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AROMATIC NITROREDUCTASE FROM THE BASIDIOMYCETE *PHANEROCHAETE CHRYSOSPORIUM*

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SUMMARY A membrane-associated aromatic nitroreductase activity was identified in cell-free extracts of the lignin-degrading fungus *Phanerochaete chrysosporium*. The enzyme catalyzed the nitro group reduction of 1,3-dinitrobenzene, 2,4-dinitrotoluene, 2,4,6-trinitrotoluene, 1-chloro-2,4-dinitrobenzene, and 2,4-dichloro-1-nitrobenzene. The corresponding hydroxylamines and/or amines were identified as reaction products by HPLC and/or GC-MS. 1-Nitroso-3-nitrobenzene and 1-hydroxylamino-3-nitrobenzene also were reduced by the enzyme, suggesting they were intermediates in the reaction. The enzyme required NAD(P)H as a cosubstrate and the optimal pH and temperature for the reaction were 6.5 and 50°C, respectively. Enzyme activity was not observed in the presence of molecular oxygen. The membrane-associated enzyme could be solubilized with the nonionic detergent Triton X-100.

Nitroaromatics are important precursors for the synthesis of a wide variety of industrial chemicals such as munitions, pesticides, herbicides, and dyes (1). Their widespread use has led to environmental contamination, and nitroaromatics are recognized as priority pollutants, owing to their toxicity (2).

The white-rot basidiomycete *Phanerochaete chrysosporium* is able to mineralize lignin, an abundant natural aromatic polymer (3-8). Two extracellular peroxidases and an H_2O_2-generating system constitute the major components of this organism's lignin-degrading system (4,5,7). *P. chrysosporium* degrades a variety of environmental pollutants (9), including 2,4-dinitrotoluene (DNT) (10) and 2,4,6-trinitrotoluene (TNT) (11). Although nitroaromatics lacking an amine or a hydroxyl group are not direct substrates for these extracellular enzymes, *P. chrysosporium* reduces the aromatic nitro group to an amine as the first step in the degradation of nitroaromatic compounds (10,12). Since the amine products are substrates for peroxidase-catalyzed oxidation, this reduction reaction is a key step in the degradation of these pollutants.

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Herein, we report the identification of an enzyme catalyzing the reduction of aromatic nitro groups to their corresponding amines.

MATERIALS AND METHODS

Cultures of *Phanerochaete chrysosporium* were grown as described previously with the omission of vanillate from the culture medium (13,14). Three-day-old agitated cultures were harvested by filtration. Mycelial mats were washed successively with ice-cold water, 0.5% (w/v) NaCl solution, and 50 mM sodium phosphate (pH 7.0). One flask (containing 1 liter of medium) yielded approximately 10 g of mycelium which could be stored at -80°C. All subsequent steps were carried out at 4°C. Mycelial mats (25 g) were resuspended in 80 ml of 50 mM sodium phosphate, 330 mM sucrose, pH 7.0 (buffer A) and homogenized for 3 × 10 s with a Polytron homogenizer. Unbroken cells and other cell debris were removed by centrifugation at 3,500 × *g* for 10 min. The resulting supernatant was filtered through two layers of Miracloth and centrifuged again at 17,000 × *g* for 30 min. The supernatant was discarded and the pellet was washed once with buffer A, then resuspended in buffer A at a final protein concentration of 5 mg/ml.

The nitroreductase activity was solubilized by adding Triton X-100 to the membrane fraction and incubating the mixture on ice for 1 h followed by centrifugation at 100,000 × *g* for 1 h. The supernatant was decanted and the pellet was resuspended in buffer A.

Nitroreductase assays were routinely carried out in glass screw-cap vials containing buffer A, protein fraction, 0.5 mM NADPH, and 0.2 mM substrate in a total volume of 1 ml. Reactions were started by flushing the head space of the vials with N_2 and incubating the vials at 47°C. Reactions were stopped by the addition of 3 N HCl (1 ml). The aromatic amine was quantitated spectrophotometrically at 535 nm after formation of a diazo dye by coupling with N-(1-naphthyl)ethylenediamine (15). The diazo dye derivative was identified by fast atom bombardment mass spectroscopy. For high pressure liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GC-MS) analysis, the addition of HCl was omitted and the reaction mixtures were extracted with ethylacetate. The ethylacetate phase was dried under a stream of nitrogen and redissolved in methanol prior to analysis.

