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New Pathway for Degradation of Sulfonated Azo Dyes by Microbial Peroxidases of *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*†

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Pathways for the degradation of 3,5-dimethyl-4-hydroxy-azobenzene-4′-sulfonic acid (I) and 3-methoxy-4-hydroxyazobenzene-4′-sulfonamide (II) by the manganese peroxidase and ligninase of *Phanerochaete chrysosporium* and by the peroxidase of *Streptomyces chromofuscus* have been proposed. Twelve metabolic products were found, and their mechanisms of formation were explained. Preliminary oxidative activation of the dyes resulted in the formation of cationic species, making the molecules vulnerable to the nucleophilic attack of water. Two types of hydrolytic cleavage were observed. Asymmetric splitting gave rise to quinone and diazene derivatives, while symmetric splitting resulted in the formation of quinone monoimine and nitroso derivatives. These unstable intermediates underwent further redox, oxidation, and hydrolytic transformation, eventually furnishing 11 organic products and ammonia.

The peroxidases produced by the white-rot fungus *Phanerochaete chrysosporium* during its secondary metabolic stage of growth are known to oxidize a wide variety of organic compounds. The ligninase and manganese peroxidase of this saprophytic organism were evolved to degrade lignin (11), but extensive research has shown that these peroxidases can also initiate the degradation of many man-made organic compounds that are toxic or harmful to the environment (25). The degradation pathways of many of these recalcitrant chemicals are now being elucidated.

Valli et al. recently proposed degradation pathways for 2,4-dichlorophenol, 2,4-dinitrotoluene, and 2,7-dichlorodibenzo-*p*-dioxin (50–52) by *P. chrysosporium*. The proposed pathways were similar in that chlorine or nitro groups were shown to be removed before ring cleavage occurred. Methylation of the resulting hydroquinone was also observed. However, because the reaction mixtures used to study degradation by ligninase contained veratryl alcohol as well as xenobiotic substrates, some of the methylated products could have come from this source. Valli et al. (51) postulated an intertwined reduction-oxidation mechanism for the degradation of trinitrotoluene by *P. chrysosporium*, which involves the reduction of nitro groups to amino groups, the oxidation of 2-amino-4-nitrotoluene to 4-nitro-1,2-quinone, and the reduction of the quinone to a hydroquinone.

There are numerous publications concerning the degradation of organic nitrogen-containing compounds in anaerobic environments (20), but we do not know much about the fate of such compounds in aerobic environments. The degradation of different azo dyes by *Pseudomonas* strains was investigated by Kulla et al. (23). An organism isolated from soil was able to grow on 4-4′-dicarboxyazobenzene as a sole carbon, nitrogen, and energy source and was able to cometabolize Orange I or II. The properties of a purified Orange II NAD(P)H-dependent reductase were described. This inducible enzyme was very substrate specific (54). *Pseudomonas* strain KF46 was not able to mineralize sulfanilic acid; moreover, the presence of sulfanilic acid or its metabolites in the medium interfered with the degradation of aminonaphthol (23). The degradative potential of the bacterial strain described above is restricted to specific dye structures (24). More recently, Haug et al. (17) described the mineralization of the azo dye Mordant Yellow 3 by a bacterial consortium. Total degradation was achieved by alternating aerobic and anaerobic culture conditions (17). Doerge and Corbett (10) recently postulated potential pathways for the oxidation of three arylamines (*p*-toluidine, 4-chloroaniline, and 3,4-dichloroaniline), first to hydroxylamine and then to aryl nitroso products via a two-electron oxidation by chloroperoxidase and pea seed peroxygenase. The authors also reported that the source of oxygen for nitroso group formation was H_2O_2. This finding suggested that peroxidases can oxidize arylamines to nitroso products.

Streptomyces spp. also produce extracellular peroxidases that contribute to their ability to solubilize lignin (1, 38). The initial oxidation of lignin by streptomycetes apparently involves a peroxidase-based mechanism (37) whereby peroxidase participates in lignin transformation by generating water-soluble polymeric products called acid-precipitable polymeric lignins (7, 29). Streptomycetes have been reported to degrade many single-ring aromatic compounds via classic aromatic catabolism pathways. Ramachandra et al. (37) showed that *Streptomyces viridosporus* was able to oxidatively cleave dimeric lignin substructure model compounds into single-ring products. Recently, Godden et al. (15) confirmed this finding for *Thermomonospora mesophila* and *Streptomyces badius*, which were able to use a β-aryl ether dimer as a carbon and energy source, producing substantial amounts of monomeric products. Extracellular peroxidase and catalase activities were detected in both strains.

The decolorization of sulfonated polymeric dyes by *P. chrysosporium* was first shown in 1983, when Glenn and Gold examined Poly B-411, Poly R-418, and Poly Y-606 as substrates

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to monitor the lignolytic activity of this fungus (14). Analogous work using Poly B-411, Poly R-418, and Remazol Brilliant Blue as substrates and *Streptomycetes* species has been done by Pasti and Crawford (28). Cripps et al. (8) first reported the ability of *P. chrysosporium* to degrade sulfonated azo dyes, in work which showed that Orange II, Tropaeolin O, Congo Red, and one heterocyclic dye, Azure B, were degraded. Azure B was further examined as a substrate for lignin-type peroxidases from various fungal sources (3). This dye appeared to have many advantages over standard veratryl alcohol assays. We have recently proposed that two of our novel azo dyes could be suitable for assaying lignin-specific peroxidases since they may distinguish between manganese peroxidase and lignin peroxidase activities (31).

