Characterization of an Intracellular Quinone Reductase from *Phanerochaete chrysosporium*

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To my family.

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ABSTRACT

Characterization of an Intracellular Quinone Reductase from *Phanerochaete chrysosporium*

Barry J. Brock, Ph.D.

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The recent elucidation of metabolic pathways for the degradation of environmental pollutants by the white-rot fungus *Phanerochaete chrysosporium* has helped in the identification of intracellular enzymes. This dissertation describes studies on the pathway for 2,4-dinitrotoluene (DNT) degradation and on the characterization of an intracellular quinone reductase involved in this and other metabolic pathways.

Under ligninolytic conditions *P. chrysosporium* mineralizes DNT. The DNT degradative pathway was elucidated by the characterization of fungal metabolites and oxidation products generated by lignin peroxidase, manganese peroxidase, and crude intracellular cell-free extracts. The multistep pathway involves a series of alternating reductive and oxidative reactions, generating the final aromatic metabolite 1,2,4-trihydroxybenzene (THB). The latter was ring cleaved by an intracellular THB dioxygenase.

One of the enzymes involved in the pathway, an intracellular soluble 1,4-benzoquinone reductase, was purified from agitated cultures of *P. chrysosporium* and characterized. The protein was purified to homogeneity using ammonium sulfate fractionation, hydrophobic interaction, ion exchange, and blue-agarose affinity chromatographies. The FMN-containing enzyme catalyzed the reduction of one- and two-electron acceptors utilizing either NADH or NADPH as an electron donor. The

steady-state kinetic parameters for a variety of electron acceptors were examined. Enzyme activity was competitively inhibited by Cibacron Blue 3GA and by dicumarol with respect to NADH.

The mechanism of the quinone reductase was investigated by spectral and kinetic methods. The native, oxidized, FMN-containing enzyme was reduced quantitatively by NADH and the resulting reduced enzyme was reoxidized in the presence of one equivalent of 2,6-dimethoxy-1,4-benzoquinone (DMBQ). The stoichiometry of NADH oxidation versus DMBQ reduction was 1:1. The enzyme catalyzes the reduction of quinones to hydroquinones by a ping-pong, steady-state mechanism. The reduction of THB autooxidation products, as well as ferricyanide and ferricytochrome *c* is described. In addition, the nonenzymatic reduction of chloroquinones, ferricyanide, and ferricytochrome *c* is described.

The regulation of quinone reductase expression also was investigated. The enzyme was constitutively expressed under both primary and secondary metabolic conditions. Under primary metabolic growth conditions, activity was induced by a variety of substituted benzoic acids, quinones, and hydroquinones. This increase in activity was associated with an increase in quinone reductase protein as determined by western blot (immunoblot) analysis, suggesting that these inducers are regulating gene expression.

CHAPTER 1
INTRODUCTION

1.1 Biodegradation of Recalcitrant Substances

As industrial processes continue to pollute the environment, new, cost-effective environmental cleanup technologies are needed to keep pace. The current practices of capping and containment and off-site disposal must be replaced by actual treatment of hazardous waste. Incineration of waste is an effective method of treatment, but it is relatively expensive (73, 205). One promising approach is the utilization of microorganisms for the large-scale, ecologically sound biodegradation of environmental pollutants. Hazardous waste treatment systems utilizing microorganisms can be manipulated to meet the demands of a wide variety of *in situ* conditions. Specific organisms or consortia could be used to treat sites contaminated by specific pollutants. One of the most important features of microbial biotransformation is its innocuous nature, i.e., toxic byproducts are not normally created.

To utilize selected microorganisms for the large-scale, on-site bioremediation of contaminated sites, fundamental knowledge needs to be acquired concerning their microbiology, biochemistry, and genetics. This entails optimization of microbial growth conditions, identification of biochemical pathways involved in biodegradation, and identification and subsequent characterization of the enzymes in these pathways. Once the enzymes have been isolated, the genes encoding these enzymes can be isolated and characterized. The information generated in these studies will enable optimization of biodegradative processes for bioremediation practices. Recombinant organisms tailored to specific needs eventually could be created by genetic engineering methods, enabling the complete degradation of toxic pollutants.

The research described in this thesis was focused on understanding an enzyme involved in biodegradative pathways for natural products and environmental pollutants in the white-rot fungus *Phanerochaete chrysosporium*. The ability of this organism to degrade the plant polymer lignin is particularly important and, hence, it is extensively studied for this purpose. *P. chrysosporium* is also studied for its ability to degrade recalcitrant priority pollutants.

1.1.1 Biodegradation of natural products

The process of photosynthesis generates 10^{11}–10^{12} tons of biomass yearly (19, 148). Approximately 95% of this biomass is composed of lignocellulosic materials from woody plant cell walls. Almost all terrestrial fixed CO_2 consists of lignified polysaccharides (57). The polysaccharides can be broken down into their constituents, such as sugars, glucose, xylose, and mannose. The constituent sugars from the degradation of cellulose and hemicellulose can be transformed to ethanol by other microorganisms. The conversion of sugars to alcohol is an established process and will become a priority when society must deal with a shortage of fossil fuels. The lignin polymer is a major impediment to microbial conversion of cellulose and hemicellulose to useful products. As will be discussed in detail below, lignin surrounds the cellulose in the wood cell wall. Therefore, an organism must degrade lignin, or rely on another organism to degrade lignin, before it can gain access to the polysaccharides. Lignin is also a major potential source of aromatics which can be utilized for a wide range of industrial processes.

1.1.2 Biodegradation of anthropogenic compounds

Materials and chemicals of anthropogenic origin are highly diverse and prevalent in the environment. Unfortunately, some of these compounds are recalcitrant and toxic. These industrial pollutants range from substituted polycyclic aromatic hydrocarbons to simple aliphatic compounds. The main sources of these pollutants are industrial wastes. However, many useful products such as herbicides, pesticides, and polychlorinated biphenyls (PCBs) accumulate or leak in the environment where they pose various environmental problems.

Halogenated aromatic and nitroaromatic compounds are two groups of pollutants that have received a great deal of attention with respect to bioremediation strategies. 750,000 and 300,000 tons, respectively, of PCBs and chlorinated benzenes have been estimated to exist in the environment (178). In addition to anthropogenic sources, chlorinated compounds are produced naturally (84, 166). As a result of exposure to naturally occurring chloroaromatics, organisms have developed the ability to degrade chlorinated pollutants (178).

In contrast to chloroaromatics, nitroaromatic compounds are almost exclusively anthropogenic in origin. Only a few natural nitro-substituted compounds have been reported (222). Nitroaromatic compounds, such as 2,4,6-trinitrotoluene (TNT) and Her Majesty's Explosive (HMX), are most notable for their use as explosives. These compounds, as well as their nitroaromatic precursors, are considered priority pollutants by the U.S. Environmental Protection Agency (195). Nitroaromatics are toxic, and they are converted to mutagenic compounds by metabolic activation in mammals (47, 134, 157, 180, 181).

Prokaryotic and eukaryotic organisms have been screened for their ability to degrade a variety of nitroaromatic compounds (103, 153, 200). Prokaryotic isolates of *Desulfovibrio* spp., use nitroaromatics as their sole source of nitrogen, and several strains of *Clostridium* can degrade nitroaromatics to small aliphatic molecules (200). Several species of bacteria can use nitrophenol as an energy source (198). Bacteria use a variety of mechanisms for degradation of nitroaromatics, including the oxygenolytic removal of the nitro group from 4-nitrophenol by an intracellular flavoprotein monooxygenase (202). A second mechanism is the dioxygenase-catalyzed replacement of the nitro group with a hydroxy group. This activity was first described by Ecker et al. (51). Subsequently, an inducible, nonspecific nitrotoluene dioxygenase has been purified and characterized (4). Another mechanism of nitro group removal is reduction of the aromatic ring of the nitroaromatic compound. This mechanism has been reported to occur during the metabolism of picric acid (2,4,6-trinitrophenol) by *Rhodococcus erythropolis* (143). However, the enzyme(s) that catalyze this reaction(s) have not been identified. The common feature

of all of these pathways is the conversion of the nitroaromatic parent compound to a 1,2-dihydroxybenzene which subsequently undergoes ring cleavage.

Although the majority of biodegradation studies have been performed with prokaryotes, fungal systems are becoming increasingly important. Since the discoveries that *P. chrysosporium* can mineralize a variety of recalcitrant dyes and pollutants, a whole new field of research has blossomed. The suggestion that *P. chrysosporium* could degrade pollutants originated from lignin degradation studies (25, 50, 74). *P. chrysosporium* most likely uses the enzymes in the lignin degradative pathway for degradation of pollutants and has not evolved specific metabolic pathways for the degradation of anthropogenic compounds (78, 79, 95). Therefore, a summary of lignin and lignin biodegradation will serve as an introduction to the biodegradation of pollutants by *P. chrysosporium*.

1.2 Environmental Significance of Lignin

After cellulose, lignin is the most abundant natural carbon source on Earth and is an essential component of the global carbon cycle. Lignin represents a vast resource of aromatic compounds and products from lignin degradation could be used as an abundant source of feedstocks for the chemical industry. Lignin degradation by microorganisms might provide an environmentally safe alternative to conventional chemical bleaching of paper pulp. The current method of chemical bleaching entails the use of chlorine and chlorine dioxide to oxidize the lignin polymer. This process generates a wide variety of toxic chlorinated byproducts (85, 232). Among the chlorinated aromatics generated and released into the environment are the highly toxic chlorinated dioxins including 2,3,7,8-tetrachlorodibenzodioxin (TCDD), a well-known pharmacologically active compound (102, 133, 220).

One of the most challenging characteristics of the lignocellulose matrix in wood is its remarkable resistance to biodegradation. Indeed, lignin protects plants from microbial degradation (221). It is this resistance to degradation that prevents the efficient microbial utilization of plant cellulose for the production of sugars, proteins, and ethanol (56).

1.2.1 Lignin structure

Lignin is synthesized by a peroxidase-catalyzed oxidative, free radical polymerization of sinapyl, coniferyl, and coumaryl alcohols. These reactions result in an amorphous, heterogeneous, polydisperse, optically inactive polymer, consisting of phenyl propanoid subunits (Figure 1.1). These subunits are connected by a variety of interunit linkages, including the arylglycerol-β-aryl ether linkage, comprising approximately 50–60% of the intermonomer linkages as well as phenylcoumaran and biphenyl linkages (2). The type of lignin structure varies with different plant species and cell types. For example, gymnosperm lignin (softwood) is composed primarily of guaiacyl lignin, whereas hardwoods are composed of both guaiacyl and syringyl lignin (5). For this reason, hardwood lignin has a higher methoxyl content than softwood lignin (191). This methoxyl composition has important consequences for the oxidation of lignin subunits by *P. chrysosporium* extracellular enzymes. By donating electron density to the aromatic rings in lignin, methoxy groups activate the ring for oxidation. 5-Methoxy groups also prevent coupling to form recalcitrant biphenyl linkages.

1.3 Lignin Degradation by *P. chrysosporium*

Only white-rot fungi can degrade lignin completely to CO_2 and H_2O. Lignin degradation by *P. chrysosporium*, is a secondary metabolic process initiated by the depletion of nutrient nitrogen, carbon, or sulfur (32, 113, 124, 131). It has been suggested that *P. chrysosporium* cannot grow on lignin but rather, by degrading lignin, gains access to the polysaccharides that are protected by lignin (129). *P. chrysosporium* preferentially degrades syringyl (hardwood) lignin; however, it is also capable of degrading guaiacyl lignin (63). Early studies by Kirk et al. demonstrated that *P. chrysosporium* metabolizes ^{14}C ring-labeled lignin and lignin model compounds to $^{14}CO_2$ under conditions that promote the onset of secondary metabolism (132).

After the culture conditions that promote lignin degradation had been defined, work on the metabolism of lignin model compounds commenced. Studies on the

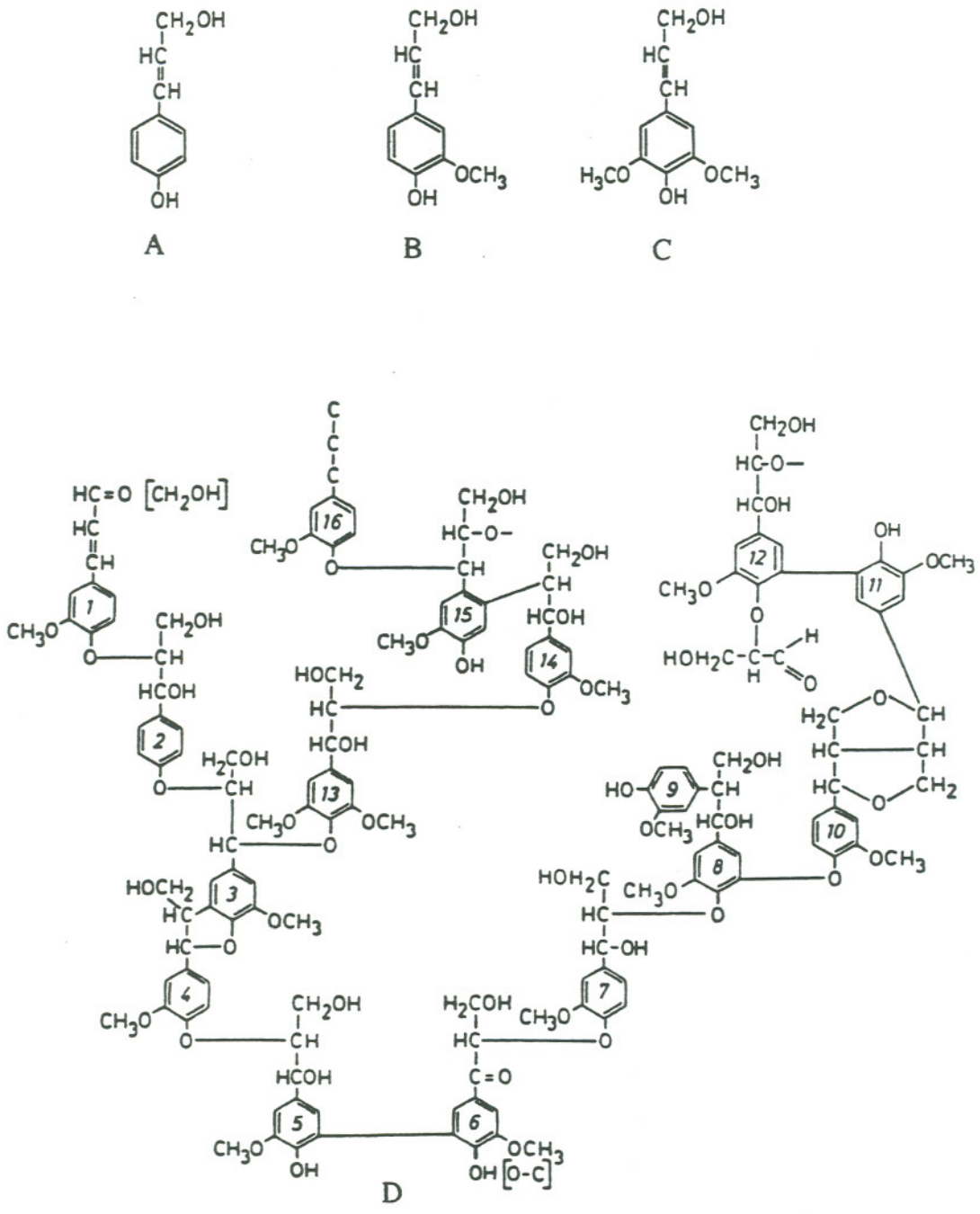

Figure 1.1 Lignin precursors and structure. (A) coniferyl alcohol; (B) sinapyl alcohol; (C) syringyl alcohol; (D) model of gymnosperm lignin [from reference (57)].

degradation of various dimeric model compounds led to an understanding of the chemistry involved (52–54). Because of the random nature of lignin, lignin degradation was first thought to be a free radical process initiated by small diffusible molecules such as the hydroxyl radical (70, 80, 90) or singlet oxygen (165). Shortly thereafter, it was discovered that H_2O_2 was important in lignin degradation and that the production of H_2O_2 in culture coincided with the onset of secondary metabolism (62, 70, 80). Although radicals can contribute to the depolymerization process, it is likely that they are not sufficient.

1.3.1 Extracellular peroxidases

It was not until 1983 that enzymes were implicated in lignin degradation by *P. chrysosporium*. Two groups isolated an extracellular enzyme that catalyzed the oxidative cleavage of lignin model compounds (76, 214). Like H_2O_2, this enzyme is produced during secondary metabolism. The enzyme is now commonly known as lignin peroxidase (LiP). LiP catalyzes the H_2O_2-dependent oxidation of nonphenolic aromatic rings found in synthetic lignin and lignin model compounds (33, 93, 131, 159). This oxidation generates aromatic radical cations that undergo subsequent nonenzymatic reactions (131, 152, 179). These reactions result in rearrangements and alkyl-phenyl and α-β cleavage of lignin model dimers (127, 159, 160). Soon after the discovery of LiP, an extracellular Mn-dependent peroxidase (MnP) was isolated from secondary metabolic cultures of *P. chrysosporium* (137). MnP catalyzes the H_2O_2- and Mn^{2+}-dependent oxidation of lignin, lignin derivatives, and phenolic lignin model compounds (83, 215, 228, 229). LiP and MnP exist as families of isozymes encoded by multiple genes (16, 78, 171, 176, 234). As many as twelve LiP isoenzymes and four MnP isoenzymes have been detected from liquid cultures of *P. chrysosporium* (142).

LiP and MnP have been extensively characterized. Both enzymes undergo a typical peroxidase-type catalytic cycle (83 , 179, 213, 225). The resting enzyme is oxidized by H_2O_2 to a two-electron oxidized ferrylporphyrin π-cation radical (Compound I). Compound I then is reduced by a variety of donors to the ferrylporphyrin (Fe^{IV}=O) Compound II. A second donor then reduces the ferryl iron

of Compound II back to the native enzyme. MnP is unique in its catalytic cycle in that it requires Mn^{2+} to reduce MnP Compound II back to native enzyme (225, 226). The oxidized Mn^{3+} chelated by oxalate or malonate then acts as a diffusable oxidant which can oxidize phenolic groups to phenoxy radicals, which undergo nonenzymatic rearrangements resulting in bond cleavage between lignin subunits (227, 228).

cDNAs and genes encoding LiP and MnP isoenzymes have been cloned (8, 10, 45, 77, 78, 184, 224, 234), and expressed in heterologous (170) and homologous (136, 155) expression systems. The expression of LiP is regulated by nutrient nitrogen and carbon depletion (8, 35, 145). Induction of LiP gene expression by exogenous agents or external stimuli has been not been observed. In contrast, the regulation of MnP expression has been characterized in some detail. The genes for manganese-dependent peroxidases are expressed in response to nitrogen depletion (176). In addition they are regulated by manganese (17, 18), chemical and oxidative stress (144), and heat shock (19, 77). Many stress-related regulatory elements have been characterized for multiple genes in diverse organisms (148, 163, 186). The response of MnP gene transcription to chemical stress and heat shock suggests that genes encoding MnP may share the same stress-related regulatory elements found in genes from other organisms.

