Glutathione-mediated mineralization of $^{14}$C-labeled 2-amino-4,6-dinitrotoluene by manganese-dependent peroxidase H5 from the white-rot fungus *Phanerochaete chrysosporium*

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**Abstract** Manganese-dependent peroxidase (MnP) H5 from the white-rot fungus *Phanerochaete chrysosporium*, in the presence of either Mn(II) (10 mM) or GSH (10 mM), was able to mineralize $^{14}$C-U-ring-labeled 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) up to 29% in 12 days. When both Mn(II) and GSH were present, the mineralization extent reached 82%. On the other hand, no significant mineralization was observed in the absence of both Mn(II) and GSH, suggesting the requirement of a mediator [either Mn(II) or GSH] for the degradation of 2-A-4,6-DNT by MnP. Using electron spin resonance (ESR) techniques, it was found that the glutathionyl free radical (GS*) was produced through the oxidation of GSH by MnP in the presence as well as in the absence of Mn(II). GS* was also generated through the direct oxidation of GSH by Mn(III). Our results strongly suggest the involvement of GS* in the GSH-mediated mineralization of 2-A-4,6-DNT by MnP.

**Introduction**

White-rot fungi like *Phanerochaete chrysosporium* are known to degrade a wide range of xenobiotic pollutants such as organochlorides, pesticides, polynuclear aromatic hydrocarbons (PAH), polychlorobiphenyls (PCB) (Barr and Aust 1994; Reddy 1995), and nitroaromatic explosives, including 2,4,6-trinitrotoluene (TNT) (Fernando et al. 1990). TNT is toxic, carcinogenic, and persistent in the environment (Won et al. 1976; Styles and Cross 1983). Production, use, and destruction of ammunition stocks have led to a severe pollution of water and soils in Europe and North America (Walsh 1990). Biodegradation of TNT by white-rot fungi involves two distinct steps: TNT is first reduced, likely through a membrane-associated redox system, to aminonitrotoluene and, to a lesser extent, to dinitrotoluenes (Stahl and Aust 1993; Rieble et al. 1994). Further transformation of the reduction products of TNT is due mainly to extracellular liginolytic enzymes: the lignin peroxidases (LiP) (Tien and Kirk 1983), the manganese-dependent peroxidases (MnP) (Kuwahara et al. 1984), and the laccases (Niku-Paavola et al. 1988). The rate-limiting step of the process is the oxidative transformation of the TNT reduction products (Bumpus and Tatarko 1994). Recently, Hofrichter et al. (1998a) and Van Aken et al. (1999) showed that MnP from white-rot fungi are able to mineralize TNT and its main products of reduction. MnP can directly oxidize some substrates, but most often MnP acts indirectly through the oxidation of Mn(II) to Mn(III), which is a strong diffusible oxidant, in its turn able to oxidize a wide range of organic molecules (Wariishi et al. 1988, 1989a). Addition of small amounts of reduced thiols like glutathione (GSH) strongly enhances the mineralization rate of TNT and of its reduction products (Hofrichter et al. 1998a; Van Aken et al. 1999). These authors have postulated the involvement of the highly reactive glutathionyl free radical (GS*) in the mineralization process.

Through in vitro experiments using purified MnP H5 from the white-rot basidiomycete *P. chrysosporium*, we investigated the requirement for a mediator – Mn(II) and/or GSH – in the MnP-catalyzed mineralization of $^{14}$C-U-ring-labeled 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT), one of the main reduction products of TNT. Using electron spin resonance (ESR) techniques, we investigated the actual involvement of the glutathionyl free radical GS* in the mineralization of $^{14}$C-2-A-4,6-DNT by MnP.
Materials and methods

Preparation of MnP

MnP isozyme H5 was from a ligninolytic culture of *P. chrysosporium* (BK-M-F-1767), purified as previously described (Tuisel et al. 1990). A 7-day extracellular fluid harvested from submerged ligninolytic cultures of the white-rot fungus *P. chrysosporium* was concentrated by ultrafiltration, dialyzed, and purified by fast protein liquid chromatography (FPLC). The collected fractions were assayed for LiP, MnP, and laccase. The purity of each main peak was analyzed through SDS-PAGE and isoelectric focusing. Purified MnP was treated three times with chelating resin (Chellex; Sigma, St. Louis, Mo.) in order to remove traces of metals, especially Mn. The purified enzyme exhibited a MnP enzymatic activity of about 30,000 U l⁻¹ (1 U = 1 µmol substrate min⁻¹), while no detectable LiP or laccase activity was recorded. Purified MnP was used to prepare MnP reaction mixture.

Mn(III)-malonate solution

Mn(III) solution was prepared according to Aitken and Irvine (1990). Briefly, Mn(III)-acetate powder (Sigma) was dissolved in pure methanol. After filtration, the mixture was stable at room temperature and was used to prepare aqueous Mn(III)-malonate solution. The concentration of Mn(III)-malonate complex was determined spectrophotometrically by measurement of the absorbance at 270 nm (Aitken and Irvine 1990). A molar extinction coefficient of 8.7 × 10⁵ M⁻¹ cm⁻¹ at 270 nm was estimated by titrating the Mn(III)-acetate stock solution.

Enzymatic analyses

Enzymatic activities of MnP, LiP, and laccase were measured photometrically by the direct oxidation of specific substrates as described elsewhere (Tien and Kirk 1983; Niku-Paavola et al. 1988; Warishii et al. 1989b). 0.1 mg ml⁻¹ catalase was added to the assay mixture for laccase in order to prevent interference with peroxidases (Van Aken et al. 1999). Oxidation product of the substrate for LiP (veratraldehyde) was monitored in parallel by HPLC at 310 nm (Van Aken et al. 1999). All activities were expressed in units per liter (U µl⁻¹).