Sulfonated azo dyes, the largest class of dyes, have great structural differences (16, 18) and consequently offer a great variety of colors. In previous work, we have shown that various sulfonated azo dyes are not equally susceptible to microbial attack. Susceptibility under aerobic conditions depended on the aromatic substitution pattern and also on the microorganism (31). We have also examined the possibility of increasing the biodegradability of azo dyes by introducing a lignin-like subunit into the chemical structure of the azo dye. Acid Yellow 9 became more degradable by *P. chrysosporium* when a guaiacyl moiety (4-hydroxy-5-methoxyphenyl) was introduced to this dye structure. Some *Streptomyces* spp. also degraded the modified dye. Acid Yellow 9 was not recognized by these bacteria as a substrate (35). Decolorization studies, however, demonstrate only the transformation of the chromophoric group of dyes, while mineralization demonstrates the conversion of the dye to carbon dioxide. We recently demonstrated the mineralization of water-soluble sulfonated azo dyes by *P. chrysosporium* and *Streptomyces chromofuscus* (36), while Spadaro et al. have confirmed the mineralization of nonsulfonated azo dyes by ligninolytic cultures of *P. chrysosporium* (43).

To understand the decolorization and degradation mechanism of azo compounds under aerobic conditions, detailed information is needed about the initial enzymatic transformation of azo linkages. This information, together with an understanding of how chemical structure influences the susceptibility of these man-made compounds to degradation, could help to develop a new generation of readily degradable dyes. Such research could also exemplify how a new generation of less recalcitrant chemicals might be produced in the future.

Here we propose for the first time the initial steps in the pathways for the aerobic, microbial degradation of recalcitrant man-made sulfonated azo dyes. Two azo dyes and three peroxidase preparations, two from *P. chrysosporium* and one from *S. chromofuscus*, were used. In all cases, the same products were detected, which suggests very similar degradation routes for the two microorganisms. Our findings support the cationic radical pathway for peroxidase-catalyzed degradation of aromatic compounds.

MATERIALS AND METHODS

Azo dye numbers, in this case 1 and 2, refer to the structures tested in our previous work (31). Azo dye 2 in this study is the amide of azo dye 3 in the previous work (31).

Chemicals. 4-Hydroxybenzenesulfonic acid, 3,4-dimethoxybenzyl alcohol, and 2,6-dimethyl-1,4-benzoquinone were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). 4-Aminobenzenesulfonamide was obtained from Sigma Chemical Co. (St. Louis, Mo.). Tetrabutylammonium hydrogen sulfate and 4-hydrazinobenzenesulfonic acid were obtained from the Eastman Kodak Company (Rochester, N.Y.). Hydroxylamine hydrochloride and hydrazine sulfate were obtained from J. T. Baker Chemical Co. (Phillipsburg, N.J.). All solvents used were high-pressure liquid chromatography (HPLC) or mass spectrometry (MS) grade, and all other chemicals were reagent grade. All other compounds were prepared as described below.

Synthesis of methyl fluorosulfate. Methyl fluorosulfate, an auxiliary reagent, was prepared as described previously (2, 42, 45). The product was distilled under vacuum and stored in 0.5-ml portions in 2-ml sealed glass vials.

Preparation of 2,6-dimethyl-4-nitrosophenol. 2,6-Dimethyl-4-nitrosophenol was synthesized as described elsewhere for the preparation of nitrosothymol (21) (mass spectrum *m/z* [relative intensity as a percentage], 153 [1], 152 [4], 151 [M^+, 100], 137 [83], 136 [31], 135 [36], 134 [31], 108 [30], 94 [22], 79 [23], 77 [25]).

Synthesis of 2,6-dimethyl-4-aminophenol. 2,6-Dimethyl-4-aminophenol was synthesized as described elsewhere for aminophenol (21) (mass spectrum *m/z* (relative intensity as a percentage], 138 [8], 137 [M^+, 100], 122 [21], 108 [15], 94 [13]).

Preparation of azobenzene-4,4′-disulfonic acid. Sulfanilic acid was oxidized with NaOCl (40). The crude product contained a mixture of azobenzene-4,4′-disulfonic acid and its mono and dichloro derivatives.

Preparation of 4,4′-azobenzenedisulfonic acid dimethyl ester. 4,4′-Azobenzenedisulfonic acid dimethyl ester was prepared by oxidation of sulfanilic acid with hydrogen peroxide (41); the disodium salt was transformed to the sulfonyl chloride (13) and then to the dimethyl ester by phase-transfer techniques (47).

Preparation of benzenesulfonic acid methyl ester. Benzenesulfonic acid methyl ester was prepared from benzene sulfonyl chloride as described previously (47).

Preparation of 4-hydroxybenzenesulfonamide. 4-Hydroxybenzenesulfonamide was synthesized from 4-hydroxybenzene sulfonic acid (sodium salt) via sulfonyl chloride (47) by a general procedure for amides (53).

Preparation of 4-hydroxybenzenesulfonic acid methyl ester. 4-Hydroxybenzenesulfonic acid methyl ester was prepared from 4-hydroxybenzene sulfonic acid (sodium salt) in a two-step reaction. The sulfonyl chloride was obtained as described previously (14), and the methyl ester was obtained by a phase-transfer procedure (47).