1.3.2 Intracellular metabolism of lignin-derived fragments

Once oxidized by the extracellular enzymes, oxidized fragments enter the cell to be metabolized further. The best-studied lignin-derived metabolite, vanillic acid (3-methoxy-4-hydroxybenzoic acid), is an important intermediate generated during the chemical oxidation of wood (2) and during the degradation of wood by white-rot fungi (39, 128). Vanillic acid also has been identified as an intermediate in the degradation of ferulic acid (3-(3-methoxy-4-hydroxyphenyl)propionic acid) (54, 86) and as a precursor to the important secondary metabolite, veratryl alcohol (150).

The metabolism of vanillic acid has been studied extensively (Figure 1.2). The first step in the catabolism of vanillic acid is an oxidative decarboxylation to yield 2-methoxyhydroquinone (26, 30) or oxidation by a phenol oxidase followed by reduction to the hydroquinone. The next step appears to be demethoxylation to yield

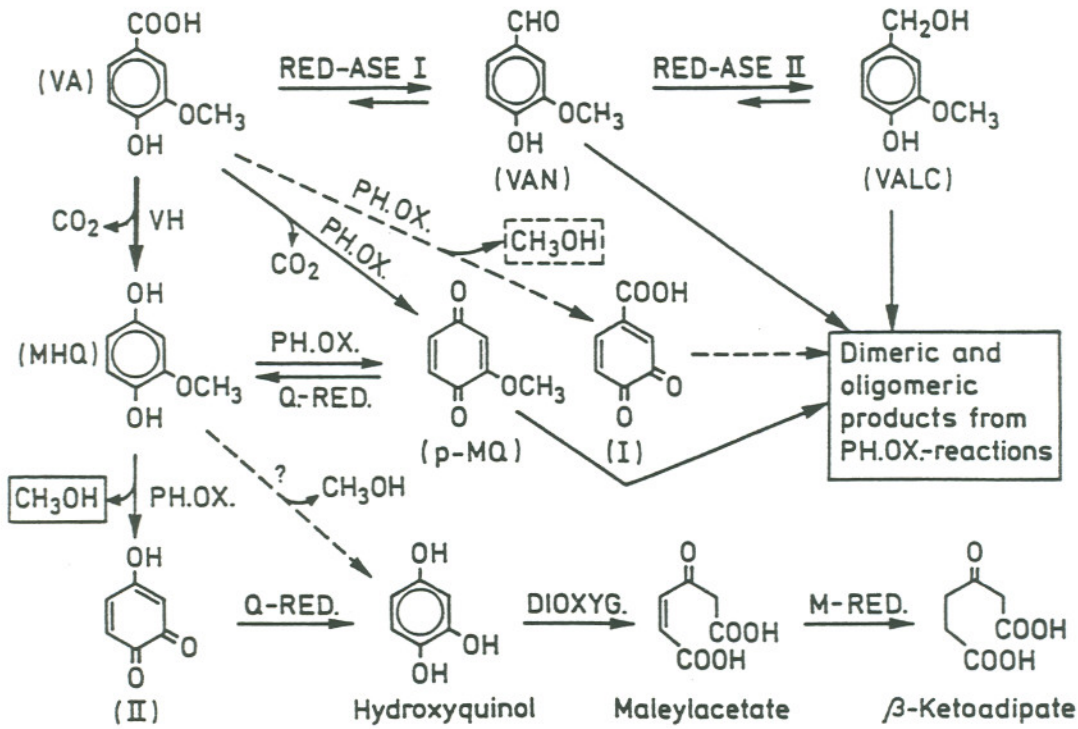

Figure 1.2 Proposed scheme for the metabolism of vanillic acid by *P. chrysosporium* [from Ander et al. (6)].

a hydroxybenzoquinone. The latter is reduced to produce THB. Alternatively, the stepwise reduction of vanillate to vanillin and subsequently to vanillyl alcohol has also been reported (7). Which of these two alternative pathways *P. chrysosporium* uses may depend on the culture conditions (7). The final step in the catabolism of vanillate is cleavage of the aromatic ring (27, 183). Similar steps can be envisaged in the degradation of syringic (3,5-dimethoxy-4-hydroxybenzoic acid) acid which is a major intermediate in the degradation of hardwood lignin (38).

Ferulic acid (3-methoxy,4-hydroxycinnamic acid) also is a main product from degradation of guaiacyl lignin by white-rot fungi (108), and degradation of ferulic acid by *P. chrysosporium* has been studied (52–54, 86). Main products identified were coniferyl alcohol, coniferyl aldehyde, dihydroferulic acid, and dihydroconiferyl alcohol. Small amounts of vanillyl alcohol, vanillic acid, and methoxyhydroquinone were also formed. It was suggested that ferulic acid is degraded via olefin saturation and subsequent α-hydroxylation followed by α,β cleavage (54).

The majority of biochemical research concerning *P. chrysosporium* has focused on the extracellular enzymes LiP and MnP involved in the initial depolymerization of the lignin polymer. However, it has been suggested that the rate-limiting step in lignin degradation by *P. chrysosporium* may be intracellular (188). The extracellular enzymes themselves cannot catalyze the complete breakdown of lignin to CO_2 and H_2O. Several intracellular enzymes involved in the further metabolism of lignin have been identified and characterized. One of the earliest intracellular enzymes to be characterized from *P. chrysosporium* crude extracts was vanillate hydroxylase (26, 231). Two years after the initial discovery of activity in crude extracts, the enzyme was purified to homogeneity (30). Vanillate hydroxylase is an NADPH-dependent hydroxylase. Another enzyme isolated from *P. chrysosporium* is an aryl-alcohol dehydrogenase (164). This enzyme catalyzes the NADPH-dependent reduction of veratryl aldehyde (3,4-dimethoxybenzaldehyde) to veratryl alcohol (3,4-dimethoxybenzylalcohol). *P. chrysosporium* cells have been reported to contain O-methyl-transferase activity (39) and an S-adenosylmethionine-dependent O-methyltransferase has recently been purified from *P. chrysosporium* (42). 1,2,4-Trihydroxybenzene-1,2-dioxygenase also has been purified from cell extracts (183).

This enzyme cleaves the aromatic ring of trihydroxybenzene to form maleyl acetate, which is reduced by an NADPH-dependent enzyme to β-ketoadipic acid which enters intermediary metabolism. There is evidence that intracellular enzymes, such as monooxygenases, epoxide hydrolases, and conjugating enzymes, exist in *P. chrysosporium* (206). Intracellular hydroxylations, reductions, group transfers, and ring cleavage probably are involved in the complete mineralization of monomeric lignin-derived compounds.

A variety of H_2O_2-generating enzymes, such as glucose oxidase (122), pyranose oxidase (44), and methanol oxidase (167), have been isolated from intracellular extracts of *P. chrysosporium*. The latter enzymes may play an important role in the metabolism of the methanol generated from methoxyl groups liberated from lignin and lignin metabolites.

1.4 Biodegradation of Environmental Pollutants by *P. chrysosporium*

Many environmental pollutants consist of substituted aromatic structures and are highly resistant to degradation. The similarities between the bond types in aromatic pollutants and lignin led to an examination of the ability of *P. chrysosporium* to degrade aromatic pollutants (25, 50, 74). The mineralization of ^{14}C-labeled aromatic pollutants and the oxidation and subsequent bleaching of dyes was observed under the conditions that promote lignin degradation. Diverse compounds, such as DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) (23), pentachlorophenol (139, 140, 161), tri- and dinitrotoluenes (67, 216), chlorophenols (115, 217), chlorophenoxyacetic acids (189, 230), and a variety of polyaromatic hydrocarbons (48, 92, 190, 206), are degraded to CO_2 and H_2O by *P. chrysosporium*. Even complex mixtures of aromatic pollutants, such as Aroclor, a mixture of polychlorinated biphenyls, and anthracene oil, are degraded (22, 50). This suggests that the lignin-degrading system of *P. chrysosporium* is very nonspecific. It also has been shown that some compounds such as phenanthrene (48, 92, 206) are transformed by a LiP- and MnP-independent pathway. Biodegradation of pollutants by *P.*

chrysosporium has been extend to *in situ* experiments with encouraging results (15, 139, 140).

1.4.1 Elucidation of metabolic pathways for aromatic pollutants

Since the discovery of mineralization of environmental pollutants by *P. chrysosporium*, metabolic pathways have been elucidated for several aromatic pollutants. We now have a better understanding of the intracellular steps involved in the metabolism of these substances by *P. chrysosporium*. Complete metabolic pathways for the degradation of 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrotoluene and 2,7-dichlorodibenzodioxin have been proposed (115, 216, 217, 219). These pathways for aromatic pollutant degradation share many features with the intracellular metabolism of lignin fragments. One of the recently proposed pathways is demonstrated in Figure 1.3 (114). Under ligninolytic conditions, *P. chrysosporium* mineralizes ring-labeled 2,4,5-trichlorophenol (IX). The pathway for the degradation of IX was elucidated by the characterization of fungal metabolites and enzyme oxidation products. The first step in the pathway is the peroxidase-catalyzed oxidative 4-dechlorination of IX to generate 2,5-dichloro-1,4-benzoquinone (X). Reduction of the quinone X yields 2,5-dichloro-1,4-dihydroxybenzene (XI). Methylation of the hydroquinone XI yields 2,5-dichloro-4-methoxyphenol (XII). The only metabolic product of XII is the quinone (X), suggesting that XII is not part of the main metabolic pathway. The hydroquinone XI is oxidized by MnP to generate 5-chloro-4-hydroxy-1,2-benzoquinone (XIII). The quinone XIII is reduced to produce 5-chloro-1,2,4-trihydroxybenzene (XIV). Finally, the chlorotrihydroxybenzene XIV undergoes another cycle of oxidative dechlorination and reduction to generate tetrahydroxybenzene (VIII). Tetrahydroxybenzene VIII is oxidatively cleaved to yield malonic acid, which is subsequently degraded to CO_2. The enzymes catalyzing the methylation and ring cleavage reactions are likely the same enzymes involved in vanillic acid catabolism (42, 183).

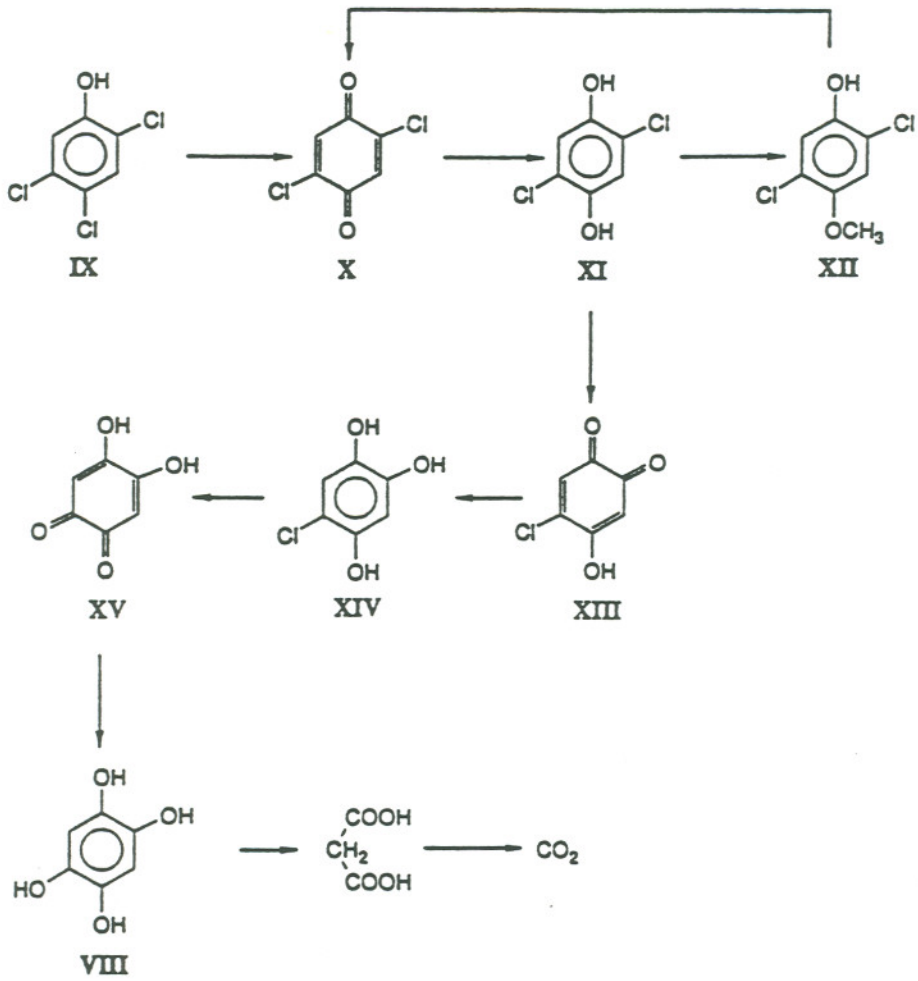

Figure 1.3 Proposed pathway for the metabolism of 2,4,5-trichlorophenol by *P. chrysosporium* [from (115)].

1.5 Quinone Metabolism and Oxidative Stress

Quinones are widely distributed in nature and lignin may represent a major source of quinones. Quinones can be classified into three groups according to Cadenas et al. (34): (i) naturally occurring quinones such as vitamin K and ubiquinone, (ii) quinones produced by metabolic conversion of xenobiotics such as benzene and benzo[a]pyrene, and (iii) chemotherapeutic quinones such as adriamycin. It is known that quinones are important metabolites in the degradation of aromatic acids derived from lignin. Substituted 1,4-benzoquinones are among the metabolites identified in both *in vivo* culture studies and *in vitro* peroxidase studies of lignin and pollutant metabolism. Quinones have been identified from the biodegradation of 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrotoluene, and chlorinated dibenzo-*p*-dioxins (79, 115, 216, 217, 219). Other strains of white-rot fungi, such as *Trametes versicolor* Paprican 52 and *Bjerkandera adusta* CBS 595.78, have been shown to generate extracellular quinone intermediates from polycyclic aromatic hydrocarbons (68, 96).

In addition to *in vivo* experiments, MnP- and LiP-catalyzed *in vitro* reactions produce substituted quinones. The LiP-catalyzed oxidations of veratryl alcohol, 3,4-dimethoxybenzylmethyl ether (192), non-phenolic arylglycerol β-aryl ether (127), pyrene (96), benzo(a)pyrene (87), chlorophenol (97, 115), dibenzodioxin (114), and 3,5-dimethyl-4-aminobenzenesulfonic acid (40) yield substituted quinone products. MnP-catalyzed oxidative cleavage of a phenolic arylglycerol β-aryl ether lignin model compound also generated quinone products (215).

Quinones are toxic to cells and the features of quinones that impart toxicological properties are their electrophilic character and their ability to undergo reversible reduction and oxidation with the concomitant production of reactive oxygen species. Quinone compounds that contain reactive leaving groups have been shown to react with DNA (210). However, the majority of the reported cytotoxic effects observed with quinones are mediated through their one-electron reduced semiquinone form (117, 197). Quinones are substrates for a diverse set of flavoproteins (34, 110). NADPH-cytochrome P-450 reductase, NADH-cytochrome *b5* reductase, and NADH-

ubiquinone oxidoreductase catalyze the one-electron reduction of quinones, whereas NAD(P)H-quinone oxidoreductase (DT-diaphorase) reduces quinones by two electrons. The one-electron reduction of a quinone produces a semiquinone radical. Semiquinones react with oxygen to regenerate the quinone and to produce the superoxide radical (169). Thus, semiquinones undergo a redox cycle that consumes O_2 at the expense of reducing equivalents such as NADH and NADPH. Superoxide may dismutate enzymatically or nonenzymatically to form H_2O_2 and O_2 (156). Superoxide can react further with H_2O_2 to produce the highly reactive hydroxyl radical (11, 100, 119). This generation of reduced oxygen species and depletion of cellular reducing equivalents results in the cytotoxic condition known as oxidative stress.

Lignin degradation by *P. chrysosporium* is an oxidative process. Oxygen concentrations as high as 100% stimulate lignin degradation (132, 233). In addition to oxygen, the lignin degrading system of *P. chrysosporium* has been shown to have an absolute requirement for H_2O_2 (62, 120). *P. chrysosporium* has a well-developed system to protect against oxidative stress, including quinone-reducing activity, catalase, and MnP. Oxidative stress is known to induce MnP transcription in *P. chrysosporium* (144). In addition, a recent report proposes that lipid peroxidation may contribute to the activity of MnP (162), suggesting that *P. chrysosporium* membrane lipids may be susceptible to peroxidative damage by active oxygen species.

1.5.1 Quinone reductases

As shown in Table 1.1, quinone reductases have been identified in a variety of tissues and organisms, and have been proposed to be involved in diverse functions. The common features of all quinone reductases are the presence of at least one flavin prosthetic group and the NAD(P)H-dependent transfer of electrons to quinones. Cytosolic quinone reductases are normally involved in biosynthetic or catabolic reactions, while the membrane-bound reductases are typically involved in electron transport and maintenance of antioxidants in the reduced form. The membrane-bound enzymes utilize NADH as the preferred electron donor, while the cytosolic forms can use either NADH or NADPH. The two-electron reduction of quinones to

Table 1.1

Quinone Reductases in Various Tissues and Organisms

Enzyme	Location	Function	Reference
quinone reductase	*P. chrysosporium*	reduction of quinones derived from aromatic precursors	this study
DT-diaphorase	liver	quinone metabolism blood clotting	(58) (208)
"	pancreatic islets	insulin secretion	(151)
"	epidermis	wound repair	(158)
sulfide quinone reductase	*Chlorobium limicola*	electron transport	(196)
"	*Oscillatoria limnetica*	anoxygenic photosynthesis	(9)
NADH-quinone oxidoreductase	*Paracoccus denitrificans*	electron transport	(231)
"	*Vibrio alginolyticus*	electron transport	(101)
zeta-crystallin	porcine lens	protection from cataracts	(177)

hydroquinones by the mammalian NAD(P)H-dependent quinone reductase DT-diaphorase, apparently provides a protective mechanism against quinone toxicity (36, 89, 146, 147). [DT-diaphorase derives its name from its ability to utilize both DPNH (NADH) and TPNH (NADPH) as electron donors (58). Diaphorases are enzymes that catalyze the nucleotide-dependent reduction of a variety of dyes (116).] The two-electron reduction of quinones to hydroquinones by DT-diaphorase prevents the formation of semiquinones and the subsequent production of superoxide. The hydroquinone products are conjugated and excreted. However, it has been suggested that hydroquinones may also produce reactive oxygen species by autooxidation (112). Indeed, the anticarcinogenic agent Diaziquone (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone) is bioactivated by the action of DT-diaphorase (69).

The induction of DT-diaphorase by xenobiotic compounds also suggests a protective role for this enzyme. It has been demonstrated that a variety of anticarcinogenic compounds induce DT-diaphorase activity in neoplastic tissue (46, 208, 209). This induction appears to be mediated through putative cis-acting antioxidant and xenobiotic response elements located in the promoter region of the DT-diaphorase gene (186). Induction also appears to be dependent on chemical and structural features of the inducer (208). Some inducing compounds require bioconversion to inducing species by phase I detoxifying enzymes, such as cytochrome P450, that catalyze the initial oxidative transformations of xenobiotics (209). Gel-shift mobility assays suggest that gene expression is controlled by a transacting protein factor (64).