Experiments with unlabeled 2-A-4,6-DNT

Experiments were conducted in 10-ml serum vials containing the following reaction mixture in a final volume of 2.0 ml: 25 mM malonate buffer (pH 4.5), 0 or 10 mM MnSO₄ · H₂O, 0 or 5 mM GSH, 127 µM 2-A-4,6-DNT (25 mg l⁻¹), 16.5 U l⁻¹ glucose oxidase and 15 mM glucose as H₂O₂-generating system, and 1,512 ± 110 U l⁻¹ purified MnP (Hofrichter et al. 1998a). Reaction mixtures were sterilized by filtration (pore size 0.22 µm, Millipore, Bedford, Mass.). The reaction flasks were incubated at 20 °C on a gyratory shaker (190 rpm). Control experiments contained 10 mM MnSO₄ · H₂O, 5 mM GSH, and were carried out with autoclaved MnP. Samples were removed periodically and analyzed for 2-A-4,6-DNT concentration and MnP enzymatic activity. Experiments were performed in triplicate.

Experiments with ¹⁴C-U-ring-labeled 2-A-4,6-DNT

Experiments were conducted in anaerobic 10-ml test tubes tightly closed with rubber septa and containing 1 ml of the reaction mixture described above except that it exhibited a final MnP activity of 3,028 ± 173 U l⁻¹. Because the ratio of GSH concentration to MnP activity is a critical factor for the degradation process (Hofrichter et al. 1998a) and in order to keep it constant (3.3 µM GSH per 1 U l⁻¹ of MnP activity), the concentration of GSH was here 10 mM. ¹⁴C-U-ring-labeled 2-A-4,6-DNT was added to a final activity of 26,250 dpm ml⁻¹. Labeled 2-A-4,6-DNT was mixed with unlabeled 2-A-4,6-DNT in order to reach a final concentration of 127 µM (25 mg l⁻¹). The mixture was sterilized and incubated as described above. The reaction tubes were flushed periodically with air for 15 min. Any gas released was bubbled into two sequential flasks. The first contained scintillation cocktail (Safety Solve Cocktail, Research Products International, Mount Prospect, Ill.) and served as a trap for volatile organic compounds. The second contained an alkaline mixture of scintillation cocktail, methanol, and ethanolamine in a 5:4:1 ratio and was used as a trap for ¹⁴CO₂.

The radioactivity trapped was measured through liquid scintillation counting (LSC) on a Beckman LS 5801 counter (Beckman Instruments, Irvine, Calif.). The mineralization extent is expressed as a percentage of the initial radioactivity of ¹⁴C-2-A-4,6-DNT released as ¹⁴CO₂.

ESR analyses

Production of glutathionyl free radical GS⁻ was recorded through the formation of glutathionyl radical-5,5-dimethyl-1-pyrroline-N-oxide (DMPO) spin adduct (Ross et al. 1985). For experiments with MnP, the reaction mixture contained in a final volume of 400 µl: 25 mM malonate buffer (pH 4.5), 0 or 10 mM MnSO₄ · H₂O, 0 or 5 mM GSH, 10 mM H₂O₂, 1,512 ± 110 U l⁻¹ purified MnP, and 50 mM DMPO. For experiments with Mn(III), the reaction mixture contained: 25 mM malonate buffer (pH 4.5), 0 or 5 mM GSH, 72 ± 126 µM Mn(III), and 50 mM DMPO. A Bruker ECS-106 spectrometer (Bruker, Billerica, Mass.) operating at 9.6 GHz with a 50 kHz-modulation frequency was used for all recordings. Other spectrometer settings are shown in the figure legend (see Fig. 3).

HPLC analyses

Quantitative determination of 2-A,2,4-DNT was performed through reverse phase HPLC (110B Solvent Delivery Modules, Analog Interface Module 406, Beckman Instruments, Berkeley, Calif.) on a C18 column (RSIL C18 HL, 250 × 4.6 mm, Alltech Associates, Deerfield, Ill.). The mobile phase was an isocratic solvent system 50% water:50% acetone (v/v), running at a flow rate of 1 ml min⁻¹. The injection volume was of 20 µl (Autosampler 507, Beckman). 2-A,4,6-DNT concentration was monitored via absorbance at 230 nm on an UV detector (Programmable Detector Module 166, Beckman).

Chemicals

Mn(III) acetate was obtained from Sigma. 2-A,4,6-DNT was synthesized from its corresponding dinitrotoluic acid (Sigma) through the Schmidt reaction (Zbarskii et al. 1971). ¹⁴C-U-ring-labeled 2-A,4,6-DNT (2.2 mCi mmol⁻¹) was generously provided by Prof. W. Fels (University of Paderborn, Germany). Other chemicals were of analytical grade and were purchased from Sigma.

Results

Figure 1 shows the disappearance of 2-A,4,6-DNT during incubation in the presence of different MnP systems. MnP alone – without Mn(II) or GSH – transformed 9% of the initial 2-A,4,6-DNT after 12 days. Addition of either 10 mM Mn(II) or 5 mM GSH led respectively to a transformation of 21% or 31% of the initial 2-A,4,6-DNT. In the presence of both 10 mM Mn(II) and 5 mM GSH, MnP converted 100% of the