Preparation of 2-methoxy-4-aminophenol. 2-Methoxy-4-aminophenol was prepared from guaiacol (2-methoxyphenol) by nitrosation and reduction as described previously for aminothymol (44).

Preparation of methoxyhydroquinone. Methoxyhydroquinone was obtained from vanillin (4-hydroxy-3-methoxybenzaldehyde) by the Dakin method according to the procedure used for pyrogallol 1-monomethyl ether (46).

Preparation of 4-nitrosobenzenesulfonic acid methyl ester. 4-Nitrosobenzenesulfonic acid methyl ester was synthesized from 4-nitrobenzene sulfonyl chloride in a multistep procedure as described by Szeja (47) and modified by Bauer and Rosenthal (4).

Preparation of sulfanilic acid methyl ester. Sulfanilic acid methyl ester was prepared as described previously (19).

Preparation of 3,5-dimethyl-4-hydroxyazobenzene-4′-sulfonic acid (azo dye 1). The preparation of azo dye 1 required three steps: (i) diazotization, (ii) coupling, and (iii) purification.

(i) Diazotization. Sulfanilic acid (8.65 g, 50 mmol) was dissolved in 1 N sodium hydroxide (50 ml, 50 mmol) and cooled to 0°C, and 4 N sodium nitrite (12.5 ml, 50 mmol) was added. The solution was stirred mechanically in a beaker immersed in an ice-salt freezing bath, and 3 N hydrochloric

TABLE 1. Analytical data for azo dyes

Compound	Elemental analysis						UV-VIS spectral data					
	% C		% H		% N		0.01 N HCl		0.01 N NaOH		Isobestic point	
	Calc[a]	Found	Calc	Found	Calc	Found	λ_{max} (nm)	ε	λ_{max} (nm)	ε	λ_{14} (nm)	ε
$C_4H_{16}N_2O_5S$ monohydrate	51.84	51.97	4.97	5.02	8.64	8.73	362	24,000	470	31,100	394	10,700
$C_{22}H_{24}N_4O_4S_2$	55.91	55.77	5.12	5.33	11.86	11.76	366	21,300	210	25,000		
$C_{13}H_{13}N_3O_4S$	50.08	50.35	4.26	4.41	13.67	13.51	374	19,800	468	29,600		

[a] Calc, calculated.

acid (34 ml, 102 mmol) was introduced at a rate that kept the temperature below 5°C. Tests for both nitrous acid (iodide starch paper) and for an excess of mineral acid (Congo Red paper, blue) were positive. The reaction mixture was kept in a cooling bath for the next step.

(ii) Coupling. 2,6-Dimethylphenol (6.1 g, 50 mmol) was dissolved in 1 N sodium hydroxide (50 ml, 50 mmol), and the solution was cooled to 0°C. A well-mixed suspension of diazotized sulfanilic acid was introduced within 10 min to the chilled, stirred solution. The reaction vessel was removed from the cooling bath, and the precipitated sodium salt of the azo dye was stirred at room temperature for 10 min. The reaction mixture was heated in a steam bath until the precipitate dissolved. Concentrated hydrochloric acid (50 ml, 582 mmol) was dropped into the hot solution while stirring was continued. After cooling to room temperature, the crystallization product was filtered off, washed with a small amount of cold water, and air dried. The resulting purple crystals with metallic luster (15.2 g) represent a yield of 93.8% as calculated for the monohydrate.

(iii) Purification. Recrystallization from water is a simple, efficient method for purification. The crude product (15 g) was dissolved in 300 ml of boiling water, cooled to room temperature, and left to crystallize slowly overnight in a refrigerator at 5°C. The crystals were then filtered off and washed with cold water. After the crystals were dried at room temperature, then in a desiccator over silica gel, 11.1 g of well-shaped purple crystals with metallic luster was obtained, representing a yield of 74%. See Table 1, row 1, for elemental analyses and UV-visible light (VIS) spectral data.

For further characterization, the *S*-benzylisothiouronium salt of the dye was prepared by the standard procedure (56). The melting point (m.p.) was 186 to 187°C (Table 1, row 2).

Preparation of 3-methoxy-4-hydroxyazobenzene-4'-sulfonamide (azo dye 2). The preparation of azo dye 2 required two steps: (i) diazotization and (ii) coupling.

(i) Diazotization. 4-Aminobenzenesulfonamide (8.6 g, 50 mmol) was dissolved in 3 N hydrochloric acid (50 ml, 150 mmol) and then cooled until copious crystallization of the hydrochloride took place. Crushed ice was added (50 g) to the solution, which was cooled in an ice-salt freezing bath, and 4 N sodium nitrite (12.5 ml, 50 mmol) was dropped in under vigorous stirring. The temperature was kept below 0°C, and the potassium iodide starch test was positive at the end of the reaction. The clear, pale yellow solution was kept in the freezing bath for the next step.