DT-diaphorase and the quinone reductase isolated from *P. chrysosporium* share many similarities. For this reason DT-diaphorase is used in this study as a working model enzyme for the *P. chrysosporium* reductase with respect to physical characteristics, mechanism, regulation of expression, and physiological function.

1.6 Summary of Research

The reactions involved in the degradation of lignin and lignin model compounds by *P. chrysosporium* and those catalyzed by the extracellular peroxidases have been extensively characterized. Much less is known about the mechanism of degradation of pollutants and the intracellular enzymes that contribute to the complete degradation of lignin and pollutants. This study focuses on the elucidation of the metabolic pathway for the degradation of 2,4-dinitrotoluene and characterization of an intracellular quinone reductase involved in the lignin and pollutants.

The second chapter of this thesis describes the elucidation of the degradative pathway for 2,4-dinitrotoluene. 2,4-dinitrotoluene was added to *P. chrysosporium* cultures and products were analyzed by a combination of HPLC and GC mass spectroscopy. By utilizing whole cultures, crude cell extracts, and purified enzymes, the degradative pathway was elucidated. In this study, substituted quinones were identified as important metabolites.

The third chapter describes the purification and preliminary characterization of the quinone reductase. Optimum culture conditions for maximum enzyme production and the purification protocol is described. The physical and kinetic characteristics of the enzyme are reported.

The fourth chapter is a more detailed study of the catalytic mechanism of the quinone reductase. The spectral intermediates of the oxidized and reduced states and the steady-state mechanism of the enzyme are presented. In addition, the reduction of one-electron acceptors and nonenzymatic reactions catalyzed by the hydroquinone products are described. The nature of the inhibition by two strong inhibitors, dicumarol and Cibacron blue 3GA, is characterized. Finally, reconstitution of the apoprotein is used to quantitate the FMN content of the enzyme.

Chapter 5 discusses studies on the regulation of expression of quinone reductase. This chapter describes the characteristics of inducing compounds. Culture conditions that are most conducive to induction also are described. We demonstrate that the increase in activity is associated with an increase in protein, rather than

stabilization of existing enzymes by the exogenous compounds. Vanillic acid-induced quinone reductase activity is demonstrated in several other species of white-rot fungi.

In summary, this research proposed a metabolic pathway for the degradation of 2,4-dinitrotoluene by *P. chrysosporium* and focused on the isolation and characterization of an intracellular quinone reductase involved in the pathway. This work indicates that quinones are important metabolites in the degradation of aromatic pollutants and lignin. Several comparisons are made between metabolism of lignin degradation products and aromatic pollutants. Finally, the mechanism and expression of quinone reductase is examined. Future directions with respect to the physiological function and regulation of expression are discussed.

CHAPTER 2

Degradation of 2,4-Dinitrotoluene by the Lignin-degrading Fungus *Phanerochaete chrysosporium*

2.1 Introduction

Nitroaromatics are important feedstocks for the synthesis of a wide range of industrial chemicals including munitions, pesticides, herbicides, and dyes (49, 99). In particular, 2,4- and 2,6-dinitrotoluene are used extensively in the production of polyurethane and explosives. In 1982, the United States alone produced approximately 720 million pounds (ca. 327 million kg) of dinitrotoluenes (49). Dinitrotoluenes have been found to be mutagenic in bacterial and mammalian assay systems and carcinogenic in animal studies (181, 195). Because of the large-scale use and toxic nature of dinitrotoluenes, these compounds have been classified as priority pollutants (118).

The white-rot basidiomycete fungus *Phanerochaete chrysosporium* is capable of effectively degrading polymeric lignin and lignin model compounds (83, 131). Two extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), as well as an H_2O_2-generating system, are thought to constitute the major components of this organism's lignin-degradative system (83, 131). The degradation of a variety of environmentally persistent pollutants by *P. chrysosporium* has been reported as well (24, 25, 94, 107). In several of these reports, the use of ^{14}C-labeled compounds demonstrated mineralization of the pollutant. Recently, we reported the complete pathway for the degradation of 2,4-dichlorophenol by *P. chrysosporium* and suggested that both LiP and MnP, as well as intracellular enzymes, are involved in this degradation (217, 218). Although the mineralization of ^{14}C-labeled trinitrotoluene by *P. chrysosporium* has been reported (67), the pathway

and mechanisms for the degradation of nitroaromatic compounds by this organism have not been elucidated previously. In this report we examine the reactions involved in the degradation of 2,4-dinitrotoluene and propose a pathway for the fungal degradation of this important pollutant.

2.2 Materials and Methods

2.2.1 Chemicals

2,4-Dinitrotoluene (I), 2-amino-4-nitrotoluene (II), 4-amino-2-nitrotoluene (III), 2,4-diaminotoluene (IV), 4-nitrocatechol (V), 2-methoxy-1,4-benzoquinone (VI), 1,2,4-trihydroxybenzene (VII), 2,5-dihydroxy-1,4-benzoquinone (VIII), 2-methoxy-1,4-hydroquinone (IX), hexafluorophosphoric acid, tetramethyltin, palladium diacetate, 2,4-dinitroaniline, chromotropic acid, 3,5-dinitrobenzoic acid, sulfanilimide and N-(1-napthyl)-ethylene diamine were obtained from Aldrich. (U)-^{14}C-ring-labeled 2,4-dinitroaniline and β-keto adipic acid (XIII) were obtained from Sigma.

2-Methoxy-1,4-benzoquinone (VI). Compound VI was prepared by the oxidation of compound IX with Ag_2O as described elsewhere (88).

1,2-Dimethoxy-4-nitrobenzene (X). 4-Nitrocatechol (V) (500 mg) was methylated with dimethylsulfate and K_2CO_3 in refluxing acetone for 5 h as described previously (66). The acetone was evaporated, 50 ml of water was added, the product mixture was extracted with chloroform, and the product was purified by preparative thin-layer chromatography (solvent system, $CHCl_3$).

4-Nitro-1,2-orthoquinone (XII). XII was prepared from 4-nitro-1,2-dihydroxybenzene (50 mg) using 1 equivalent of $NaIO_4$ in water (5 ml) at room temperature for 2 min (1). The reaction mixture was extracted with ethyl acetate. The organic fraction was dried over Na_2SO_4 and the quinone was isolated via silica gel chromatography (solvent system, CH_3Cl).

1,2,4,5-Tetahydroxybenzene (XI). XI was prepared from 2,5-dihydroxy-1,4-benzoquinone (VIII) as previously described (217).

(U)-^{14}C-ring-labeled 2,4-dinitrotoluene (I). The specific activity of the U-^{14}C-labeled 2,4-dinitroaniline was adjusted to 4.5 nCi/mmole. The diazonium salt of

(U)-^{14}C-labeled 2,4-dinitroaniline was prepared by adding a slight molar excess of $NaNO_2$ solution to labeled 2,4-dinitroaniline in HCl (1.75 N) at -10°C with vigorous stirring. Subsequently, 2 equivalents of hexafluorophosphoric acid (60%) were added, the mixture was stirred for 5 h, and the hexafluorophosphate diazonium salt of 2,4-dinitroaniline was collected by centrifugation and dried under nitrogen (187). The diazonium hexafluorophosphate (15 mg) thus prepared was added to tetramethyltin (0.5 ml), a catalytic amount of palladium diacetate, and dry CH_3CN (1 ml), and the mixture was stirred at rt for 3 h (125). The reaction mixture was added to 10 ml of water and extracted with CH_3Cl. The organic fraction was dried over Na_2SO_4 and the final product was purified by preparative TLC (solvent system, CH_3Cl). The final product had identical chromatographic properties with the unlabeled standard on TLC and HPLC.

2.2.2 Culture conditions

The organism was grown from a conidial inoculum at 38°C in 25-ml stationary cultures as described elsewhere (52, 82). Unless indicated otherwise, the medium used in this study was as previously described (82, 132) with 2% glucose and either 1.2 or 12 mM ammonium tartrate as the carbon and nitrogen sources, respectively. The medium was buffered with 20 mM sodium 2,2-dimethyl succinate (pH 4.5). Cultures were incubated under air for 3 days, after which they were purged with 99.9% O_2 every three days.

2.2.3 Mineralization of 2,4-dinitrotoluene (I)

^{14}C-labeled substrate (4.5×10^4 cpm, 4.5 nCi/μmol) in N,N-dimethylformamide (25 μl) was added to three replicate cultures on day 6 to avoid inhibition of growth by I. Flasks were fitted with ports which allowed periodic purging with O_2 and trapping of $^{14}CO_2$ (82, 132) in a basic scintillation fluid as previously described (132). The efficiency of $^{14}CO_2$ trapping after purging for 10 min was greater than 98%. Counting efficiency (>70%) was monitored with an external standard.

2.2.4 Metabolism of 2,4-dinitrotoluene and metabolic intermediates

After six days of incubation, the substrates in acetone (20 μl) were added to cultures to a final concentration of 250 μM. After the indicated additional intervals (2, 24 or 48 h), cultures were filtered through a Buchner funnel. The mycelial mat and the extracellular medium then were extracted separately with ethyl acetate (82). The total organic fraction was washed with 10 ml of water, dried over sodium sulfate, and evaporated under reduced pressure. Products were dissolved in methanol (250 μl) and the quinone products were reduced with sodium dithionite. The products were analyzed either directly or after derivatization. Trimethylsilylation of the reduced products was carried out in bis (*N,O*-trimethylsilyl)trifluoroacetimide:pyridine (2:1). Acetylation of reduced products was carried out in acetic anhydride:pyridine (1:1).

2.2.5 Peroxidases

LiP and MnP were purified from the extracellular medium of an acetate-buffered agitated culture of *P. chrysosporium* strain OGC101 (3) as described elsewhere (75, 81, 226, 227). LiP concentration was determined at 408 nm using an extinction coefficient of 133 mM^{-1} cm^{-1} (81). The specific activity of the purified LiP was 18 nmol mg^{-1} min^{-1} by the veratryl alcohol oxidation assay (81, 213). MnP concentration was determined at 406 nm using an extinction coefficient of 129 mM^{-1} cm^{-1} (75). The specific activity of the purified MnP was 85 nmol μg^{-1} min^{-1} by the Mn(II) lactate assay (75, 226).

2.2.6 Intracellular enzyme preparation

Six-day-old cells grown under nitrogen-limiting conditions were filtered and washed with ice-cold 0.5% NaCl. The cells (10 g [wet wt]) were ground with 15 g of acid-washed sand with a mortar and pestle. Subsequently, 50 mM Na-phosphate buffer (pH 7.0) (20 ml) was added and the mixture was stirred at 4°C for 15 min. The crude extract was centrifuged at 15,000 $\times$ *g*, and the supernatant was concentrated by ultrafiltration using an Amicon PM-10 membrane. The final protein concentration was determined to be 1 mg/ml (199).

2.2.7 Enzyme reactions

LiP reaction mixtures (2 ml) consisted of enzyme (5 μg), substrate (100 μM), and H_2O_2 (100 μM) in 20 mM sodium succinate (pH 3.0). MnP reaction mixtures consisted of enzyme (5 μg), substrate (100 μM), $MnSO_4$ (100 μM), and H_2O_2 (200 μM) in 50 mM Na-malonate (pH 4.5). Reactions were carried out at 30°C for 15 min. Enzyme reactions were conducted in duplicate.

2.2.8 Ring cleavage of 1,2,4-trihydroxybenzene

The reaction mixture (10 ml) contained 1 ml of concentrated cell extract, 1,2,4-trihydroxybenzene (25 μmol), NADPH (10 μM), glucose-6-phosphate (25 μM), and glucose-6-phosphate dehydrogenase (15 U) in 50 mM sodium phosphate (pH 7.0). The reaction was stirred slowly at 28°C for 30 min as described elsewhere (27). At the end of the reaction, the pH was adjusted to 2.0 and the mixture was immediately extracted with ethyl acetate. The organic fraction was washed with water, dried over sodium sulfate, and evaporated. After silylation, the product was analyzed by GC-mass spectrometry. Control reactions with boiled extracts were also conducted.

2.2.9 Detection of methanol released in enzyme reactions

Two different procedures were used to detect methanol as a product of the enzyme reactions. (i) Reaction mixtures contained substrate (500 μM), $MnSO_4$ (500 μM), MnP (25 μg), and H_2O_2 (1 mM) in 5 ml of 50 mM sodium malonate (pH 4.5). Reactions were carried out at 30°C for 60 min. Control reactions in which either MnP or H_2O_2 was omitted were also conducted. Subsequently, 500 μl of H_2SO_4 (18 M), followed by 3,5-dinitrobenzoic acid (500 μM final concentration) was added to form the corresponding methyl benzoate. The reaction mixture was incubated at 90°C for 3 h. The mixture was extracted with ethyl acetate, washed with water, dried over Na_2SO_4, evaporated under reduced pressure and analyzed for methyl-3,5-dinitrobenzoic acid (123). (ii) A 0.5-ml volume of 5% phosphoric acid and 1.0 ml of 5% $KMnO_4$ were added to 1 ml of the reaction mixture described above. The solution was stirred slowly at room temperature for 10 min to ensure the oxidation of methanol to formaldehyde. Sodium bisulfite (saturated solution) was

added dropwise to remove the excess $KMnO_4$. The solution was cooled in an ice bath, after which 4 ml of concentrated H_2SO_4 followed by 0.5 ml of 2% chromotropic acid were added. The mixture was incubated at 60°C for 15 min, the A_{570} was measured, and the amount of methanol generated was determined as described elsewhere (13).

2.2.10 Detection of nitrite released in enzyme reactions

The reaction mixture used for the detection of nitrite was identical to that used for the detection of CH_3OH. Identical control reactions in which either MnP or H_2O_2 was omitted were also carried out. A 100-μl volume of sulfanilamide (1.7% in 2N HCl) was added at the end of the enzymatic reaction, and the resultant mixture was incubated at room temperature for 5 min. Subsequently, N-(1-napthyl)-ethylenediamine dihydrochloride was added, and the A_{540} was measured after 10 min. The concentration of NO_2^- was determined using a standard curve (98).

2.2.11 Chromatography and spectrometry

GC-mass spectrometry was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A gas chromatograph and a 25-m-long fused silica column (DB-5; J & W Science). The oven temperature was programmed from 80–320°C at 10°C/min. Quantitation of aromatic products was carried out on an HP gas chromatograph with a flame ionization detector and calculated by using standard curves. Substrates and products were also analyzed by HPLC with a Beckman C8 ultrasphere column and a linear gradient from 30% methanol in water to 100% methanol. The 4-nitro-1,2-benzoquinone was analyzed by HPLC (retention time = 2.8 min) and quantitated by using standard curves.

2.3 Results

2.3.1 Mineralization of dinitrotoluene

Mineralization of 2,4-[^{14}C]dinitrotoluene (I) by cultures of *P. chrysosporium* is demonstrated in Figure 2.1. After a 24-day incubation period, approximately 34% of

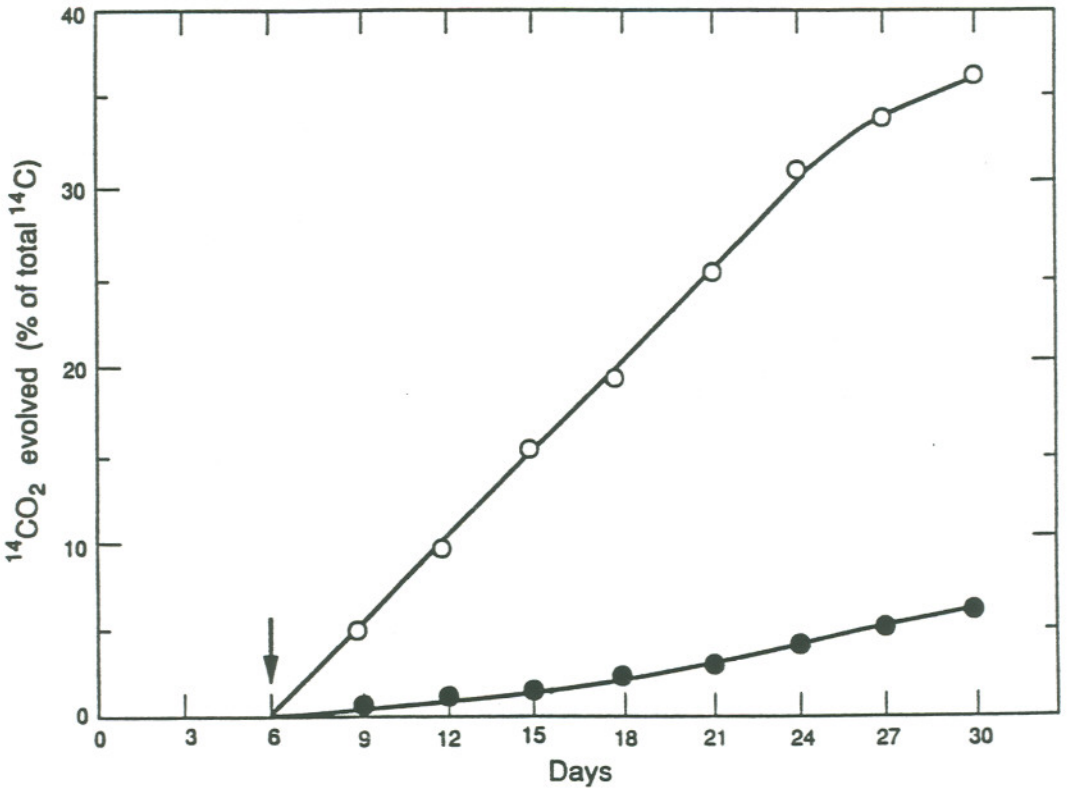

Figure 2.1 Effect of nitrogen concentration on the mineralization of U-[^{14}C]-ring-labeled-2,4-dinitrotoluene. Three stationary cultures containing 1.2 mm (open circles) and 12 mm (closed circles) ammonium tartrate were inoculated with conidia and incubated for 6 days at 37°C after which radiolabeled substrate was added (arrow). Flasks were purged with O_2, and evolved $^{14}CO_2$ was trapped and counted as described in the text. Results are averages obtained from triplicate cultures. Variance in individual flasks from the average was less than 10% of the radioactivity counted.

the substrate was degraded to $^{14}CO_2$ in nitrogen-limited (1.2 mM ammonium tartrate) cultures, whereas only about 7% of the substrate was mineralized in nitrogen-sufficient (12 mM ammonium tartrate) cultures.