HPLC analysis was carried out with an HP LiChrospher 100 RP-18 (5 μm) column, using a linear gradient of methanol (30-75%, 12 ml) in 1% acetic acid at a flow rate of 1 ml/min. GC-MS was performed as described previously (10,14).

1-Hydroxylamino-3-nitrobenzene was prepared by a modification of a previously described procedure (16). Ammonium chloride (0.5 g in 10 ml of water) was added to 1,3-dinitrobenzene (0.84 g in 30 ml of dioxane). Zinc (0.65 g) was added and the mixture was stirred for 30 min (rt), then extracted with ethylacetate and dried. The product was purified by chromatography on a silica gel column using ethylacetate:hexane (1:3, v/v) as eluent. 1-Nitroso-3-nitrobenzene was prepared as described previously (17). Protein concentration was determined by the Bradford method (18). All other chemicals were reagent grade.

RESULTS

A cell-free membrane-associated fraction able to reduce aromatic nitro groups was isolated from *P. chrysosporium* mycelia harvested during the primary growth phase. For routine nitroreductase assays, 1,3-dinitrobenzene (DNB) was used as the substrate. 1-Hydroxylamino-3-nitrobenzene and 1-amino-3-nitrobenzene were identified as products by HPLC and GC-MS. Under

standard 1-ml reactions (0.5 mM NADPH, 0.2 mM DNB, 0.75 mg protein, 47°C for 30 min), more than 90% of the product was identified as 1-amino-3-nitrobenzene and less than 10% as 1-hydroxylamino-3-nitrobenzene. Enzyme activity routinely was measured as 1-amino-3-nitrobenzene formation using the diazotization assay. Enzymatic activity was linear with respect to time and enzyme concentration and heat-denatured enzyme was inactive. Removal of oxygen by flushing the incubation vial head space with N_2 was necessary for activity. A reduced pyridine nucleotide was required as a cosubstrate; the specific activity for 1-amino-3-nitrobenzene formation was 3.6 and 1.9 nmoles $\cdot$ min^{-1} $\cdot$ mg^{-1} using NADPH and NADH, respectively. The reaction had a temperature optimum of 50°C and a pH optimum of 6.5 (Figure 1).

Chemically prepared 1-hydroxylamino-3-nitrobenzene was reduced quantitatively to 1-amino-3-nitrobenzene by the enzyme preparation. The enzyme also reduced 1-nitroso-3-nitrobenzene to 1-hydroxylamino-3-nitrobenzene and 1-amino-3-nitrobenzene; however, the latter conversion was not quantitative owing to the substrate instability under the incubation conditions.

Reduction of 2,4-dinitrotoluene yielded the hydroxylamino-, amino-nitrotoluene, and diaminotoluene isomers with the amino-nitrotoluene isomers being the most abundant products. 2,4,6-Trinitrotoluene was reduced to the hydroxylamino- and amino-dinitrotoluene isomers. 2,4-Dichloro-1-nitrobenzene and 1-chloro-2,4-dinitrobenzene also served as substrates but only their respective amino derivatives were observed.

After cell breakage, some enzyme activity was found in the supernatant upon centrifugation at 17,000 $\times$ *g*. Subsequent centrifugation at 100,000 $\times$ *g* did not pellet this activity. The partitioning of the activity between pellet and supernatant was dependent on the duration of homogenization and composition of the breakage buffer. Prolonged homogenization increased the amount of activity in the supernatant. All of the nitroreductase was located in the supernatant when sucrose was omitted from the breakage buffer. Membrane-associated nitroreductase could not be released into the supernatant by increasing the ionic strength in the fraction with sodium chloride (0.5 M final concentration).

The membrane-associated enzyme activity could be solubilized by Triton X-100 (Figure 2). The optimal detergent concentration was 2.5% (v/v) at a protein concentration of 5 mg/ml. The recovery of activity in the supernatant was about 80%. When active membrane fractions were digested with Proteinase K (Sigma) for 20 min at 37°C prior to assaying, 85% loss of activity was observed as compared to controls without Proteinase K.