(ii) Coupling. Guaiacol (6.2 g, 50 mmol) was dissolved in 1 N sodium hydroxide (50 ml, 50 mmol), and 1 N sodium acetate (50 ml, 50 mmol) was added. The solution was vigorously stirred and cooled in an ice-salt freezing bath until the temperature dropped below −5°C. The diazonium solution was added dropwise while stirring was continued. A dark cherry red color developed immediately, and a brown-yellow precipitation began when one-third of the diazonium salt solution was introduced. The coupling reaction was continued at −5°C, and vigorous stirring was necessary to keep the thick suspension sufficiently agitated. At the end of the coupling operation, the color of the reaction mixture changed from red to pale yellow. The reaction vessel was removed from the cooling bath, and stirring was continued at room temperature for 1 h. The mobility of the reaction mixture increased substantially as soon as crystallization in the suspension was easily seen. The precipitate was filtered off on a Büchner funnel, and the filter cake was well pressed and washed with water. After drying, 14.2 g of the crude product (m.p., 234 to 235°C) was obtained (yield, 92.5%). Recrystallization from glacial acetic acid (1 g in 40 ml of boiling CH_3COOH) resulted in a product with an m.p. of 235 to 236°C and yield of 84%. Recrystallization from ethanol (1 g in 50 ml of boiling C_2H_5OH) gave orange needles (m.p., 237 to 238°C; yield, 68%) (Table 1, row 3).

HPLC-MS analyses. A Hewlett-Packard 1050 HPLC equipped with a UV-VIS detector and operating at either 210, 260, or 394 nm was used to deliver samples to a particle beam vacuum desolvation interface connected to an MS quadruple detector (Hewlett-Packard 5989A MS controlled by HP 59940A MS ChemStation software, HP-UX series). The following adjustments were made to the electron impact sample ionization mode: repeller, 7 V; emission, 300 V; and electron energy, 70 eV. The source temperature was 250°C. Perfluorotributylamine was used as the calibration standard for the MS engine.

A PhaseSep microbore Spherisorb S5 ODS2 (25 by 0.2 cm) C-18 column was used for separation. A linear gradient from 20% acetonitrile in water (isocratic for the initial 4 min) to 100% acetonitrile in 25 min, and isocratic from 25 to 30 min at the flow rate of 0.3 ml/min, was applied. Before use, solvents were saturated with helium. Products were identified by comparing their retention times with those of standards and/or their fragmentation patterns with those of chemically prepared compounds as well as with the mass spectra of known compounds (Wiley data base) stored in an MS ChemStation library. The library was searched by using probability-based matching or parametric retrieval.

GC-MS. The instrument used was an HP series II 5890 gas chromatograph (GC) equipped with a capillary-fused silica DB-5MS column (25 m by 0.21 mm by 0.33 μm; J&W Scientific, Folsom, Calif.) and MS interface. The interface temperature was set at 280°C. The MS detector was set as it was for the HPLC analyses except for the source temperature, which was 175 to 200°C. A linear gradient of the oven temperature from 100 to 300°C at 10°C/min was used.

Culture conditions and enzyme preparation. *P. chrysosporium* BKM 1767 (ATCC 55184) was maintained and grown as

described in our previous work (34). For enzyme production, a medium with an increased concentration of microelements was used (5). Fungal mycelia were grown at 37°C on the roughened interior walls of a 20-liter carboy filled with 100% oxygen and containing 1 liter of medium which was rotated at about 0.2 rpm (33). A crude preparation of peroxidases was prepared from freshly filtered media as described earlier (32). The specific activities of ligninase and manganese peroxidase were determined by using veratryl alcohol and vanillyl acetone, respectively, as substrates (32, 49).

The stock culture of wild-type *S. chromofuscus* A11 (ATCC 24725) was maintained as described previously (29). A crude enzyme preparation was prepared from shaken, 2-day-old cultures. The extracellular enzyme was prepared from culture filtrate as described previously (31).

Product extraction and derivatization after enzymatic oxidation of azo dye 1, sulfanilic acid, and 4-hydrazinobenzenesulfonic acid. The manganese peroxidase reaction mixture (10 ml) contained 50 mM sodium tartrate buffer (pH 5), 1 mM $MnSO_4$, 156 μM dye, 200 μM H_2O_2, and 50 U of enzyme. To increase yields of the reaction products, multiple additions of substrate, enzyme, and hydrogen peroxide were made. Up to 5 mg of azo dye was added to each reaction mixture. Each addition was performed after the reaction mixture showed decolorization. The control reactions did not contain hydrogen peroxide or enzyme.

The ligninase reaction mixture (10 ml) contained 50 mM sodium tartrate buffer (pH 2.5), 156 μM dye or 290 μM sulfanilic acid, 200 μM H_2O_2, and 15 U of enzyme. To increase the concentrations of reaction products, multiple additions of substrate, enzyme, and hydrogen peroxide were made as described above.

The streptomycete peroxidase reaction mixture (10 ml) contained 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 500 μM hydrogen peroxide, peroxidase (160 absorbance units, where 1 U of enzyme is expressed as the amount of enzyme required for an increase of 1 absorbance unit/min at 510 nm), and 156 μM of dye. The reaction process was performed as described above, and incubation was carried out at room temperature for 2 h.

At the end of the incubation period, 10 mg of tetrabutylammonium hydrogen sulfate was dissolved in the sample. The reaction products and remaining substrate were removed with three extractions of methylene chloride (60 ml in total). The extract was dried with sodium sulfate, filtered, and concentrated to about 0.5 ml. One to three drops of methylfluorosulfate were added to the solution, and the mixture was left for 1 h at room temperature or in a heat block at 60°C (45). To remove the tetrabutylammonium salt and hydrolyze the remaining methyl fluorosulfate, the sample was washed five times with an equal volume of water (45). The solution was dried, evaporated, and dissolved in 0.5 ml of acetonitrile. The sample was immediately chromatographed on the GC and/or HPLC and analyzed by MS.