2.3.2 Metabolism of substrates

Products and yields obtained from the *P. chrysosporium* metabolism of various substrates and intermediates are diagrammed in Figure 2.2. Three aromatic products were identified as *P. chrysosporium* metabolites of (I): 2-amino-4-nitrotoluene (II), 4-amino-2-nitrotoluene (III), and 2,4-diaminotoluene (IV). Since 2-amino-4-nitrotoluene was found in the greatest yield, the metabolism of this intermediate was examined further. As shown in Figure 2.2, 4-nitrocatechol (V), 1,2-dimethoxy-4-nitrobenzene (X) and 2,4-diaminotoluene (IV) were identified as fungal metabolites of 2-amino-4-nitrotoluene (II). Several of the metabolites identified above were also added to fungal cultures. 4-Nitrocatechol (V) was metabolized to 1,2-dimethoxy-4-nitrobenzene (X) and 1,2,4-trihydroxybenzene (VII). 1,2-Dimethoxy-4-nitrobenzene (X) was metabolized to 2-methoxy-1,2-hydroquinone (IX) and 1,2,4-trihydroxybenzene (VII). Finally, 2-methoxy-1,4-hydroquinone (IX) was metabolized to 1,2,4-trihydroxybenzene (VII) and 1,2,4,5-tetrahydroxybenzene (XI). Metabolites were identified by comparing their retention times on GC and by comparing their mass spectra with standards. The mass spectra and GC retention times of these metabolites and enzyme reaction products or their derivatives are listed in Table 2.1. In all cases, the mass spectra and retention times of metabolites and enzyme reaction products were essentially identical to those of chemically synthesized standards.

2.3.3 Enzymatic oxidation of substrates and metabolic intermediates

The peroxidase oxidation products of 2-amino-4-nitrotoluene (II) and several identified metabolites are diagrammed in Figure 2.3. MnP, but not LiP, oxidized 2-amino-4-nitrotoluene (II) to 4-nitro-1,2-benzoquinone. Methanol was also detected as a product of this reaction. The release of methanol was detected via the formation of methyl-3,5-dinitromethylbenzoate. Mass spectrum *m/z* (relative intensity) values are 226 (10.7), 197 (32), 195 (100), 183 (1.9), 149 (15.2), 75 (38), and 74 (10). No

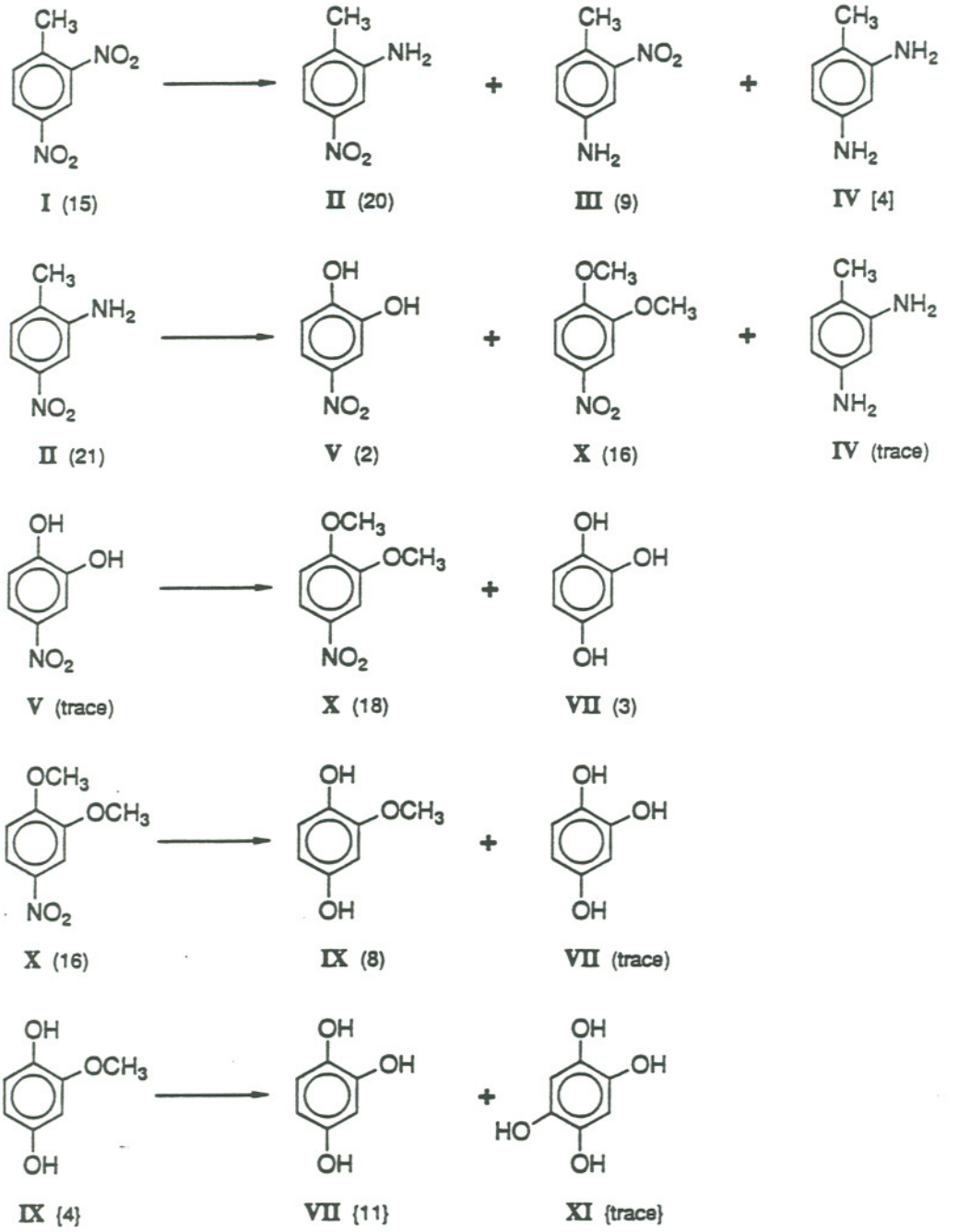

Figure 2.2 Metabolites identified from the degradation of 2,4-dinitrotoluene and pathway intermediates. Duplicate cultures were incubated and extracted, and products were analyzed as described in the text. HPLC and GC were used to determine yields. Average mole percent yields from duplicate cultures of products or remaining substrate for incubations of 2 h (in braces), 24 h (in parentheses), and 48 h (in brackets) are indicated. Variance from the average was less than 10%.

Table 2.1

Mass Spectra of Fungal Metabolites and Enzyme Reaction Products and Their Derivatives[a]

Substrate	GC retention time (min)	Mass spectrum m/z (relative intensity)
2,4-Dinitrotoluene (I)	12.67	182 (16), 165 (100), 119 (10.6), 89 (36.2), 77 (13.8), 63 (19.1)
2-Amino-4-nitrotoluene (II)	13.50	152 (100), 106 (70.2), 94 (7.5), 79 (38.3), 77 (44.6)
4-Amino-2-nitrotoluene (III)	12.78	152 (100), 135 (64.9), 122 (13.8), 107 (72.3), 94 (7.5), 79(36.2), 77 (79.7)
2,4-Diaminotoluene (IV)	10.70	122 (100), 121 (89.4), 105 (13.8), 94 (13.8), 77 (10.6), 67 (5.3), 61 (8.5), 57 (8.5)
4-Nitro-1,2-di(TMS)benzene[b]	15.40	299 (10.6), 284 (8.5), 269 (6.4), 104 (6.4), 74 (41.5), 73 (100)
1,2-Diacetoxy-4-nitrobenzene	15.78	239 (14.9), 197 (100), 155 (71.3),139 (21.3), 125 (33), 109 (14.9), 79 (45.7)
1,2-Dimethoxy-4-nitrobenzene (X)	13.54	183 (100), 168 (5.3), 152 (16), 137 (22.3), 125 (10.6), 107 (25.5), 92 (36.2), 79 (100), 77 (87.2), 63 (35)
2-Methoxy-1,4-di(TMS)benzene	12.84	284 (72), 269 (122), 254 (80), 239 (10.2), 112 (10.2), 89 (9.1), 73 (100)

1,4-Diacetoxy-2-methoxybenzene	14.11	224 (5.2), 182 (15.2), 140 (100), 135 (26.2), 97 (10.2), 69 (15.2)
Tri(TMS)benzene	13.43	342 (28.6), 327 (26), 312 (1.2), 239 (12.1), 73 (100)
1,2,4-Triacetoxybenzene	15.20	252 (3.2), 210 (10.6), 197 (10.6), 168 (25.5), 155 (7.4), 126 (100), 97 (53)
1,2,4,5-Tetra(TMS)benzene	15.45	430 (26.9), 415 (2.0), 355 (1.5), 342 (2.6), 215 (4.6), 179 (4.6), 147 (21.9), 73 (100)
1,2,4,5-Tetraacetoxybenzene	18.23	310 (10.6), 268 (20.2), 226 (69.1), 184 (84.0), 142 (100), 113 (10.6), 69 (20)
β-Keto adipic acid (tri-TMS derivative)[c] (XIII)	14.84	376 (15.4), 361 (73), 317 (15.4), 286 (38.5), 259 (15.4), 231 (38.5), 169 (88.5), 147 (65.4), 125 (15.4), 73 (100)

[a] Products identified from the *P. chrysosporium* metabolism of 2,4-dinitrotoluene and intermediates. Cultures were incubated and extracted and products were analyzed as described in the text. Also, products from the oxidation of various intermediates by LiP, MnP, and crude cell-free extracts were identified. Reaction conditions and analysis were as described in the text. In all cases, the retention times and mass spectra of standard compounds were essentially identical to those of the substrates and metabolites.

[b] TMS, trimethylsiloloxy

[c] Under the basic conditions used in derivatization, β-ketoadipic acid exists in its enolic form, 3-hydroxy-hex-2-ene-1,6-dioic acid; hence, we obtain the tri(TMS) derivative of β-ketoadipic acid for both the experimentally produced and the standard compounds.

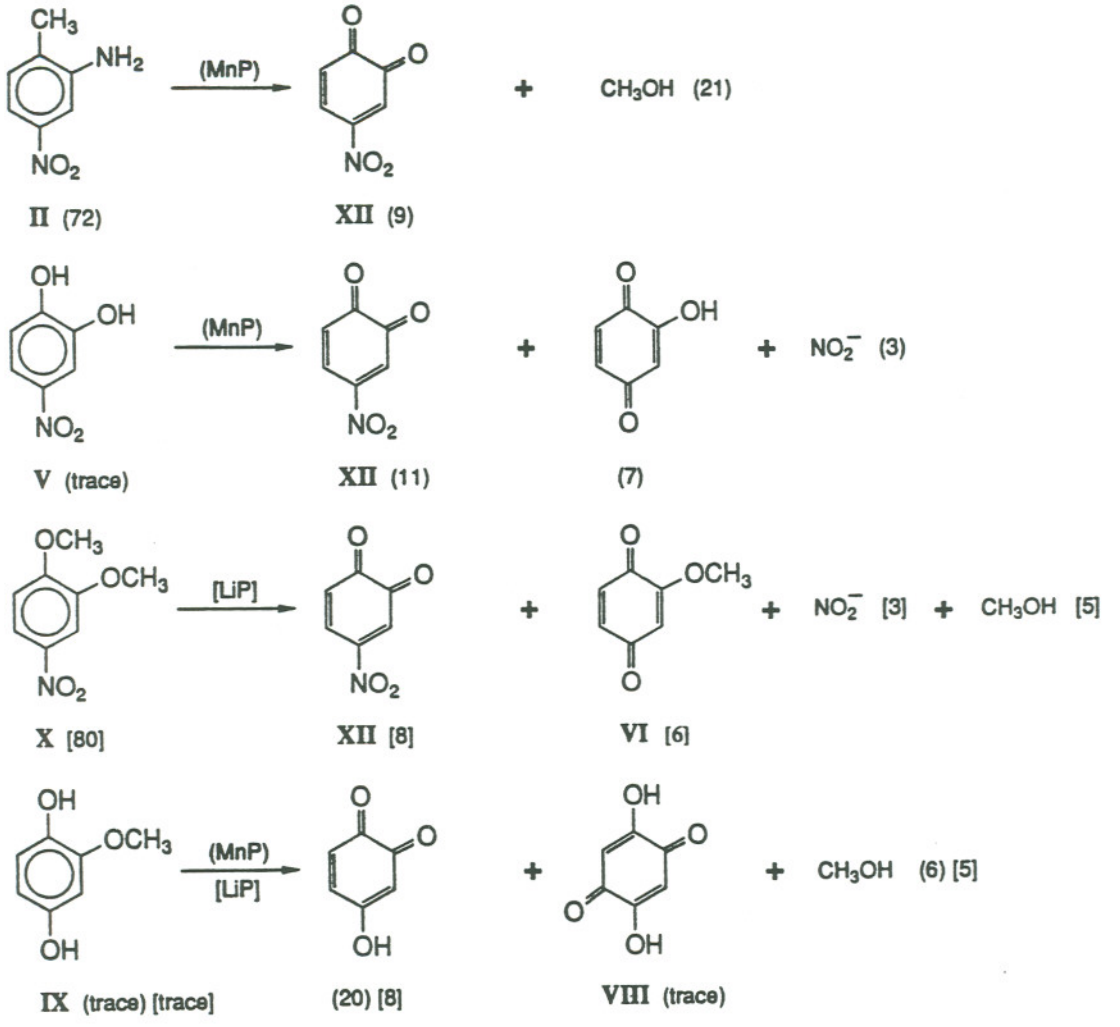

Figure 2.3 Products identified from the oxidation of 2-amino-4-nitrotoluene and several other intermediates by purified LiP and MnP. Reaction conditions and identification of products were as described in the text. Mole percent product yields from the reaction with MnP (in parentheses) and LiP (in brackets) are indicated. No oxidized aromatic products, NO_2^- or methanol, were detected when either H_2O_2 or enzyme was omitted from the reactions.

methanol was detected when either enzyme or H_2O_2 was omitted from the reaction mixture. A quantitative spectrophotometric assay demonstrated that 0.8 equivalents of methanol was released for each equivalent of substrate oxidized. MnP, but not LiP, also oxidized 4-nitrocatechol (V) to 4-nitro-1,2-benzoquinone (XII) and 2-hydroxy-1,4-benzoquinone. The latter was detected after reduction as 1,2,4-trihydroxybenzene (VII). NO_2^- was also detected as a product of this reaction. Under the conditions used, LiP was not able to oxidize either of the metabolic intermediates II or V. Because of their instability, no attempt was made to detect 2-hydroxy-1,4-benzoquinone or 4-hydroxy-1,2-benzoquinone prior to reduction and derivatization.

LiP, but not MnP, slowly oxidized 1,2-dimethoxy-4-nitrobenzene (X) to 4-nitro-1,2-benzoquinone (XII) and 2-methoxy-1,4-benzoquinone (VI). Both methanol and NO_2^- were detected as products of the reaction. Finally, both LiP and MnP oxidized 2-methoxy-1,4-hydroquinone (IX) to 4-hydroxy-1,2-benzoquinone and 2,5-dihydroxy-1,4-benzoquinone (VIII). Methanol was also detected as a product of this reaction. For each of the reactions described above, no oxidized aromatic product, NO_2^-, or methanol was detected when H_2O_2 or enzyme was omitted from the reaction mixture or when the enzyme was boiled for 2 min prior to the reaction.

When the oxidation of 1,2,4-trihydroxybenzene was carried out with the crude cell extract in the presence of NADPH and an NADPH-regenerating system, the formation of β-ketoadipic acid was demonstrated by GC-mass spectrometry (Table 2.1). The yield of β-ketoadipic acid was 24 mol% of the starting substrate. A small amount (1.9 mol%) of levulinic acid was also obtained. Mass spectrum *m/z* (relative intensity) values for di(trimethylsilyloxy) derivative are 260 (9.5), 245 (4.3), 143 (83), and 73 (100). The same ratio of β-ketoadipic acid to levulinic acid was observed when standard β-ketoadipic acid was incubated in the reaction buffer and then extracted under identical conditions. The nonenzymatic decarboxylation of β-ketoadipic acid to levulinic acid has been reported previously (37).

2.4 Discussion

White-rot basidiomycetous fungi are primarily responsible for the initiation of the depolymerization of lignin in wood (33, 83, 131). The best-studied white-rot fungus, *P. chrysosporium*, degrades lignin during secondary metabolic (idiophasic) growth (33, 83, 131). Under ligninolytic conditions *P. chrysosporium* secretes two heme peroxidases (LiP and MnP) in addition to an H_2O_2-generating system (83, 131). These two peroxidases appear to be primarily responsible for the oxidative depolymerization of this heterogeneous, random phenylpropanoid polymer. Recent work has also demonstrated that *P. chrysosporium* is capable of mineralizing many persistent environmental pollutants (24, 25, 50, 94) including trinitrotoluene (67). However, to date, only the *P. chrysosporium* degradation pathway for the pollutant 2,4-dichlorophenol has been elucidated in detail (217). In our report, we showed that oxidative, reductive, and methyl transfer reactions were involved in the degradation of 2,4-dichlorophenol (217).

The metabolism of 2,4-dinitrotoluene in both mammalian and microbial systems has been examined previously (181). Reduction of the *ortho* and the *para* nitro groups by rat hepatic postmitochondrial supernatant and microsomal fractions has been reported (47, 134). However, the major 2,4-dinitrotoluene metabolite in rats appears to be 2,4-dinitrobenzyl alcohol, which is excreted as its glucuronide conjugate (47, 180). Both the yeast *Rhodotorula glutinis* (134) and the fungus *Microsporium* sp. (157) reduced 2,4-dinitrotoluene to 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene. Thus, in these studies an important first step in the metabolism of 2,4-dinitrotoluene is reduction of the 2- or 4-nitro group to the amino function. Beyond this initial step, little has been reported about the degradation of this compound.

