding to cytochrome P-450 (14, 17), and the binding of substrate to cytochrome P-450 is considered the first step in the transformation reaction catalyzed by P-450 (11). In the present work, binding atrazine to the microsomal fraction showed a reverse type I interaction, indicating the binding of atrazine to high-spin P-450 [F(III) heme S = 5/2] (17), and it was more significant in microsomes obtained from the Mn-induced mycelium (Fig. 7). The type of interaction between atrazine and components from the soluble fraction of the Mn-treated mycelium was changed to type II interactions, indicating the binding of atrazine by the ammonium ion (18). Although these shifts could indicate interaction between atrazine and components other than P-450, changes in the type of interaction with P-450 were reported for other P-450 inducers, such as phenobarbital (35). In that report, it was claimed that since P-450 is found in multiforms in the tissue, the shift in the binding spectrum was a result of a change in the P-450s induced or destroyed and their interaction with the substrate. The type of interactions between substrates or inhibitors and P-450 is known to be influenced in higher organisms by a number of factors, such as enzyme and substrate concentrations, type of tissue, age of the organism, pretreatment, etc. (17). In the present work, the binding spec-

tra of atrazine were observed in the crude enzyme extract, and further isolation of P-450 should be conducted in order to prove the direct interaction of atrazine with P-450. P-450 could be responsible for the N-dealkylation and propylhydroxylation of atrazine or for only the latter, while N-dealkylated metabolites could be catalyzed by the increased activities of other oxygenases or peroxidases. This may be the reason for the greater enhancement in the production of N-dealkylated metabolites than hydroxylated metabolites.

The role of oxidative conditions and the involvement of free radicals in atrazine transformation were also indicated by its inhibition in the presence of antioxidants. Transformation was inhibited most strongly by the addition of NDGA, a lipooxygenase, peroxidase, and P-450 inhibitor (8, 24), and PBO, a P-450 inhibitor, which was found to act as a synergist to the herbicidal activity of atrazine in plants (44). NDGA also inhibited the enhanced activity of Mn-induced culture, showing that the stimulating activity of Mn could be inhibited by this antioxidant.

In conclusion, the enhancement by Mn of atrazine transformation by *P. pulmonarius* was probably due to an increase in nonspecific activity, which could enhance the transformation and detoxification of other organic xenobiotic compounds as well. Mn enhancement has been studied in a *Pleurotus* sp. and other ligninolytic fungi only with regard to ligninolytic activity (6, 20). We have shown here that it also regulates P-450, which has a role in the oxidative activity of this fungus. However, since lipooxygenases (monooxygenase) and P-450 have been reported to have peroxidative activities (11, 21), further work on cell-free degradation of atrazine, purification of the enzyme, and inhibitor studies are required to clarify the specificities of the enzymes involved in Mn enhancement of atrazine transformation by *P. pulmonarius*.

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