Product extraction after enzymatic oxidation of azo dye 2 and 4-hydrazinobenzenesulfonamide. The reaction mixture and conditions were as for azo dye 1. After the reaction, the sample was extracted twice with methylene chloride and twice with ethyl ether. Extracts were dried and evaporated, and the residue was dissolved in 0.5 ml of acetonitrile. The sample was analyzed without derivatization.

Determination of oxygen concentration in reaction mixtures. Dissolved oxygen was measured with an oxygen meter equipped with a Clark-type oxygen probe as described earlier (34, 39).

Determination of dissolved ammonia. A Fisher model Accumet 950 pH meter equipped with an Orion model 95-12 ammonia electrode was used to measure dissolved ammonia in the reaction mixtures (27, 48).

RESULTS

Nearly complete decolorization of both dyes was observed after 2 h of incubation. Substantial amounts of specific reaction products accumulated in the reaction mixtures (Fig. 1 and 2). Each new peak detected by HPLC was analyzed by a particle beam interface and MS detector as well as by MS with the GC interface. During oxidation of dyes and sulfanilic acid, the oxygen concentration remained unchanged; no oxygen consumption or release was observed. This observation suggested that water or hydrogen peroxide was the hydroxylation agent that acted on the azo dyes. In the reaction mixture with *P. chrysosporium* enzymes, ammonia was detected (about 25% of the theoretical amount expected from sulfanilic acid and about 20% of the amount expected for azo dye 1).

Figures 1 and 3 show the proposed pathways of the degradation of azo dyes 1 and 2 by peroxidatic enzymes of *P. chrysosporium* and *S. chromofuscus*. Compounds found in significant amounts in reaction mixtures are marked by brackets.

Peroxidase metabolic intermediates detected for azo dye 1. All sulfonated products from azo dye 1 (3,5-dimethyl-4-hydroxy-azobenzene-4'-sulfonic acid) were derivatized and analyzed as sulfomethyl esters. For azo dye 1, mass spectrum *m/z* (relative intensity [percent] shown in parentheses) of the methyl ester was 322 (3), 321 (8), 320 (M^+, 38%), 289 (1), 225 (1), 171 (4), 149 (30), 121 (100), 91 (29), 77 (31). In Mn(II) peroxidase and streptomycete peroxidase reaction mixtures, 2,6-dimethyl-1,4-benzoquinone having mass spectrum *m/z* (relative intensity [percent]) 138 (17), 137 (13), 136 (M^+, 100%), 108 (62), 96 (26), 79 (41), 68 (96) was found by using the GC interface. In the same reaction mixture, 4-hydroxybenzenesulfonic acid methyl ester having mass spectrum *m/z* (relative intensity [percent]) 190 (4), 189 (6) 188 (M^+, 70%), 171 (30), 157 (100), 109 (48), 93 (72), 65 (53), methyl ester of 4-aminobenzenesulfonic acid methyl ester having mass spectrum *m/z* (relative intensity [percent]) 189 (4), 188 (6), 187 (M^+, 63), 156 (100), 108 (49), 92 (56), 65 (32), methyl ester of 4-nitrosobenzenesulfonic acid methyl ester having mass spectrum *m/z* (relative intensity [percent]) 203 (5), 202 (11), 201 (M^+, 100), 170 (43), 122 (71), 106 (48), 79 (31), and benzenesulfonic acid methyl ester having mass spectrum *m/z* (relative intensity [percent]) 174 (2), 173 (5), 172 (19^+, 38) 142 (11), 141 (31), 108 (12), 77 (100), 51 (43) were found. Several aromatic dimeric and trimeric products were found in larger amounts in the *S. chromofuscus* peroxidase reaction mixtures than in the *P. chrysosporium* Mn(II) peroxidase reaction mixture (31).

Peroxidase metabolic intermediates detected for azo dye 2. Azo dye 2, 3-methoxy-4-hydroxyazobenzene-4'-sulfonamide, was characterized by mass spectrum *m/z* (relative intensity [percent]) 309 (3), 308 (8), 307 (M^+, 43), 172 (68), 156 (68), 151 (27), 139 (91), 124 (61), 123 (100), 108 (48), 96 (47). After the incubation of this compound with *S. chromofuscus* peroxidase or *P. chrysosporium* peroxidases, the following structures were detected: 4-aminobenzenesulfonamide (sulfanilamide), characterized by mass spectrum *m/z* (relative intensity [percent]) 174 (6), 172 (10), 172 (M^+, 100), 156 (75), 140 (4), 108 (62), 92 (68), 65 (67); benzenesulfonamide, characterized by mass spectrum *m/z* (relative intensity [percent]) 158 (6), 157 (M^+, 57), 141 (28), 93 (41), 77 (100), 64 (25), 54 (19), 51 (47); 4-hydroxybenzenesulfonamide, characterized by mass spectrum *m/z* (relative intensity [percent]) 175 (4), 174 (7), 173