Our results demonstrate that *P. chrysosporium* extensively mineralizes 2,4-dinitrotoluene only under nutrient nitrogen-limiting conditions (Figure 2.1), suggesting that the lignin-degradative system is (at least in part) responsible for the degradation of this pollutant. Sequential identification of the primary metabolites produced during 2,4-dinitrotoluene degradation and subsequent identification of the secondary metabolites after addition to cultures of synthesized primary metabolites have enabled us to propose a pathway for the degradation of 2,4-dinitrotoluene (Figure 2.4). The

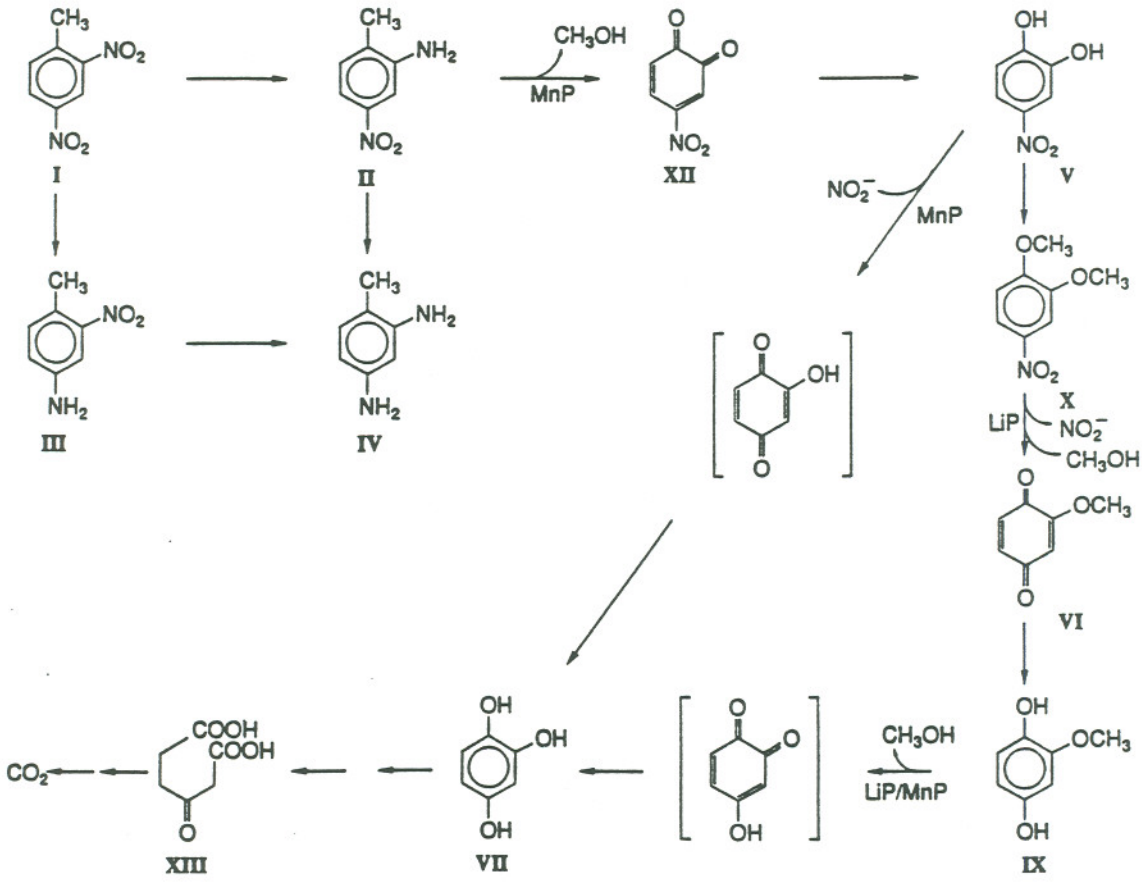

Figure 2.4 Proposed pathway for the degradation of 2,4-dinitrotoluene by *P. chrysosporium*.

first step in the pathway is the reduction of I to either 2-amino-4-nitrotoluene (II) or 4-amino-2-nitrotoluene (III). The subsequent reduction of the monoamino-mononitrotoluenes apparently also takes place, since we have identified 2,4-diaminotoluene (IV) as a *P. chrysosporium* metabolite of I. Presumably, the organism takes up the substrate and the reduction takes place intracellularly. Enzymes involved in the reduction of aromatic nitro groups have been characterized from microorganisms (20, 21, 126). In addition, oxygen-insensitive and oxygen-sensitive aromatic nitroreductases from *E. coli* (173) and mammalian microsomes (168) have been studied. The oxygen-sensitive enzymes catalyze the one-electron reduction of the substrate to form the nitroaromatic-anion free radical which reacts with molecular oxygen to form superoxide and the parent nitroaromatic in a futile cycle (173).

P. chrysosporium cultures convert 2-amino-4-nitrotoluene to 4-nitro-1,2-benzoquinone (XII) and 4-nitro-1,2-dimethoxybenzene (X). Results of enzyme reactions (Figure 2.3) suggests that MnP oxidizes 2-amino-4-nitrotoluene (II) to 4-nitro-1,2-benzoquinone (XII). Nitroquinones are not substrates for either LiP or MnP, and, like chloroquinones, they are strong oxidizing agents. Thus, the nitroquinone intermediates in fungal cultures could be converted to the corresponding hydroquinone either nonenzymatically or enzymatically. Methylation of hydroquinone intermediates was previously observed in our study of 2,4-dichlorophenol degradation (217).

Both 4-nitro-1,2-hydroquinone (V) and 1,2-dimethoxy-4-nitrobenzene (X) are metabolized by *P. chrysosporium* (Figure 2.2) suggesting that they are substrates for either LiP or MnP. MnP oxidizes 4-nitro-1,2-hydroquinone to 4-nitro-1,2-benzoquinone (XII) and to 2-hydroxy-1,4-benzoquinone, releasing NO_2^- in the process. LiP oxidizes, 1,2-dimethoxy-4-nitrobenzene (X) to 4-nitro-1,2-benzoquinone (XII) and 2-methoxy-1,4-benzoquinone (VI) also releasing NO_2^- and methanol in the process. This demonstrates that LiP is capable of oxidizing nitrodimethoxybenzenes and chlorodimethoxybenzenes (217), although at a rate lower than that for dimethoxybenzene (123).

P. chrysosporium also rapidly metabolizes 4-nitro-1,2-hydroquinone (V) to 1,2-dimethoxy-4-nitrobenzene (X) and 1,2,4-trihydroxybenzene (VII), confirming that 2-amino-4-nitrotoluene is probably first oxidized to 4-nitro-1,2-benzoquinone (XII) by MnP and then the latter is reduced to the hydroquinone (V). Subsequently, 4-nitro-1,2-hydroquinone (V) is oxidized by MnP to yield 2-hydroxy-1,4-benzoquinone, which is reduced to form 1,2,4-trihydroxybenzene (VII) (Figure 2.4). V is also methylated to yield 1,2-dimethoxy-4-nitrobenzene (X), which is in turn a substrate for LiP. Oxidation of 1,2-dimethoxy-4-nitrobenzene (X) by LiP yields 2-methoxy-1,4-benzoquinone (VI) (Figure 2.3), which is subsequently reduced to the hydroquinone (IX) (Figure 2.2). This intermediate is oxidized by both LiP and MnP to 2-hydroxy-1,4-benzoquinone (Figure 2.3), which is subsequently reduced to 1,2,4-trihydroxybenzene (VII). Using a crude intracellular enzyme preparation, we confirmed (27) that trihydroxybenzene is ring cleaved by a 1,2-dioxygenase to produce, after subsequent reduction, β-ketoadipic acid. The likely intermediate maleylacetic acid, not identified in this crude system because of its presumed instability, was probably reduced to yield β-ketoadipic acid (27, 201, 202). β-ketoadipic acid would be readily metabolized to CO_2. The biodegradation of 4-amino-2-nitrotoluene (III) and 2,4-diaminotoluene (IV) by *P. chrysosporium* cultures was not pursued, but presumably similar reactions are involved.

Our results suggest that *P. chrysosporium* elaborates a general pathway for the degradation of nitroaromatic compounds which involves the initial reduction of an aromatic nitro group to an aromatic amine. Subsequent oxidation of the amine by MnP generates 4-nitro-1,2-benzoquinone (XII). This intermediate undergoes a cycle of reduction and methylation generating 4-nitro-1,2-hydroquinone (V) and 1,2-dimethoxy-4-nitrobenzene (X). The former is a substrate for MnP, and the latter is a substrate for LiP. Oxidation of compound V by MnP yields 2-hydroxy-1,4-benzoquinone which is reduced to form 1,2,4-trihydroxybenzene (VII). Oxidation of X by LiP yields 2-methoxy-1,4-benzoquinone (VI), which is subsequently metabolized to VII.

Presumably, parts of the proposed pathway evolved for the degradation of lignin metabolites. For example, the oxidative decarboxylation of the lignin

metabolite vanillic acid yields 2-methoxy-1,4-hydroquinone (26, 231) and 2-methoxy-1,4-benzoquinone (135). 2-Methoxy-1,4-benzoquinone (VI) is also a product of LiP-catalyzed oxidation of the *P. chrysosporium* metabolite veratryl alcohol (194). Reduction of 2-methoxy-1,4-benzoquinone to its corresponding hydroquinone (IX) in cell extracts has been measured (28). It is likely that the peroxidase oxidations described above would also result in the formation of oligomeric products. However, *P. chrysosporium* is well adapted to degrade the aromatic polymer lignin. Thus, it is likely that any oligomers which are formed would be degraded subsequently.

In the pathway described above, both nitro groups are removed before ring cleavage by a 1,2-dioxygenase occurs. An initial reduction of one nitro group to an aromatic amine activates the pollutant for attack by a peroxidase. The oxidized product, an orthoquinone, undergoes a cycle of reduction and methylation which again generates several peroxidase substrates. A second oxidation of 4-nitro-1,2-hydroquinone (V) by MnP removes the second nitro group from the ring. Alternatively, a second oxidation of the intermediate 1,2-dimethoxy-4-nitrobenzene (X) by LiP removes the second nitro group from the ring. We recently demonstrated *P. chrysosporium* activation of compounds by cycles of reduction and methylation in the degradation of 2,4-dichlorophenol (217). Preliminary results (218) suggest that 2,4,6-trinitrotoluene is degraded via similar pathways. We are attempting to isolate and characterize the nitroreductase(s), quinone reductase(s), methyl transferase(s), and 1,2-dioxygenase(s) implicated by the results of this study.

CHAPTER 3

Purification and Characterization of a 1,4-Benzoquinone Reductase from the Basidiomycete *Phanerochaete chrysosporium*

3.1 Introduction

The wood-rotting basidiomycete *Phanerochaete chrysosporium* degrades lignin (33, 78, 131) and a variety of aromatic pollutants (24, 79, 94). Under secondary metabolic conditions, *P. chrysosporium* secretes two extracellular heme peroxidases, lignin (LiP) and manganese (MnP) peroxidase, which catalyze the initial steps in the depolymerization of lignin (76, 137, 164), generating a wide variety of metabolic intermediates, including substituted benzoquinones, benzaldehydes, aromatic acids, and ring-opened fragments (33, 57, 131, 192, 215, 229). Vanillate (4-hydroxy-3-methoxy-benzoic acid) is an abundant product of the fungal degradation of wood (7, 29, 39, 57). Quinones are derived from both lignin and vanillate, as well as from the fungal degradation of a variety of aromatic pollutants including 2,4-dinitrotoluene (216), 2,4,5-trichlorophenol (115), and 2,7-dichorodibenzo-*p*-dioxin (219). Whereas the role of extracellular peroxidases in lignin and aromatic pollutant degradation has been well characterized, much less is known about the intracellular enzymes involved in the further breakdown of monomeric intermediates such as quinones, hydroquinones, and aromatic acids. The recent elucidation of metabolic pathways for the degradation of several aromatic pollutants by *P. chrysosporium* has suggested roles for intracellular enzymes in the reduction of quinones and in the ring cleavage of hydroquinones (115, 183, 216, 217, 219). In this regard, we recently purified and characterized a 1,2,4-trihydroxybenzene-1,2-dioxygenase which catalyzes the ring cleavage of a lignin-derived and pollutant-derived trihydroxybenzene (183).

Since quinones generated by the extracellular peroxidase oxidation of both lignin and aromatic pollutants (115, 183, 192, 215–217, 219, 228, 229) appear to be key intermediates in aromatic degradation by *P. chrysosporium*, we have examined the subsequent metabolism of these intermediates, particularly the role of quinone-reducing enzymes. Herein we report the purification to apparent homogeneity and the characterization of an intracellular NAD(P)H-dependent quinone reductase from *P. chrysosporium*.

3.2 Materials and Methods

3.2.1 Culture conditions

Stock cultures of *P. chrysosporium* strain OGC101 (a derivative of strain BKM 1767 (183; unpublished observations) were maintained on malt agar slants as described previously (3). The organism was grown from a conidial inoculum at 38°C in stationary culture (50 ml/1-L flask) for 3 days (38). The medium was as described elsewhere (132) and supplemented with 2% glucose, 12 mM (excess N) or 1.2 mM (limiting N) ammonium tartrate, and 10 mM dimethyl succinate (pH 4.5). Mycelial mats from two flasks were homogenized for 20 s in a blender and used to inoculate a 2-L flask containing 1 L of medium supplemented with 2% glucose, 12 mM ammonium tartrate, and 20 mM sodium acetate (pH 4.5). Cultures were grown at 28°C on a rotary shaker (150 rpm) for 92 h. Either 2 mM vanillic acid or 100 μM 1,4-benzoquinone were added to the cultures 72 h after inoculation.

3.2.2 Preparation of enzyme extracts

Cells (ca. 40 grams) were harvested by filtration, washed in ice-cold distilled water, vacuum filtered through Miracloth, and stored at -80°C. All subsequent steps were carried out at 4°C. Frozen cells initially were broken in a Waring blender, then transferred to a bead beater (BioSpec Inc.) and homogenized 3 times in 2-min bursts. Extraction buffer consisted of 50 mM sodium phosphate (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.004% phenylmethylsulfonyl fluoride.

The homogenate was centrifuged at 17,300 × *g* for 20 min and the supernatant from the first spin was centrifuged at 105,000 × *g* for 30 min.

3.2.3 Purification of the quinone reductase

The 105,000 × *g* supernatant was fractionated by sequential additions of solid ammonium sulfate, with each addition followed by centrifugation at 17,000 × *g*. The quinone reductase activity precipitated between 45 and 65% ammonium sulfate saturation and was redissolved in extraction buffer containing 20% glycerol.

3.2.3.1 *Phenyl Sepharose chromatography.* A phenyl Sepharose column (6 × 2.5 cm) was equilibrated in 50 mM sodium phosphate (pH 7.0) containing 0.5 M ammonium sulfate. The protein was applied in equilibration buffer and the column was washed with 3 volumes of equilibration buffer followed by elution with a descending gradient consisting of 100 ml 0.5 M ammonium sulfate and 100 ml of 0 M ammonium sulfate in 50 mM sodium phosphate (pH 7.0). The column was subsequently washed with 100 ml each of 50 and 4 mM sodium phosphate buffer (pH 7.0), followed by 10% ethylene glycol in H_2O. Active fractions were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane.

3.2.3.2 *Ion exchange chromatography.* The active fractions from the phenyl Sepharose column were applied to a Pharmacia HR 5/5 Mono Q column equilibrated in 10 mM sodium phosphate, pH 7.0 (Buffer A). The column was washed with 2 ml of Buffer A and eluted with a linear gradient of phosphate (10–125 mM in 25 min) at a flow rate of 1.0 ml/min. Active fractions were pooled and concentrated as described above.

3.2.3.3 *Blue-agarose chromatography.* The active fractions from the Mono Q column were concentrated and applied to a blue-agarose column (0.5 × 5 cm) equilibrated in Buffer A, containing 5 mM $MgSO_4$. The column was washed with equilibration buffer and eluted with 2 volumes of equilibration buffer containing 0.5 mM NADPH. Active fractions from the NADPH step were pooled and stored in 20% glycerol at −80°C.

3.2.4 Enzyme assays

Quinone reductase activity was determined by following the oxidation of NAD(P)H at 340 nm. Standard reaction mixtures in 1 ml consisted of 50 mM sodium citrate buffer (pH 6.0), 100 μM 2,6-dimethoxy-1,4-benzoquinone (2,6-DMBQ) and enzyme. Reactions were initiated by the addition of 200 μM NADH unless otherwise indicated. Enzyme assays were carried out at room temperature using a Shimadzu UV-260 spectrophotometer. One enzyme unit is defined as the amount of enzyme required to oxidized 1 μmole NADH min^{-1} mg^{-1} protein.

3.2.5 Analytical methods

Protein concentration was measured using the BCA method (199), using bovine serum albumin as a standard. An extinction coefficient of 12.2 cm^{-1} mM^{-1} at 450 nm (61) was used to quantitate enzyme-bound flavin. The native molecular weight was determined by gel filtration on a Superose 12 HR 10/30 column (Pharmacia) with a flow rate of 0.5 ml/min in 30 mM Tris-Cl (pH 7.0), containing 100 mM NaCl and 2% glycerol (41). Purity and subunit molecular weight were determined by analytical isoelectric focusing on a Pharmacia Phast system and by sodium dodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-PROTEAN™; Bio Rad), respectively (138).

Flavin identification was performed by fluorescence spectroscopy and high performance liquid chromatography (HPLC). The excitation wavelength was 450 nm and the emission was recorded at 535 nm (61). HPLC analysis was performed on an HP LiChrospher 100 RP-8 column. The mobile phase consisted of an acetonitrile–0.05% phosphoric acid gradient, starting at 10:90 for 5 min, followed by a linear gradient from 10% acetonitrile in 0.05% phosphoric acid to 100% acetonitrile over 20 min with a flow rate of 1 ml/min (216).

3.2.6 Chemicals

Flavin standards, vanillic acid, menadione (2-methyl- 1,4-naphthoquinone), 2,6-dichlorophenolindophenol (2,6-DCPIP), dicumarol (3,3′-methylene-bis(4-hydroxycoumarin), Cibacron blue 3GA, NADH, NADPH, and

p-chloromercuribenzoic acid were obtained from Sigma and used without further purification. 2-Methoxy-1,4-benzoquinone (2-MBQ), 2,6-DMBQ, and 2,5-dimethoxy-1,4-benzoquinone were prepared by the AgO oxidation of the respective hydroquinones (88). The purity of substrates was determined by silica gel thin layer chromatography in $CHCl_3$–CH_3OH [99:1].

3.3 Results

3.3.1 Expression of quinone reductase

As shown in Table 3.1, low constitutive levels of enzyme activity were detected in high-carbon high-nitrogen (HCHN) and high-carbon low-nitrogen (HCLN) cultures when measured after 92 and 164 h of incubation, respectively. The addition of vanillate or benzoquinone to HCHN cultures after 72 h of incubation resulted in 13.9- and 6.18-fold increases in enzyme specific activity, respectively, following an additional 20 h of incubation. In contrast, addition of these inducers to 144-h HCLN cultures resulted in increases in specific activity of 1.4- and 1.7-fold, respectively, for vanillate and benzoquinone, following an additional 20 h of incubation. Since vanillate-induced HCHN 92-h cultures yielded the highest activity, these conditions were used for routine enzyme preparation.

3.3.2 Purification of quinone reductase

Table 3.2 summarizes the purification procedure from 40 g (wet weight) of HCHN vanillate-induced 92-h cells. The overall enzyme yield was 4.8% with a concomitant 124-fold purification. The phenyl Sepharose chromatographic profile is shown in Figure 3.1. The quinone reductase activity routinely eluted in the water fraction. This fraction was concentrated and stored at -80°C in 20% glycerol.

Mono Q ion-exchange (Figure 3.2) and blue-agarose column (data not shown) chromatographies were performed immediately before the protein was required. Typically 1–5 mg were purified in these steps. After blue-agarose chromatography the protein was more than 97% homogeneous by analytical IEF (data not shown) and SDS-PAGE (Figure 3.4C, lane 2).

Table 3.1

Constitutive and Induced Expression of Quinone Reductase Activity under Primary and Secondary Metabolic Growth Conditions[a]

Culture conditions	Activity (units/ml)[b]	Protein (mg/ml)	Specific activity (units/mg)	Induction (fold)
Primary metabolic cells				
Control	.595	5.05	.118	1.00
2-Methoxy-*p*-quinone	3.47	4.76	.729	6.18
Vanillate	12.2	7.43	1.64	13.9
Secondary metabolic cells				
Control	.600	3.39	.177	1.00
2-Methoxy-*p*-quinone	.600	1.93	.311	1.76
Vanillate	1.00	4.02	.249	1.41

[a] Details of the growth conditions are described in the text.

[b] 1 unit = μmole NAD(P)H oxidized min^{-1}.