DISCUSSION

Previous studies have shown that the white-rot fungus *P. chrysosporium* is able to mineralize nitroaromatic compounds such as 2,4-dinitrotoluene (10) and 2,4,6-trinitrotoluene (11) under ligninolytic conditions. The initial degradation products of TNT metabolism have been identified as hydroxylamino- and amino-dinitrotoluene isomers (12,19,21). Although many microorganisms

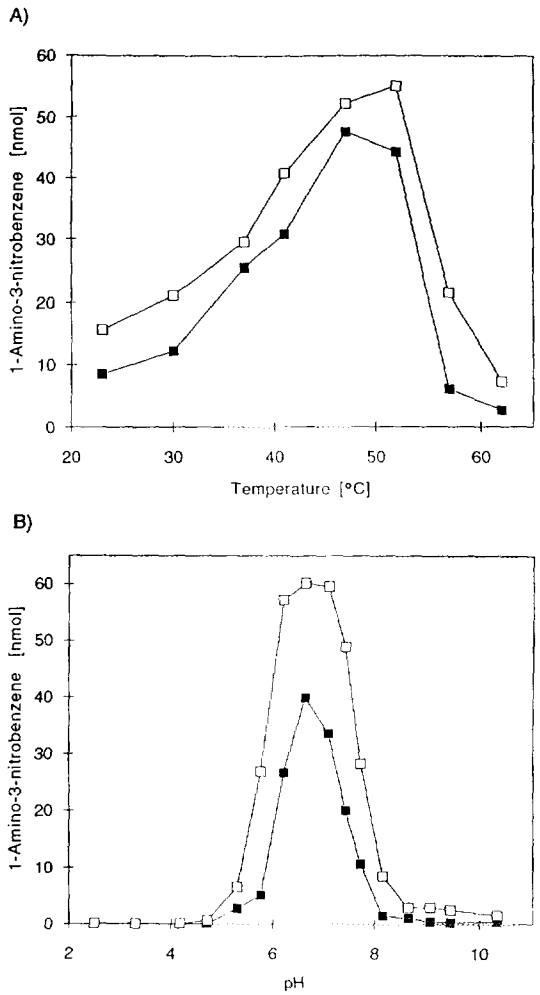

Figure 1. (A) Temperature dependence of the nitroreductase activity. Reaction mixtures contained 0.75 mg protein, 0.5 mM NADPH, and 0.2 mM 1,3-dinitrobenzene in 1 ml total volume. Incubations were carried out for 30 min at 47°C. 1-Amino-3-nitrobenzene concentrations were determined as described in the text. Membrane-associated (□) and solubilized (■) nitroreductase fractions were tested. (B) pH dependence of the nitroreductase activity. pH was adjusted by using a universal buffer of boric acid, citric acid and trisodium orthophosphate as described elsewhere (24). Incubation mixtures contained 850 µl universal buffer and 0.75 mg protein in buffer A. Incubations were carried out as described under A. Membrane-associated (□) and solubilized (■) nitroreductase fractions were tested.

catalyze the initial reduction of nitroaromatics (22), they appear to lack the capacity of *P. chrysosporium* for complete degradation of these pollutants. In this study, we have investigated the enzymatic activity of *P. chrysosporium* that is responsible for the reduction of nitroaromatics.

An NAD(P)H-dependent nitroreductase activity is detectable in cell-free extracts of *P. chrysosporium*. The enzyme catalyzes the reduction of a variety of nitroaromatics including DNT