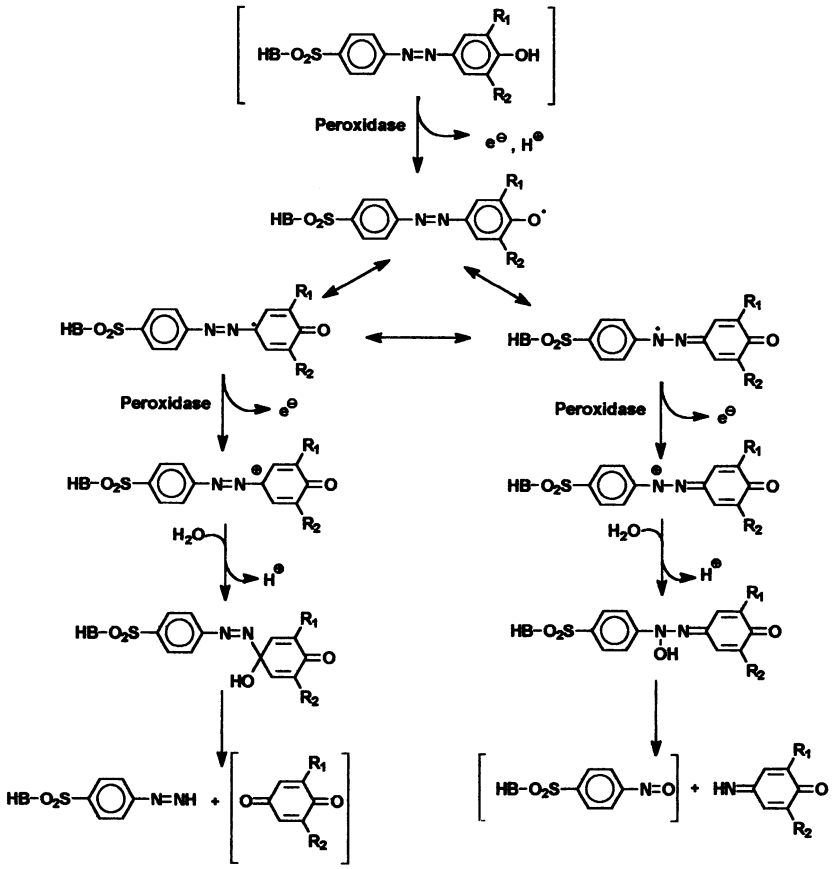

FIG. 1. Proposed mechanism for alternative asymmetrical and symmetrical cleavages of sulfonated azo dyes by *P. chrysosporium* and *S. chromofuscus* peroxidases (ligninases). The compounds represented by structures in brackets were found in the reaction mixture. Azo dye 1, $R_1 = R_2 = CH_3$ and B = O; azo dye 2, R_1 = H, $R_2 = OCH_3$, and B = NH.

(M^+, 76), 157 (100), 125 (9), 109 (30), 108 (11), 93 (61), 65 (73); 2-methoxyhydroquinone, characterized by mass spectrum *m/z* (relative intensity [percent]) 141 (7), 140 (M^+, 100), 125 (69), 111 (4), 97 (59), 79 (5), 69 (7), 55 (7); and 2-methoxy-4-aminophenol, characterized by mass spectrum *m/z* (relative intensity [percent]) 140 (11), 139 (M^+, 100), 124 (41), 96 (37), 80 (6), 68 (19), 52 (24). One oligomeric compound, the product of dye 2 coupling with methoxyhydroquinone, was identified for the Mn(II) peroxidase degradation mixture: mass spectrum *m/z* (relative intensity [percent]) 430 (12), 429 (M^+, 56), 261 (98), 245 (100), 154 (38), 138 (32).

Ligninase transformation of sulfanilic acid. Sulfanilic acid and 4-hydrazinebenzenesulfonic acid were included in these investigations as possible intermediates in the sulfonated azo dye degradation pathway. We have previously shown that an agitated culture of *P. chrysosporium* mineralized sulfanilic acid to about 17% after 21 days of growth (36). At the beginning of degradation, a brown color accumulated in the culture. In the present ligninase reaction mixture, sulfanilic acid was rapidly oxidized to a similar dark-brown-colored product, which in higher concentration tended to precipitate. The precipitate contained azobenzene-4,4′-disulfonic acid. Its dimethyl ester, characterized by mass spectrum *m/z* (relative intensity [percent]) 372 (4), 371 (6), 370 (M^+, 30), 199 (35), 185 (40), 171 (100), 156 (33), was one of the components of the reaction mixture (Fig. 3). The azobenzene-4,4′-disulfonic acid was also detected in the azo dye 1 reaction mixture with Mn(II) peroxidase and with *Streptomyces* peroxidase preparations. We did not detect 4-hydroxybenzenesulfonic acid during degradation of sulfanilic acid.

DISCUSSION

Our earlier results (30–32, 35, 36) suggested that in azo dyes in which the whole molecule represents a fully conjugated electronic system, an access site with a lignin-like structure is sufficient to provide an enzyme-dependent excitation state, from which the stepwise propagation of cleavage processes usually resulted in the biodegradation of the entire molecule. The oxidative character of the cleavage suggested the formation of intermediates different from those postulated for anaerobic transformation mechanisms whereby aromatic amines accumulated in the cultures (6, 24, 26). Thus, a new synthetic class of biodegradable azo dyes with lignin-like features, in which the formation of carcinogenic amines as intermediates during the aerobic degradation of the dyes might be avoided, may possibly be developed.