Table 3.2

Purification of Quinone Reductase

Step	Total Volume (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Total Activity (units)[a]	Spec. Activity (units/mg)	Yield (%)	Purification (-fold)
Clarified Extract	670	3.83	2570	4500	1.75	100	1
45–65 % $(NH_4)_2SO_4$ Fraction	48	28.2	1350	4340	3.22	96	1.8
Phenyl-Sepharose	67.5	1.38	93.2	3410	36.7	76	21
Ion exchange	10	2.63	26.3	1600	60.9	35.6	35
Blue agarose	5.0	1.18	5.90	1280	217	28.5	124

[a] 1 unit = μmoles NAD(P)H oxidized min^{-1} ml^{-1}

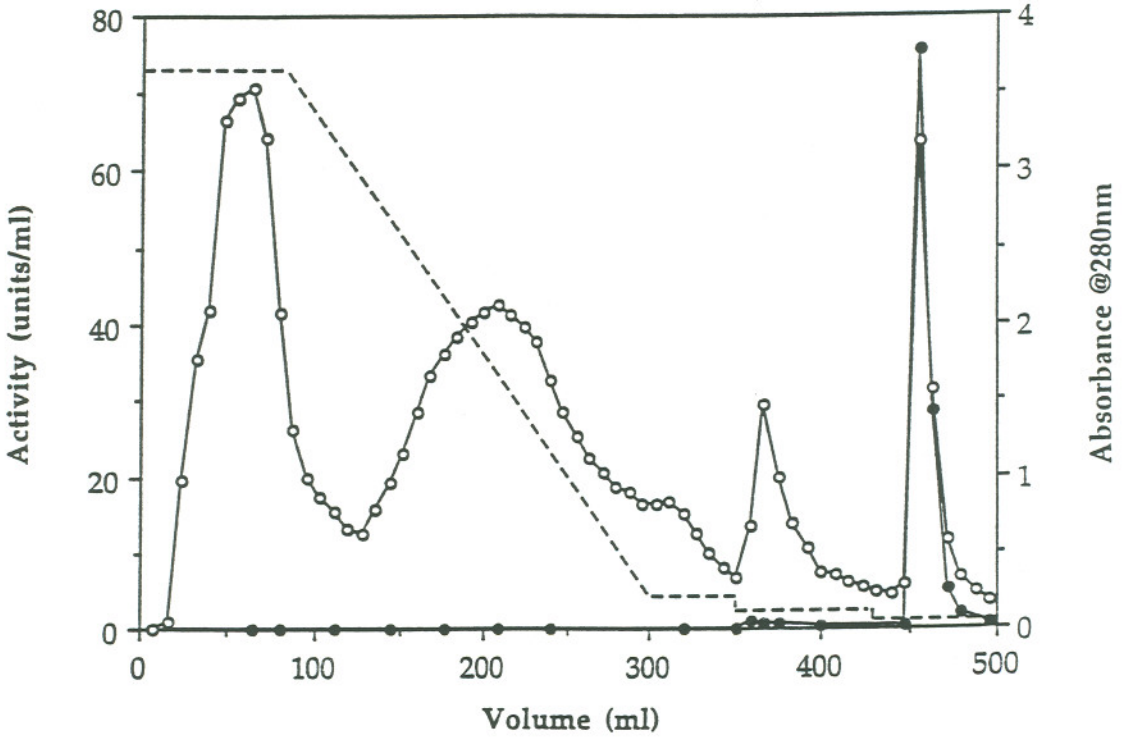

Figure 3.1 Phenyl Sepharose CL-4B chromatography of the quinone reductase. The enzyme was applied to the column in 50 mM sodium phosphate (pH 7.0), containing 0.5 M ammonium sulfate. The column contents were eluted with a combination of linear reverse ammonium sulfate gradient and descending phosphate step gradient (---): 0 to 100 ml (0.5 M ammonium sulfate); 100 to 200 ml (0.5 to 0.0 M ammonium sulfate); 300 to 350 ml (0.05 M sodium phosphate); 350 to 450 ml (0.004 M sodium phosphate); and 450 to 500 ml (H_2O). Absorbance at 280 nm (○); enzyme activity (●).

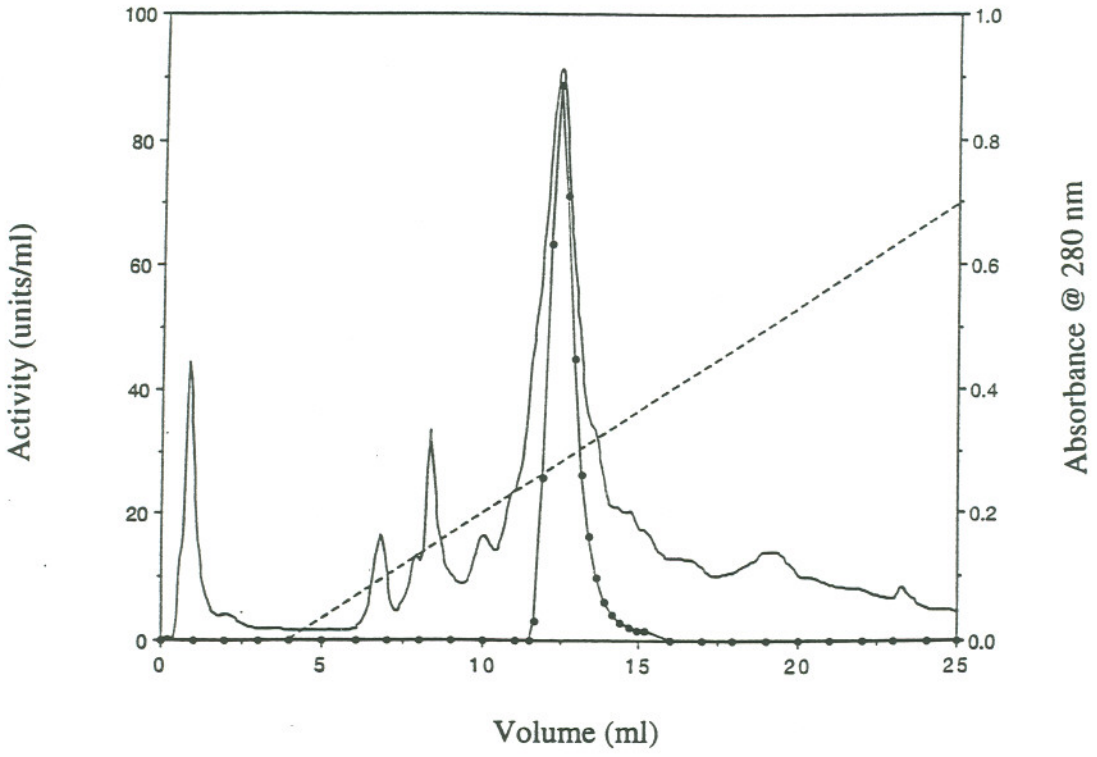

Figure 3.2 Mono Q ion-exchange chromatography of the quinone reductase. The gradient was from 10 mM sodium phosphate, pH 7.0 to 125 mM sodium phosphate (pH 7.0) in 25 min. A_{280} (—); enzyme activity (●); phosphate gradient (---).

3.3.3 Characterization of quinone reductase

3.3.3.1 *Prosthetic group.* The purified enzyme exhibited a typical oxidized flavin spectrum with visible maxima at 375 and 450 nm (Figure 3.3). The flavin spectrum was sharply decreased upon reduction of the enzyme with sodium dithionite. The identity of the enzyme-bound flavin was determined by two independent methods. First, the released flavin from a boiled enzyme preparation was isolated by ultrafiltration. HPLC analysis of the ultrafiltered flavin had the same retention time as that of standard FMN (6.8 min), whereas an FAD standard had a retention time of 6.3 min. Second, fluorescence of the flavin extracted from the enzyme exhibited a pH dependence identical to that of FMN but clearly different from that of FAD (Table 3.3).

3.3.3.2 *Physical properties.* The purified enzyme exhibited a pI value of 4.3 as determined by analytical isoelectric focusing. The enzyme exhibited a native molecular weight of approximately 44 kDa as determined by gel filtration on a Superose 12 column (Figure 3.4A). The subunit molecular weight was estimated by SDS PAGE to be 21.4 kDa (Figure 3.4B).

3.3.4 Substrate specificity

Several different electron acceptors were tested as substrates for the purified enzyme (Table 3.4). Using NADH as electron donor, the k_{cat} ranged from 4.4×10^5 s^{-1} for 2-MBQ to 3.3×10^2 s^{-1} for 2,6-DCPIP. The bimolecular steady-state rate constants, k_{cat}/K_m, ranged from 1.8×10^8 for 2-MBQ to 3.1×10^4 for 2,6-DCPIP. Owing to its relative stability 2,6-DMBQ was used as the substrate in routine assays. NADH oxidation was not detected when hydroxy-1,4- benzoquinones or nitroaromatics were used as potential electron acceptors. In addition to the para- and orthoquinones, as well as 2,6-DCPIP, the enzyme reduced the one-electron acceptor ferricyanide.

3.3.5 pH and temperature dependence of quinone reductase activity

The effect of pH on activity was measured using a citric acid/sodium phosphate buffer (172). The enzyme had a broad pH range with the optimum

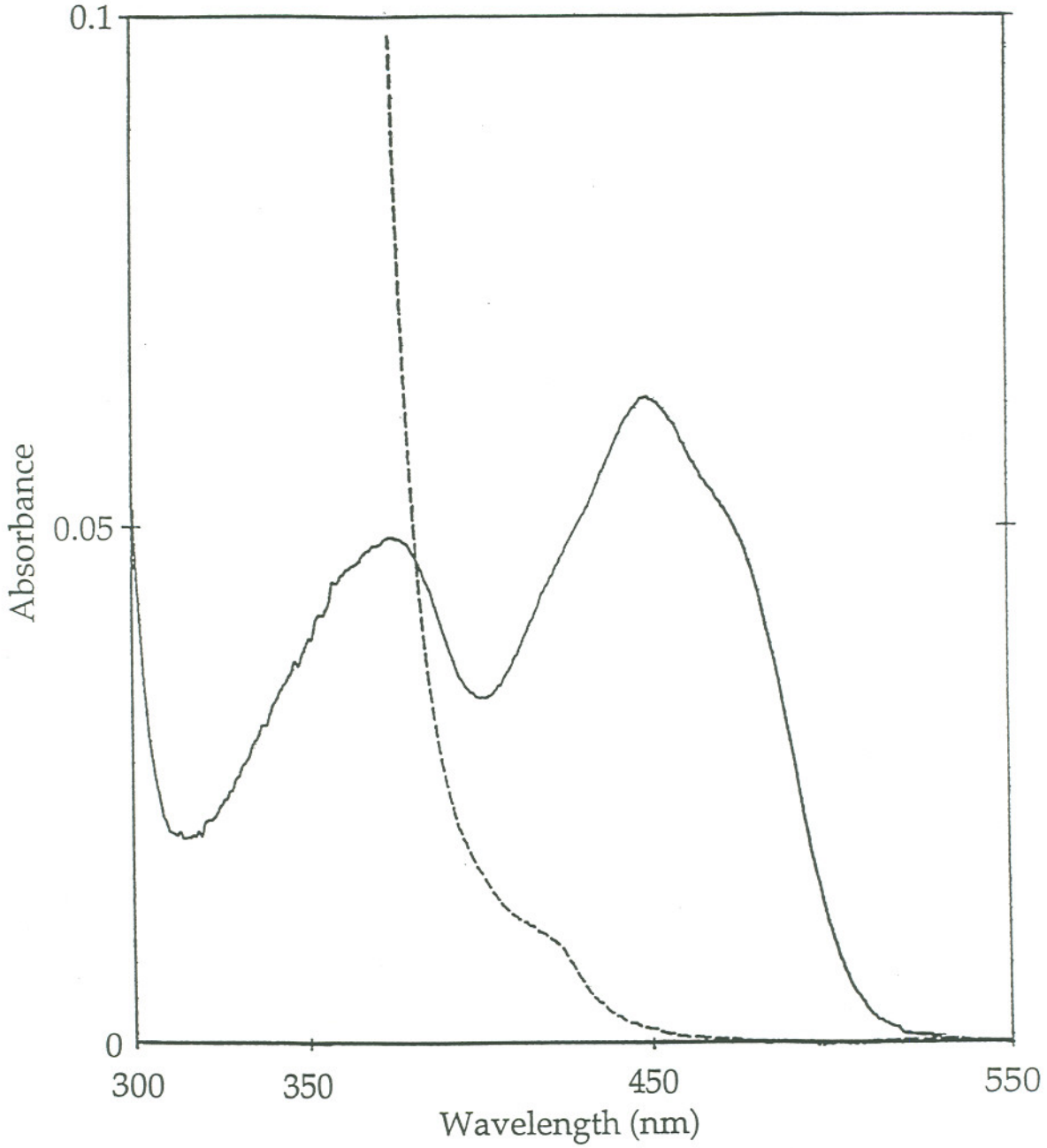

Figure 3.3 Visible spectra of the native oxidized (—) and sodium dithionite-reduced quinone reductases (---). The strong absorption below 400 nm in the reduced-enzyme spectrum is due to sodium dithionite.

Table 3.3

pH Dependence of the Fluorescence of FAD, FMN, and the Quinone Reductase Flavin[a]

Sample	Fluorescence		Ratio
	pH 7.7	pH 2.6	
FAD	44	85	.52
FMN	150	47	3.2
quinone reductase flavin	170	51	3.3

[a] Fluorescence emissions at 535 nm of FAD, FMN, and flavin extracted from quinone reductase were recorded using an excitation wavelength of 450 nm. Concentrations of FAD, FMN, and enzyme-bound flavin were 0.37, 0.2, and 0.23 μM, respectively.

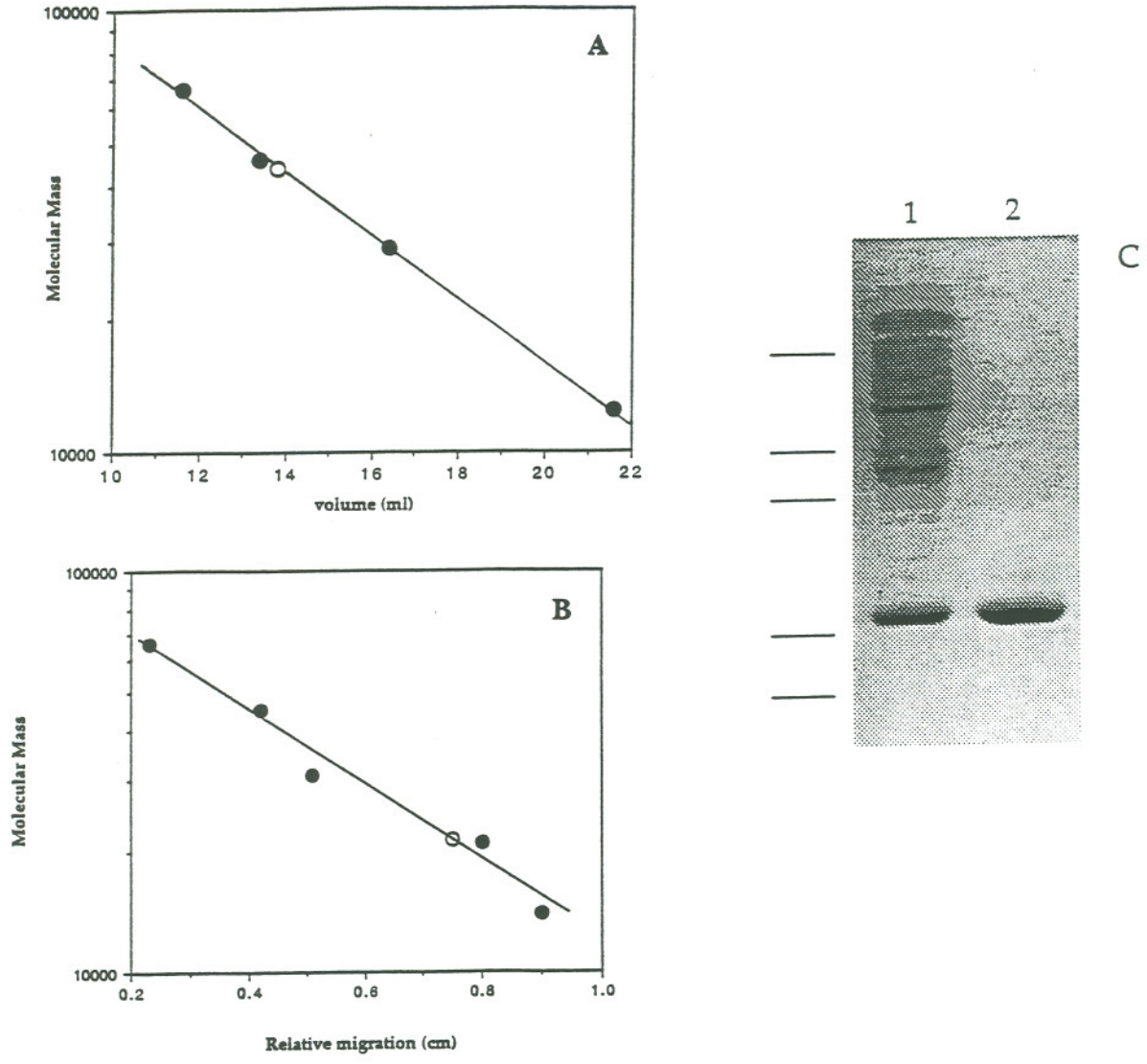

Figure 3.4 (A) Molecular mass determination of quinone reductase by size exclusion chromatography. The molecular mass standards were bovine serum albumin (66 kDa), manganese peroxidase (46 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa); o, molecular mass of the quinone reductase. (B) Molecular mass determination by SDS-PAGE. Molecular mass markers bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa). (C) Stained gel: lane 1, phenyl Sepharose fraction; lane 2, blue-agarose fraction. Molecular mass markers are indicated.

Table 3.4

Steady State Kinetic Parameters for the Quinone Reductase[a]

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
2-MBQ	2.4	4.4×10^5	1.8×10^8
2,6-DMBQ	7.4	5.6×10^3	7.6×10^5
2,6-DCPIP	10.5	3.3×10^2	3.1×10^4
2,5-di-*tert*-butyl-1,2-benzoquinone	20.8	1.4×10^3	6.7×10^4
Menadione	32.0	4.9×10^3	1.5×10^5
$K_3Fe(CN)_6$	380	6.0×10^3	1.6×10^4
NADH	55	8.0×10^2	1.5×10^4
NADPH	48	7.6×10^2	1.6×10^4

[a] Assays were carried out as described in the text. A fixed NADPH concentration of 200 μM was used in determining the K_m for the electron acceptors. A fixed 2,6-DMBQ concentration of 100 μM was used in determining the K_m for the electron donors.

between pH 5.4 and 6.2. Enzyme activity was linearly dependent on temperature between 5 and 30°C. The temperature optimum was between 30 and 40°C with rapid inactivation occurring above 40°C. Using 2,6-DMBQ as the electron acceptor, the activation energy for the oxidation of NADH was 22.5 kJ $mole^{-1}$ as calculated from an Arrhenius plot.

3.3.6 Inhibitors

The enzyme was not inhibited by 1 mM concentrations of KCN, NaN_3, $ZnSO_4$, $MgSO_4$, or $CuSO_4$. $MnSO_4$ (1 mM) and EDTA (1 mM) inhibited the enzyme less than 10%. Potassium cyanide and sodium azide have been reported to inhibit the activity of flavoproteins (55, 174). Dicumarol and Cibacron blue, both inhibitors of the mammalian NAD(P)H:(quinone acceptor)oxidoreductase (DT diaphorase) (59, 60, 175), were efficient inhibitors of this fungal enzyme. In the presence of 4 μM dicumarol, the enzyme activity was lowered 37%. In the presence of 2 μM Cibacron blue, enzyme activity was lowered 53%.