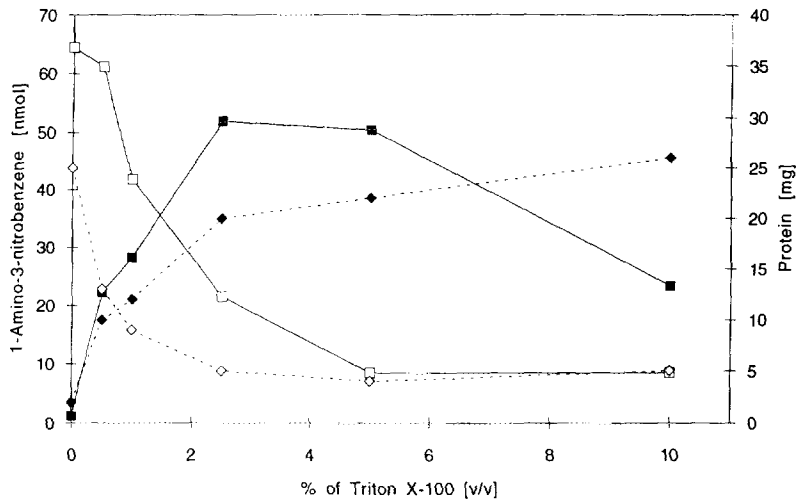

Figure 2. Solubilization of the membrane-associated nitroreductase. Various amounts of Triton X-100 were added to 5 ml of membrane fraction (5 mg protein/ml). The mixtures were incubated on ice for 1 h and then centrifuged at 100,000 × *g* for 1 h. The pellet was resuspended in 5 ml of buffer A. Symbols: Amount of protein in the pellet fraction (-- ◇ --) and the supernatant (-- ◆ --); nitroreductase activity in the pellet fraction (—□—) and the supernatant (—■—). 150 μl of each fraction was assayed. Incubations were carried out at 47°C for 30 min; 1-amino-3-nitrobenzene concentrations were determined by diazo dye formation as described in the text.

and TNT. Our standard assay employs 1,3-dinitrobenzene which would be expected to generate fewer stereoisomers, facilitating the identification and quantification of the reaction products. Furthermore, 1-amino-3-nitrobenzene concentrations are easily determined by coupling the amino group to N-(1-naphthyl)ethylenediamine which produces a strongly colored diazo dye that can be quantitated spectrophotometrically (15).

The reduction of nitro groups to their corresponding amines appears to proceed in a stepwise manner via nitroso and hydroxylamino intermediates. However, only the hydroxylamine intermediates could be detected. Chemically synthesized 1-hydroxylamino-3-nitrobenzene was stable and was reduced to 1-amino-3-nitrobenzene. Chemically synthesized 1-nitroso-3-nitrobenzene was found to be unstable in the presence of NADPH and/or protein. Nevertheless, some of the 1-nitroso-3-nitrobenzene was converted to 1-hydroxylamino-3-nitrobenzene and 1-amino-3-nitrobenzene upon incubation with the enzyme fraction, suggesting that it is an intermediate in nitroaromatic reduction. The apparent multistep nature of the nitroreduction suggests that more than one enzyme may be involved in the pathway for full reduction to the amine, although some bacterial nitroreductases are apparently single component systems. Our preliminary results do not allow a determination of the number of components required for this fungal-mediated reduction.

Enzyme activity is only observed when the incubation vial head space is flushed with N_2. Oxygen-sensitive nitroreductases have been described in prokaryotic and mammalian systems (23).

Oxygen inhibition has been shown to be a direct result, at least in part, of the oxidation by air of the nitroaromatic anion radical formed via an initial one-electron reduction (23). Oxygen-insensitive nitroreductases catalyze an initial two-electron reduction, thereby preventing the formation of oxygen-sensitive anion radicals (23). Further purification of this enzyme system will be required to establish the mechanism of oxygen sensitivity of the nitroreductase from *P. chrysosporium*.

The *P. chrysosporium* nitroreductase system is membrane associated and the activity is not released by increasing the salt concentration. However, omission of sucrose from the buffer during breakage results in the release of the activity into the supernatant fraction, suggesting that the enzyme is unlikely to contain membrane-spanning regions. The membrane-associated nitroreductase activity is susceptible to protease digestion, indicating that the activity is not simply being trapped in membrane vesicles during breakage, and that at least a part of the protein is exposed on the surface of the membrane. The detergent-solubilized nitroreductase has the same temperature and pH dependence as the membrane-associated enzyme, indicating that the solubilization process does not significantly alter these two parameters.

A previous report on TNT reduction by *P. chrysosporium* claims that a membrane potential is required for this reaction (20,21). The nitroreductase described here does not have this requirement since the enzyme is active in the supernatant fraction devoid of membranes and in the detergent-solubilized form, where a membrane potential would not be maintained. This strongly suggests that a membrane potential does not drive *P. chrysosporium* nitroaromatic reduction.