Because methylation with methyl fluorosulfate could have created artifacts, causing inaccuracies in interpreting analytical results, we used a second substrate in the sulfonamide form. This less polar compound is soluble in both water and organic solvents and does not need derivatization for analysis. We realized that introducing the sulfonamide group as a substituent would diminish the electron-withdrawing effect and could

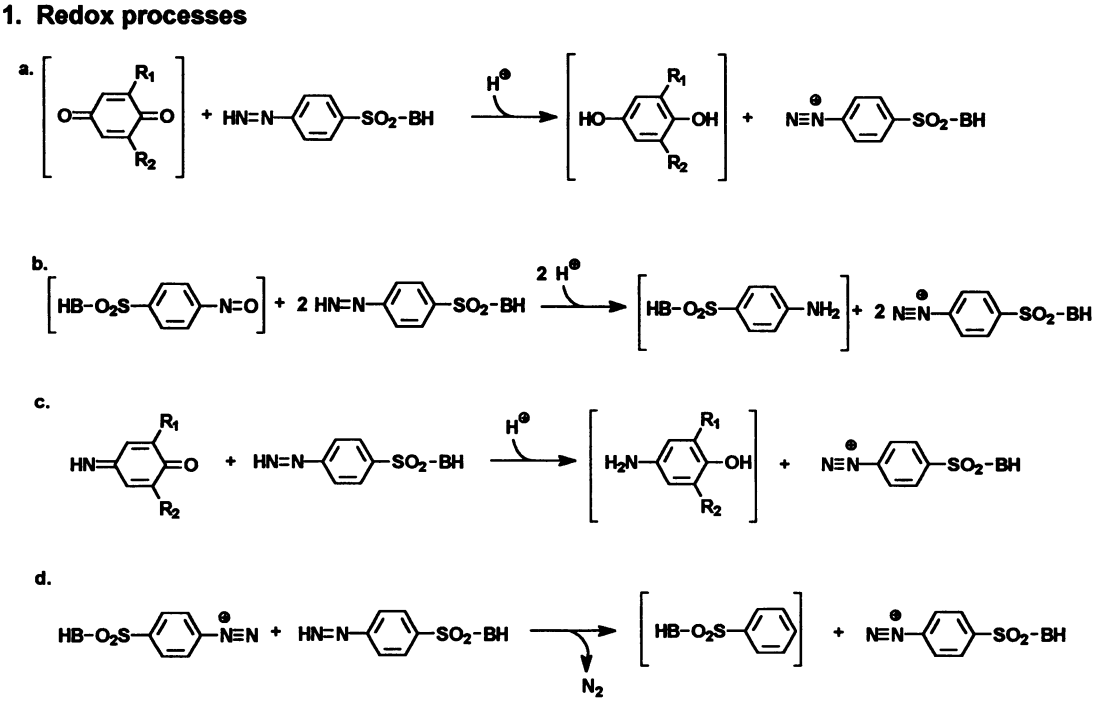

FIG. 2. Succeeding transformations of the products of the initial azo dye degradation. The compounds represented by structures in brackets have been found in the azo dye reaction mixtures. Original azo dye 1, $R_1 = R_2 = CH_3$ and B = O; azo dye 2, $R_1 = H$, $R_2 = OCH_3$, and B = NH.

change the reaction rate, but we also realized that the reaction pathway should not be altered.

Our findings about the intermediates and mechanisms of azo dye degradation by ligninolytic peroxidases of *P. chrysosporium* and *S. chromofuscus* lead us to conclude that the enzymes convert the azo dye to a cation radical (32) that is susceptible to the nucleophilic attack of water or hydrogen peroxide molecules. The results support the conclusion that the azo linkage is split both asymmetrically and symmetrically (Fig. 1). The resulting reactive products (intermediates) will undergo several redox reactions that produce a more stable intermediate (Fig. 2). To find support for the possibility of symmetrical cleavage, we synthesized 2,6-dimethyl-4-nitrosophenol and 2,6-dimethyl-4-aminophenol. Neither 2,6-dimethyl-4-nitrosophenol (molecular weight, 151) nor 2,6-dimethyl-4-aminophenol (molecular weight, 137) was observed in any of the reaction mixtures. The absence of these two compounds and the abundance of 2,6-dimethyl-1,4-benzoquinone suggested nonsymmetric cleavage of the azo linkage. On the other hand, the presence of 4-nitrosobenzenemethylsulfonate having an HPLC retention time of 14.7 min in the azo dye 1 reaction mixture and the presence of 2-methoxy-4-aminophenol in the azo dye 2 reaction mixture indicated some symmetrical cleavage of the azo linkage.

Sulfanilic acid was oxidized only by ligninase. The formation of a brown-yellow product was observed immediately after the substrate was added. The same phenomenon was observed when sulfanilic acid was used in a degradation study with a whole culture of *P. chrysosporium* (36).

In support of our proposed mechanism of asymmetric cleavage of the azo linkage (Fig. 1) are the well-known condensation reactions of quinones with arylhydrazines, which