3.4 Discussion

Various substituted quinones have been identified as intermediates in the degradation of lignin model compounds, polymeric lignin, and aromatic pollutants (115, 183, 192, 215–217, 219, 229); thus, quinone metabolism is likely to be involved in the complete degradation of these compounds. Several intracellular quinone reductases have been identified in *P. chrysosporium* extracts (194), and one such enzyme has been purified (41) but not characterized extensively.

In the present study, an NAD(P)H-dependent quinone reductase has been isolated from primary metabolic cultures of *P. chrysosporium*. Although the enzyme activity is found under both primary and secondary metabolic conditions, the effect of inducers is much more profound during primary metabolism. The addition of vanillate or methoxy-*p*-quinone to primary metabolic cells results in an increase in enzyme activity (13.9-fold and 6.2-fold, respectively), whereas the addition of vanillate or quinone to secondary metabolic cells has a much smaller effect. These

results suggest that this quinone reductase is regulated independently of lignin and manganese peroxidase (33, 78, 131). The latter enzymes only are expressed during the secondary metabolic phase of growth, and their expression is not induced by aromatic substrates (78). The regulation of this quinone reductase appears to be similar to that described for vanillate hydroxylase (30, 231), and it is likely that the quinone reductase is involved in vanillate metabolism. However, the quinone reductase also is present during the ligninolytic phase of growth, suggesting that it also plays a role in the reduction of quinones produced during lignin degradation. The response of the cells to exogenously added quinones suggests that this organism exhibits a sensitive mechanism for removing metabolically generated quinone toxins.

The quinone reductase was purified to apparent homogeneity by a combination of ammonium sulfate fractionation, hydrophobic interaction, ion exchange, and blue-agarose affinity chromatography. The homogenous enzyme has a molecular mass of 44 kDa and is composed of two similar 21.4-kDa subunits as demonstrated by SDS-PAGE. This dimeric composition is similar to that reported for DT diaphorase (104, 106), a mammalian quinone reductase. In contrast, Constam et al. (41) have isolated a *P. chrysosporium* quinone reductase that is apparently composed of a single polypeptide chain. We have identified the cofactor as FMN and, using a molecular mass of 44 kDa, we calculate a 0.9:1 ratio of FMN to holoenzyme. In contrast, DT diaphorase contains 2 FAD molecules per holoenzyme (104). The cofactor for the enzyme(s) isolated by Constam et al. (41) was not identified. Isoelectric focusing demonstrates a pI of 4.3 for the quinone reductase. In contrast, the pI values calculated for the quinone reductase(s) isolated by Constam et al. (41) were 5.7 to 6.3.

The purified quinone reductase utilizes either NADH or NADPH as electron donors, whereas the purified enzyme of Constam et al. (41) utilized only NADH. The K_m for NADH and NADPH are 55 and 48 mM, respectively. This quinone reductase is capable of reducing both para- and orthoquinones. The substituted paraquinones, 2-MBQ and 2,6- DMBQ, have both been identified as fungal metabolites of lignin model compounds and aromatic pollutants (215–217, 228). 2,6-DMBQ was chosen as the routine substrate, because it is relatively stable and it is

a breakdown product of the manganese peroxidase-catalyzed oxidation of lignin model compounds and synthetic lignins (57, 215, 228). The high turnover numbers (4.4×10^5 s^{-1}) exhibited by the enzyme for 2-MBQ and 2,6-DMBQ suggests that the fungus has a very efficient mechanism for conversion of this metabolic intermediate, which is probably derived from vanillic acid, syringic acid (57, 183), and soft- and hardwood lignins (39, 215, 228). This also suggests that the enzyme is involved in lignin degradation. However, the enzyme also effectively catalyzes the reduction of other substituted quinones and nonphysiological substrates such as ferricyanide and 2,6-DCPIP. The broad substrate specificity of this enzyme, as well as that of the previously isolated quinone reductase (41), suggests that this organism does not produce multiple specific quinone reductases, as has been proposed previously (194).

The inhibition of the quinone reductase by a variety of compounds was examined. Zn^{2+}, Cu^{2+}, and Mg^{2+} have no effect, whereas Mn^{2+} is slightly inhibitory. Only 6% inhibition is observed with 1.0 mM EDTA, suggesting that the enzyme does not require a transition metal for activity. As is true for DT diaphorase (59, 60, 175), dicumarol and Cibacron blue 3GA are efficient inhibitors, suggesting that the mechanism of this quinone reductase may be similar to that of DT diaphorase. The potency of the inhibition by Cibacron blue and dicumarol also suggests that these compounds may be useful for studying the role of this enzyme *in vivo*.

The existence of other *P. chrysosporium* reductive enzymes has been established. In addition to quinone reductases, Muheim et al. (164) have characterized an aryl aldehyde reductase which can regenerate veratryl alcohol from veratraldehyde. Likewise, quinone reductases regenerate hydroxyquinones, which are substrates for extracellular peroxidases (79, 216, 217). The intracellular location of this quinone reductase suggests that monomeric quinones generated during lignin degradation are taken up by the cells for subsequent metabolism.

In addition to being degradative intermediates, quinones also act as redox-active toxins. Exogenous quinones, as well as those formed by metabolic conversion, undergo facile one-electron reductions to generate semiquinone radicals (34, 109, 110). Semiquinones then are oxidized by O_2 to generate superoxide anion (100).

Superoxide may be converted to H_2O_2, either enzymatically by superoxide dismutase or non-enzymatically (71, 100). In the presence of suitable electron donors, H_2O_2 can give rise to highly reactive hydroxyl radical (91). Therefore, quinone reduction in *P. chrysosporium* also may act to protect the organism from oxidative stress created by metabolically generated or exogenous quinones.

In conclusion, we have characterized a quinone reductase from *P. chrysosporium*. Further studies are planned to understand in more detail the regulation and kinetic mechanism of this enzyme.

CHAPTER 4

1,4-Benzoquinone Reductase from the Basidiomycete *Phanerochaete chrysosporium*: Spectral and Kinetic Analysis

4.1 Introduction

The wood-rotting basidiomycete *Phanerochaete chrysosporium* is capable of degrading polymeric lignin (33, 78, 131) and a variety of aromatic pollutants (24, 79, 94). During secondary metabolic (idiophasic) growth, *P. chrysosporium* secretes two families of extracellular peroxidases—lignin peroxidase (LiP) and manganese peroxidase (MnP) (76, 130, 137, 142, 214). These enzymes, along with an H_2O_2-generating system (121), catalyze the initial steps in the depolymerization of lignin, generating a wide variety of metabolic intermediates, including substituted quinones, hydroquinones, benzaldehydes and ring-opened fragments (33, 57, 131, 192, 215, 229).

Whereas the role of extracellular peroxidases in lignin and pollutant degradation has been well characterized, much less is known about the intracellular enzymes involved in the further breakdown of monomeric intermediates such as quinones, hydroquinones, and benzaldehydes. The elucidation of metabolic pathways for the degradation of several aromatic pollutants has suggested roles for intracellular enzymes in the reduction of quinones and in the ring cleavage of hydroquinones (115, 183, 216, 217, 219). With regard to the latter activity, we have recently purified and characterized a 1,2,4-trihydroxybenzene-1,2-dioxygenase which catalyzes the ring cleavage of lignin and pollutant-derived trihydroxybenzene (183). Since quinones—which are generated by the extracellular peroxidase oxidation of both lignin and aromatic pollutants—appear to be key intermediates in aromatic degradation by *P. chrysosporium* (33, 43, 57, 79, 94, 115, 131, 183, 192, 193, 215-217, 219, 228,

229), we have examined the subsequent metabolism of these intermediates, particularly their reduction.

The reduction of methoxylated, lignin-derived quinones by *P. chrysosporium* is apparently catalyzed by intracellular quinone reductases (14, 26, 41, 194). Recently we purified and carried out the preliminary characterization of an intracellular NAD(P)H-dependent quinone reductase from this organism. This soluble, intracellular flavoprotein is a 44,000-kDa dimer, consisting of two similar 22,000-kDa subunits. A variety of methoxylated quinones and other electron acceptors serve as substrates for this enzyme (14). NADH and NADPH are equally efficient as electron donors, as observed previously for the mammalian quinone reductase DT-diaphorase (60). Herein, we have examined further the catalytic mechanism and substrate specificity of this intracellular quinone reductase.

4.2 Materials and Methods

4.2.1 Culture conditions and enzyme preparation

P. chrysosporium strain OGC101 was maintained on malt agar slants as described (3). The quinone reductase was isolated from three-day-old, high-carbon (1% glucose), high-nitrogen (12 mM ammonium tartrate) agitated cultures, and purified by a combination of ammonium sulfate precipitation, hydrophobic interaction, ion exchange, and affinity chromatography as described (14).

4.2.2 Identification of spectral intermediates

The electronic absorption spectra of oxidized and reduced quinone reductase (10–15 μM) in 50 mM sodium phosphate (pH 7.0) were recorded with a Shimadzu UV-260 spectrophotometer. Where indicated, anaerobic conditions were maintained by purging the contents of the cuvettes with scrubbed argon. O_2-free electron donor or acceptor then was added and spectra were recorded.

4.2.3 Stoichiometry of NADH oxidation versus acceptor reduction

Quinone reductase can utilize either NADH or NADPH as an electron donor (14). For this study, NADH was used throughout. Reaction mixtures contained either 2,6-dimethoxy-1,4-benzoquinone (DMBQ) (300 μM) or ferricyanide (500 μM) and enzyme (1 μg) in 50 mM sodium citrate, pH 6.0. Extinction coefficients for DMBQ and ferricyanide were $\epsilon_{390} = 0.5\ cm^{-1}\ mM^{-1}$ and $\epsilon_{420} = 1.08\ cm^{-1}\ mM^{-1}$, respectively. Reactions were initiated by the addition of NADH (100–250 μM). Reduction of DMBQ or ferricyanide was measured by monitoring the decrease in absorbance at the appropriate wavelength.

For HPLC analysis, reaction mixtures contained 50 mM sodium citrate, pH 6.0, enzyme (1 μg), DMBQ (200 μM), and NADH (25–175 μM). The reactions were carried out at room temperature for 5 min and stopped by acidification. HPLC analysis was conducted on an HP LiChrospher 100 RP-8 column with a mobile phase of acetonitrile:0.05% phosphoric acid (15:85), with a flow rate of 1 ml/min. The remaining quinone substrates and hydroquinone products were detected at 275 nm. The 2,6-dimethoxy-1,4-hydroquinone (DMHQ) standard was prepared by reduction of DMBQ with sodium dithionite.

4.2.4 Steady-state kinetic studies

Reactions were conducted in 1.0 ml of 50 mM sodium citrate, pH 6.0, with 1 μg of enzyme at room temperature. Quinone and NADH concentrations ranged from 2–20 μM and 10–150 μM, respectively. Initial velocities were measured by monitoring the decrease in absorbance at 340 nm ($\epsilon_{340} = 6.22\ cm^{-1}\ mM^{-1}$) owing to NADH oxidation. Ferricytochrome *c* (40 μM) reduction was measured by following the increase in absorbance at 550 nm ($\epsilon_{550} = 21.1\ cm^{-1}\ mM^{-1}$) (72), and ferricyanide (100 μM) reduction was monitored at 420 nm as described above.

4.2.5 Reduction of the autooxidation products of trihydroxybenzene and chlorinated trihydroxybenzenes

1,2,4-Trihydroxybenzene (THB) and 5-chloro-1,2,4-trihydroxybenzene (Cl-THB) were synthesized as described (115). Stock solutions (10 mM) of THB and Cl-

THB were prepared in 50 mM sodium citrate (pH 3.0), and assays were conducted immediately. Reaction mixtures (1.0 ml) in 50 mM sodium citrate (pH 6.0) contained 100 μM THB or Cl-THB, and 200 μM NADH. Autooxidation of THB or Cl-THB was monitored by following the increase in absorbance at 475 nm. Autooxidation was reversed by the subsequent addition of quinone reductase to the reaction mixture.

4.2.6 Inhibition by dicumarol and Cibacron blue 3GA

The initial rate of NADH oxidation was determined in the presence of various concentrations of dicumarol and Cibacron blue 3GA. Reaction mixtures (1 ml) contained 50 mM sodium citrate (pH 6.0), 10 μM DMBQ, 10–100 μM NADH, and various concentrations of inhibitor. The reactions were initiated by addition of enzyme (1–2 μg). A stock solution of 10 mM dicumarol was prepared in 0.1 N NaOH. Cibacron blue (10 mM) was prepared in H_2O.

4.2.7 Reconstitution of apoprotein

Apoenzyme was prepared as described (211). The native enzyme (12 μM) was dialyzed for 72 h at 4°C against 200 mM potassium phosphate (pH 6.0), containing 0.3 mM EDTA, 20% glycerol, 2 M potassium bromide, and activated charcoal (2 g/l). After removal of FMN, as determined by measuring the absorbance at 450 nm, the dialysis buffer was changed to 200 mM potassium phosphate (pH 7.0), containing 0.3 mM EDTA and 20% glycerol. Holoenzyme was reconstituted from the apoenzyme at 4°C by the stepwise addition of 0.5-1.0 nmole aliquots of FMN. After each addition of FMN, the sample was incubated for 5 min at 4°C. Subsequently, a 2-μl aliquot was analyzed for activity.

4.3 Results

4.3.1 Spectral intermediates of quinone reductase

A spectral analysis of the oxidized and reduced forms of quinone reductase was utilized to determine the order of substrate binding. The addition of 1 equivalent of NADH to the oxidized, native enzyme yielded the fully reduced enzyme as

monitored by the disappearance of the oxidized flavin bands at 375 and 450 nm (Figure 4.1A). The reduced enzyme spectrum is stable for up to 30 min in the absence or presence of oxygen (data not shown). However, the addition of 1 equivalent of DMBQ to the reduced enzyme regenerates the fully oxidized enzyme spectrum (Figure 4.1A). In contrast, addition of DMBQ to the oxidized, native enzyme does not alter its spectrum. Addition of one equivalent of ferricyanide to the reduced enzyme only partially regenerates the oxidized enzyme spectrum (Figure 4.1B). However, neither a shift in the spectral peaks nor the appearance of new peaks at longer wavelengths were observed. The addition of two equivalents of ferricyanide to the reduced enzyme regenerates the fully oxidized enzyme spectrum (Figure 4.1B).

4.3.2 Reaction stoichiometry

The stoichiometry of DMBQ reduction was determined by two methods. In the first method, the number of moles of acceptor reduced was measured spectrophotometrically in the presence of limiting concentrations of NADH (Figure 4.2). A plot of the moles of acceptor reduced versus NADH added yields slopes of 1.0 and 2.0 for DMBQ and ferricyanide, respectively (Figure 4.2C). Similar results were obtained when NADH oxidation was monitored in the presence of various concentrations of acceptor.

In the second method, HPLC was used to determine the amount of hydroquinone produced as a function of NADH added to the enzyme in the presence of 200 μM DMBQ. Under the conditions described above, DMHQ and DMBQ eluted at 2.4 and 4.1 min, respectively. As shown in Figure 4.3, the amount of DMHQ produced is proportional to the amount of NADH added. Furthermore, the disappearance of DMBQ is stoichiometric with appearance of DMHQ.

The steady-state rates of ferricyanide reduction and NADH oxidation also were compared. The ratio of the ferricyanide reduction rate to the NADH oxidation rate was 1.8:1 (58.4 μmoles min^{-1}:32.4 μmoles min^{-1}).

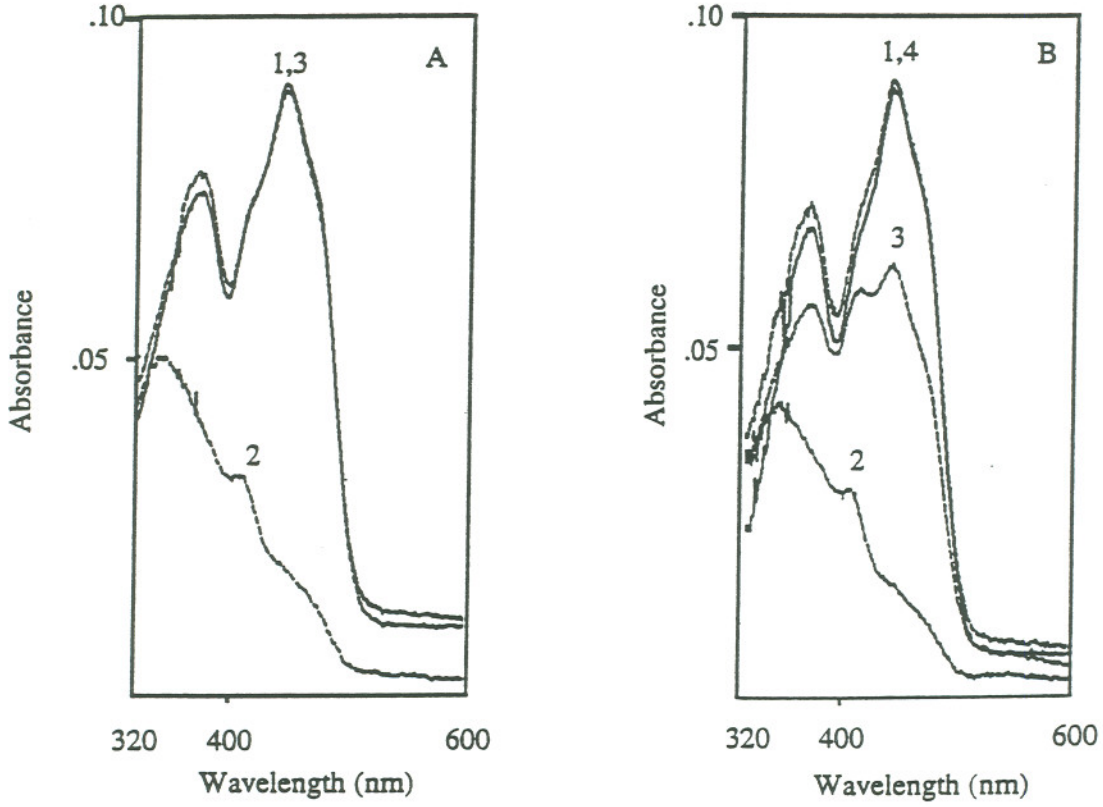

Figure 4.1 Reduction and reoxidation of quinone reductase. (A) The native enzyme (spectrum 1, solid line) was reduced with one equivalent of NADH to produce spectrum 2. The reduced enzyme was subsequently reoxidized with DMBQ to produce spectrum 3 (dashed line). (B) The native enzyme, spectrum 1 (solid line), was reduced with one equivalent of NADH to produce spectrum 2. The fully reduced enzyme was then reoxidized with one (spectrum 3) and then two (spectrum 4, dashed line) equivalents of ferricyanide. All spectra were recorded immediately after the additions.