The *P. chrysosporium* extracellular lignin-degrading system is expressed during secondary metabolic (idiophasic) growth which is triggered by nutrient nitrogen limitation (5,7). Since the nitroreductase described here is isolated from cells grown under primary metabolic conditions, the regulation of nitroreductase expression appears to be different from that of the lignin-degrading peroxidases. However, even under nitrogen-limited conditions, sufficient nitroreductase is available for the degradation of nitroaromatics (10,11). Purification and further characterization of this aromatic nitroreductase is underway.

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REFERENCES

1. Hartter, D. R. (1985) In Toxicity of Nitroaromatic Compounds (D. E. Rickert, Ed.), Chemical Industry Institute of Toxicology Series, pp. 1-13, Hemisphere Publishing, Washington, DC.
2. Keither, L. H., and Telliard, W. A. (1979) Environ. Sci. Technol. 13, 416-423.
3. Buswell, J. A., and Odier, E. (1987) Crit. Rev. Biotechnol. 6, 1-60.

4. Eriksson, K.-E., Blanchette, R. A., and Ander, P. (1990) In Microbial and Enzymatic Degradation of Wood and Wood Components, pp. 225-333, Marcel Dekker, New York.
5. Gold, M. H., and Alic, M. (1993) Microbiol. Rev. 57, 605-622.
6. Higuchi, T. (1990) Wood Sci. Technol. 24, 23-63.
7. Kirk, T. K., and Farrell, L. F. (1987) Annu. Rev. Microbiol. 41, 465-505.
8. Schoemaker, H. E. (1990) Recl. Trav. Chim. Pays-Bas Netherlands 109, 255-272.
9. Hammel, K. E. (1992) In Metal Ions in Biological Systems (H. Sigel and A. Sigel, Eds.), Vol. 12, pp. 41-60, Academic Press, New York.
10. Valli, K., Brock, B. J., Joshi, D. K., and Gold, M. H. (1992) Appl. Environ. Microbiol. 58, 221-228.
11. Fernando, T., Bumpus, J. A., and Aust, S. D. (1990) Appl. Environ. Microbiol. 56, 1666-1671.
12. Michels, J., and Gottschalk, G. (1994) Appl. Environ. Microbiol. 60, 187-194.
13. Yajima, Y., Enoki, A., Mayfield, M. B., and Gold, M. H. (1979) Arch. Microbiol. 123, 319-321.
14. Rieble, S., Joshi, D. K., and Gold, M. H. (1994) J. Bacteriol. 176, 4838-4844.
15. Zucker, M., and Nason, A. (1955) Methods Enzymol. 2, 406-411.
16. Furniss, B. S., Hannaford, A. J., Smith, P. W. G., and Tatchell, A. R. (1989) In Vogel's Textbook of Practical Organic Chemistry, 5th Edition, p. 955, Longman Scientific & Technical, Longman Group UK Limited.
17. Furniss, B. S., Hannaford, A. J., Smith, P. W. G., and Tatchell, A. R. (1989) In Vogel's Textbook of Practical Organic Chemistry, 5th Edition, p. 956, Longman Scientific & Technical, Longman Group UK Limited.
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
19. Bumpus, J. A., and Tatarko, M. (1994) Curr. Microbiol. 28, 185-190.
20. Stahl, J. D., and Aust, S. D. (1993) Biochem. Biophys. Res. Commun. 192, 471-476.
21. Stahl, J. D., and Aust, S. D. (1993) Biochem. Biophys. Res. Commun. 192, 477-482.
22. Rickert, D. E. (1985) In Toxicity of Nitroaromatic Compounds (D. E. Rickert, Ed.), Chemical Industry Institute of Toxicology Series, pp. 87-101, Hemisphere Publishing, Washington, DC.
23. Peterson, F. J., Mason, R. P., Hovsepian, J., and Holtzman, J. L. (1979) J. Biol. Chem. 254, 4009-4014.
24. Perrin, D. D., and Dempsey, B. (1974) Buffers for pH and Metal Ion Control, p. 156, Chapman and Hall, New York.