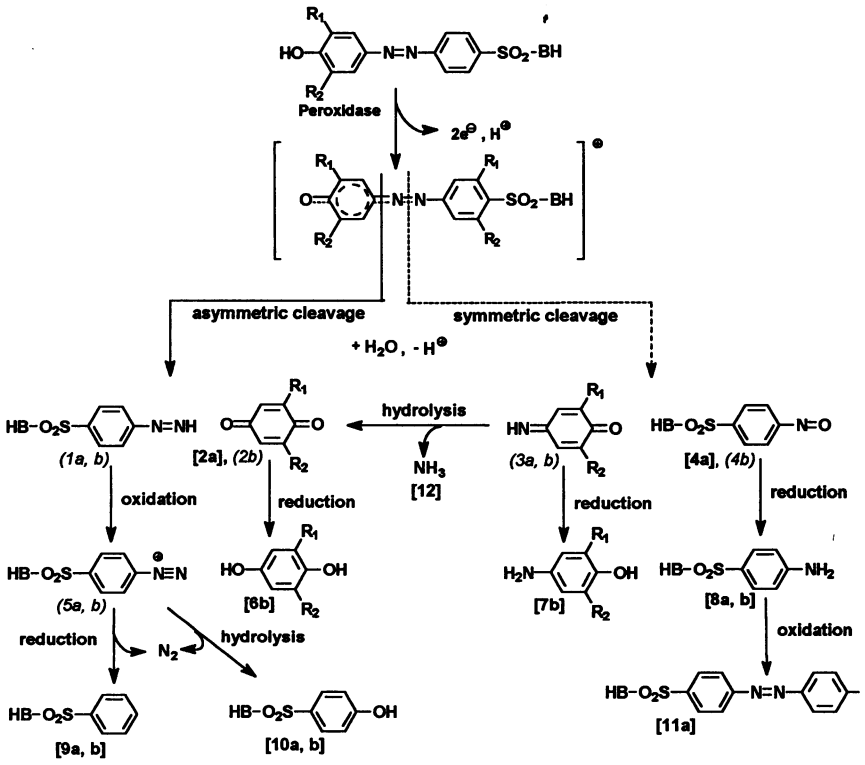

FIG. 3. Proposed pathway for peroxidase-catalyzed degradation of sulfonated azo dyes. The compounds represented by numbers in parentheses have not been found, but their existence is rationalized as necessary intermediates for the final products found. The compounds represented by numbers in brackets have been found in reaction mixtures. Substitution pattern a [as in I], $R_1 = R_2 = O$ and B = O; substitution pattern b [as in II], R_1 = H, R_2 = OCH, and B = NH. [2a], 2,6-dimethyl-1,4-benzoquinone; [4a], 4-nitrosobenzenesulfonic acid; [6b], 2-methoxyhydroquinone; [7b], 2-methoxy-4-aminophenol; [8a], sulfanilic acid; [8b], sulfanilamide; [9a], 4-hydroxybenzenesulfonic acid; [9b], 4-hydroxybenzenesulfonamide; [10a], benzenesulfonic acid; [10b], benzenesulfonamide; [11a], azobenzene-4,4′-disulfonic acid; [12], ammonia.

are used for synthesis of hydroxyazo compounds. For example, *o*-naphthoquinone will condense with phenylhydrazine to form 1-phenylazo-2-hydroxynaphthalene. The condensation is reversible, and the backward reaction is asymmetric hydrolysis. It is also well known that hydroxyazobenzenes exist in tautomeric equilibrium between hydroxyazo and hydrazono (quinone) forms. The hydrazones are susceptible to hydrolytic cleavage by a variety of processes that always produce asymmetric splitting between the nitrogen of the azo group and the carbon of the aromatic ring substituted with a hydroxy group (Fig. 1). Our observation of methoxyhydroquinone as a product of enzymatic attack on guaiacyl-containing azo dyes agrees with these known asymmetric cleavage mechanisms. Asymmetric cleavage catalyzed chemically was observed by Desai and Giles (9) in 1949. This unexpected observation could not be explained at that time. Stiborova et al. (44) observed the transformation of Sudan I (1-phenylazo-2-hydroxynaphthalene) by microsomal enzymes (P-450-type heme-containing oxygenases) to a variety of products. One product was benzenediazonium ion, which could be produced only by asymmetric cleavage, probably by an oxidative mechanism similar to that proposed by us for heme-containing peroxidases.

Symmetric cleavages of azo linkages might also yield some of the products that we observed. The observed products might arise from oxidation-reduction interactions between intermediates formed by mixed types of hydrolytic cleavages of the azo linkage. Symmetric cleavage could form a nitroso group that is easily reduced to an amine. If simultaneous asymmetric cleavage provided reducing species in the form of hydrazine derivatives, sulfanilic acid or sulfanilamide could be formed through interactions of various products present. In another such scenario, 3,5-dimethyl-4-hydroxyaniline (produced by symmetric cleavage of dye 2) might reduce 2,6-dimethyl hydroquinone and 2,6-dimethyliminoquinone. Upon hydrolysis by water, this last compound would yield ammonia and 2,6-dimethylbenzoquinone.

However, additional support for a primarily asymmetric cleavage mechanism comes from our observations of 4-hydroxybenzenesulfonic acid and 4-hydroxybenzenesulfonamide in reaction mixtures. Phenylhydrazine sulfonic acid produced by asymmetric hydrolysis could be oxidized rather easily to a diazonium salt. Hydrolysis of the diazonium salt would yield the 4-hydroxybenezenesulfonic acid that we observed. Chemical hydrolysis of hydrazo dyes similar to that shown in the proposed pathways has already been reported (9, 12).

The results indicate that azo dyes can be cleaved both symmetrically and asymmetrically, as presented in Fig. 1. Our summary of how peroxidases catalyze the azo dye transformations observed in our study is shown in Fig. 3. These results, along with our previous data (30–32, 35, 36), show that the accumulated knowledge of the mechanisms of azo dye biodegradation can be used for the rational explanation of oxidative splitting mechanisms of azo linkages.

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