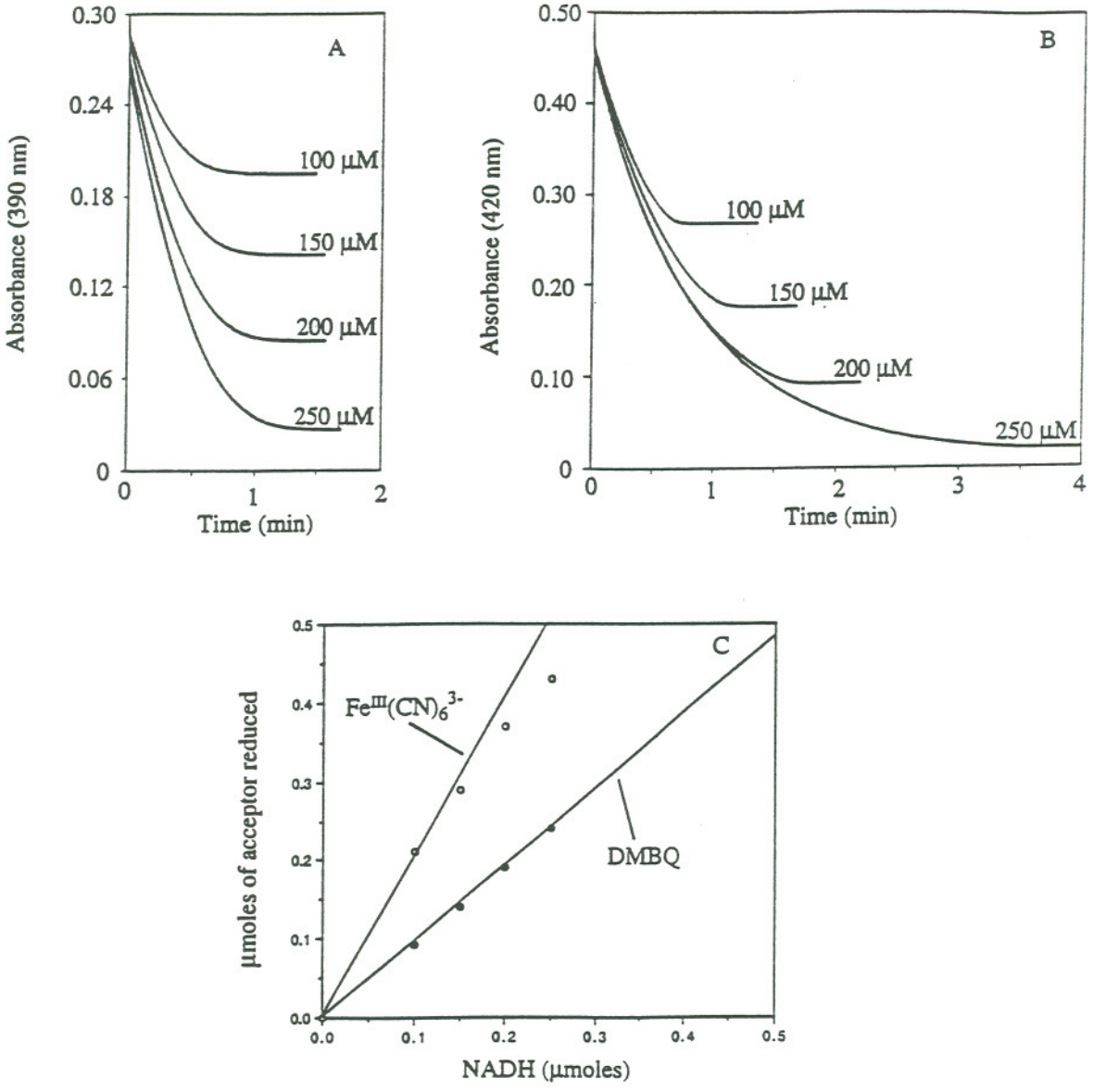

Figure 4.2 Stoichiometry of enzyme-catalyzed electron acceptor reduction in the presence of various concentrations of NADH. (A) DMBQ reduction was measured in the presence of the indicated concentrations of NADH. (B) Ferricyanide reduction was measured in the presence of the indicated concentrations of NADH. (C) Stoichiometry of the reduction of DMBQ (○) and ferricyanide (●) versus NADH concentration.

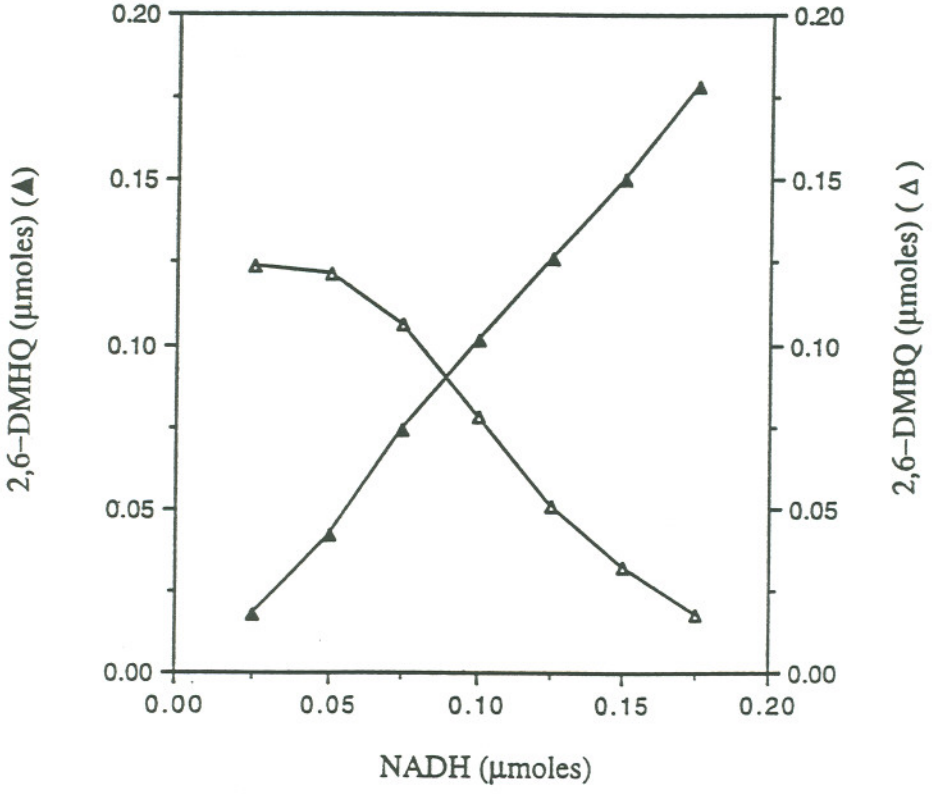

Figure 4.3 Enzymatic reduction of DMBQ (△) to hydroquinone (▲) in the presence of various concentrations of NADH. Enzymatic reactions were conducted as described in the text. The remaining substrate and product formed were analyzed by HPLC as described in the text.

4.3.3 Steady-state reaction mechanism

A double reciprocal plot of 1/activity versus 1/quinone concentration in the presence of various concentrations of NADH exhibits parallel lines in the concentration range of 50–100 μM NADH (Figure 4.4). At NADH concentrations below 50 μM, an increase in slope is observed.

4.3.4 Reduction of chlorinated quinones

One possible role of the quinone reductase is to maintain hydroxylated aromatics in the reduced form. This was examined by monitoring the subsequent enzymatic reduction of THB and chlorinated THB autooxidation products. As shown in Figure 4.5, THB autooxidizes rapidly as measured by an increase in absorption at 475 nm. The rate of autooxidation of these trihydroxybenzenes varies with the extent of chlorination. Cl-THB and 3,5-dichloro-1,2,4-trihydroxybenzene undergo autooxidation more rapidly than THB. The autooxidation of 3,5-dichloro-1,2,4-trihydroxybenzene was too fast to measure by this method. The addition of NADH alone did not prevent or reverse autooxidation of either THB or Cl-THB. However, the addition of quinone reductase and NADH reverses the autooxidation process (Figure 4.5). Furthermore, autooxidation was prevented by preincubating THB and Cl-THB with enzyme plus NADH (data not shown).

4.3.5 Nonenzymatic reduction of quinones

The observation that quinone reductase is capable of reducing the autooxidation products of chlorinated trihydroxybenzenes prompted us to examine the reduction of a variety of substituted quinones. All the quinones listed in Table 4.1 serve as substrates for the enzyme. However, rapid nonenzymatic NADH oxidation was observed with each chloroquinone tested. Indeed, this nonenzymatic reduction appears to account for the majority of chloroquinone-dependent NADH oxidation. In contrast, efficient reduction of methoxybenzoquinones requires the enzyme. In a separate experiment, a *P. chrysosporium* intracellular crude extract was ultrafiltered using a 10,000-kDa cutoff membrane (Amicon). The protein-free filtrate readily reduced 2-chloro-1,4-benzoquinone to 2-chlorohydroquinone.

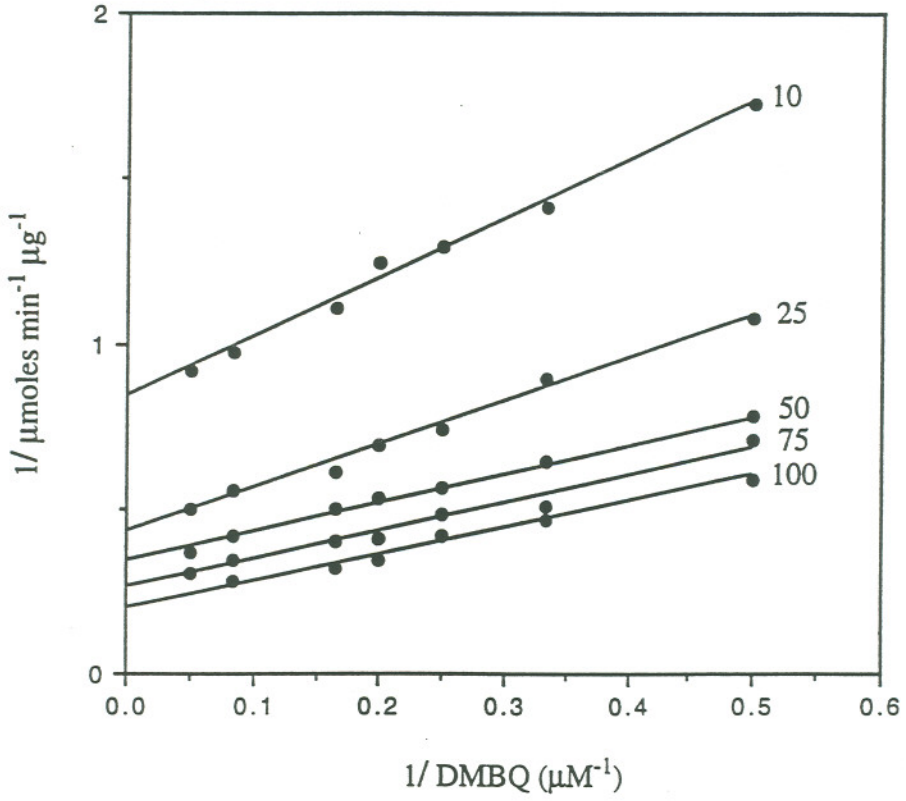

Figure 4.4 Steady-state kinetic analysis of quinone reductase activity. 1/V versus 1/[DMBQ] at the various indicated fixed concentrations of NADH. The DMBQ concentrations ranged from 2–20 μM in the presence of the indicated μM concentrations of NADH.

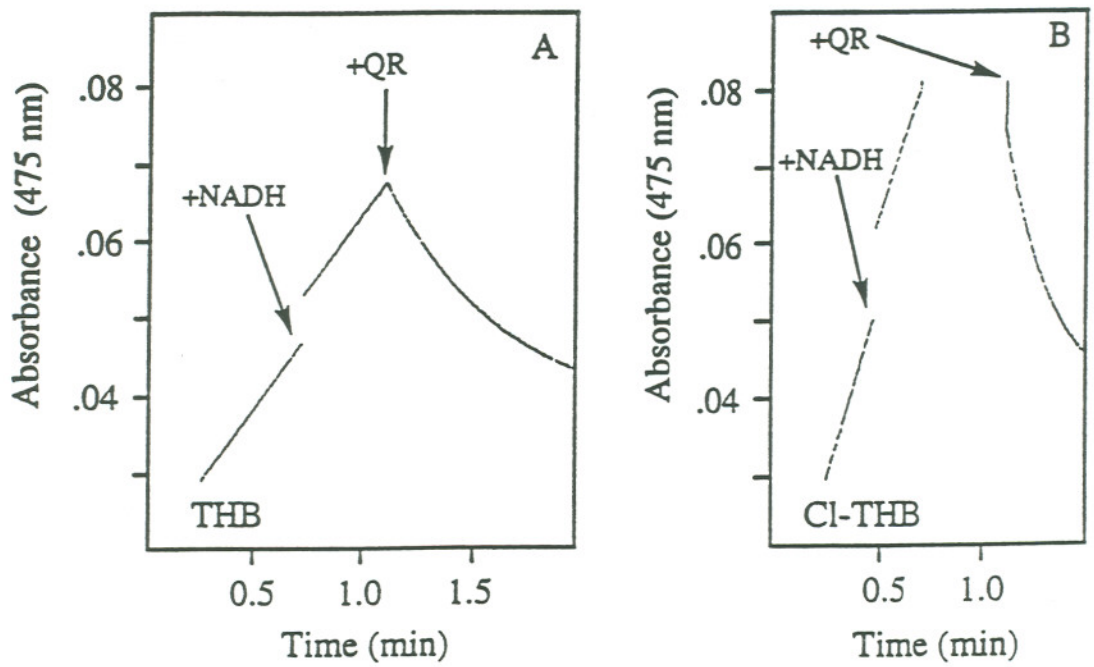

Figure 4.5 Reduction of the autooxidation products of THB and Cl-THB by quinone reductase. (A) 100 μM THB was incubated in 50 mM sodium citrate (pH 6.0) for the indicated period. Subsequently, 100 μM NADH was added followed by 2 μg of quinone reductase (QR). (B) 100 μM Cl-THB was incubated for the indicated period. Subsequently, 100 μM NADH was added followed by 100 μg of quinone reductase.

Table 4.1

Nonenzymatic and Enzymatic Reduction of Various Quinones[1]

Quinone substrate	NADH oxidation		Nonenzymatic rate/
	nmoles · min^{-1} (minus enzyme)	nmoles · min^{-1} (plus enzyme)	enzymatic rate (%)
1,4-benzoquinone	7.23	17.1	42
2-methoxy-1,4-benzoquinone	5.06	28.7	18
2,6-dimethoxy-1,4-benzoquinone	0	5.79	0
1,4-napthoquinone	0	6.22	0
2-chloro-1,4-benzoquinone	72.3	78.4	92
2,6-dichloro-1,4-benzoquinone	t.f.[2]	t.f.	-
2,3,5,6-tetrachloro-1,4-benzoquinone	11.6	12.1	96

[1] Reaction mixtures (1.0 ml) contained 50 mM sodium citrate, pH 6.0, 100 μM quinone, and 200 μM NADH. NADH oxidation was measured in the absence and presence of 0.89 μg of quinone reductase.

[2] t.f. NADH oxidation was too fast to measure by the method used.

4.3.6 Reduction of ferricytochrome *c* and ferricyanide

The results in Table 4.2 show that the enzyme is able to reduce both ferricyanide and ferricytochrome *c* (Cc^{3+}). The rate of NADH oxidation by the enzyme in the presence of ferricyanide was 58.4% of that for DMBQ. However, when one considers the number of electrons transferred, the ferricyanide rate is decreased by 50%. The presence of DMBQ stimulates the rate of ferricyanide reduction by the enzyme. The enzyme also slowly reduces Cc^{3+}. When the reaction is conducted in 50 mM sodium citrate (pH 6.0), the rate of Cc^{3+} reduction is 0.08% of the rate with DMBQ. The Cc^{3+} reduction rate is ~3 times faster in H_2O than in citrate buffer. In the presence of DMBQ, the rate of Cc^{3+} reduction is increased tenfold. The rate of Cc^{3+} reduction in either the presence or absence of quinone was not affected by the presence of molecular O_2 in the reaction mixture (data not shown). In the absence of DMBQ, the ratio of the rate of Cc^{3+} reduction versus NADH oxidation was 1.8:1 (0.081 μmol min^{-1}:0.045 μmol min^{-1}).

The results in Table 4.3 show that a variety of hydroquinones effectively reduce both ferricyanide and Cc^{3+} in the absence of enzyme. In particular, ferricyanide was reduced efficiently by 1,4-hydroquinone, 2-methoxyhydroquinone, DMHQ, and 1,4-napthohydroquinone, while Cc^{3+} was slowly reduced by 2-methoxyhydroquinone, DMHQ, and 1,4-dihydroxynapthalene.

4.3.7 Inhibition by dicumarol and Cibacron blue

We reported previously (14) that the DT-diaphorase inhibitors dicumarol and Cibacron blue 3GA also inhibited quinone reductase activity. As shown in Figure 4.6, both compounds competitively inhibit quinone reductase with respect to NADH. A replot of the slope versus inhibitor concentration yields K_i values of 2.1 and 0.30 μM for dicumarol and Cibacron blue 3GA, respectively.

4.3.8 Reconstitution of apoenzyme

We reported (14) that the enzyme contains one molecule of FMN per dimer. This FMN content was obtained by incubating the protein with FMN, removing excess FMN by gel filtration, and finally determining the FMN content of a known

Table 4.2

Relative Efficiencies of DMBQ, Ferricyanide, and Ferricytochrome *c* (Cc^{3+}) as Electron Acceptors in the Quinone Reductase Reaction

Substrate	Units mg^{-1}[a]	Relative activity (%)
DMBQ	101	100
Ferricyanide	58.4[b]	58[b]
Ferricyanide + DMBQ	121	121
Cc^{3+} in citrate	0.08	0.08
Cc^{3+} in H_2O	0.25	0.25
Cc^{3+} + DMBQ	0.80	0.80

[a] 1 unit = 1 μmol of acceptor reduced $\cdot$ min^{-1}. Acceptor concentrations were 100 μM (DMBQ), 500 μM (ferricyanide) and 40 μM (Cc^{3+}). The NADH concentration was 200 μM in all experiments.

[b] The reduction of DMBQ is a two-electron reaction. In contrast, the reduction of $Fe^{III}(CN)_6^{3-}$ and Cc^{3+} are one-electron reactions. Thus, the rate of single electron transfer with DMBQ is two times the rate shown.

Table 4.3

Reduction of Ferricyanide and Ferricytochrome *c* by Various Hydroquinones[1]

Hydroquinone	Nonenzymatic ferricyanide reduction (nmoles min^{-1})	Nonenzymatic Cc^{3+} reduction (nmoles min^{-1})
1,4-Hydroquinone	39	0
2-Methoxyhydroquinone	251	22.6
2,6-Dimethoxyhydroquinone	311	15.5
1,4-Napthohydroquinone	t.f.[2]	39.3

[1] Hydroquinone (100 μM) was added to a 1.0-ml reaction mixture containing 50 mM sodium citrate, pH 6.0, and ferricyanide (100 μM) or Cc^{3+} (40 μM). Ferricyanide and Cc^{3+} reduction were measured as described in the text.

[2] t.f. This reaction is too fast to follow by the methods used.

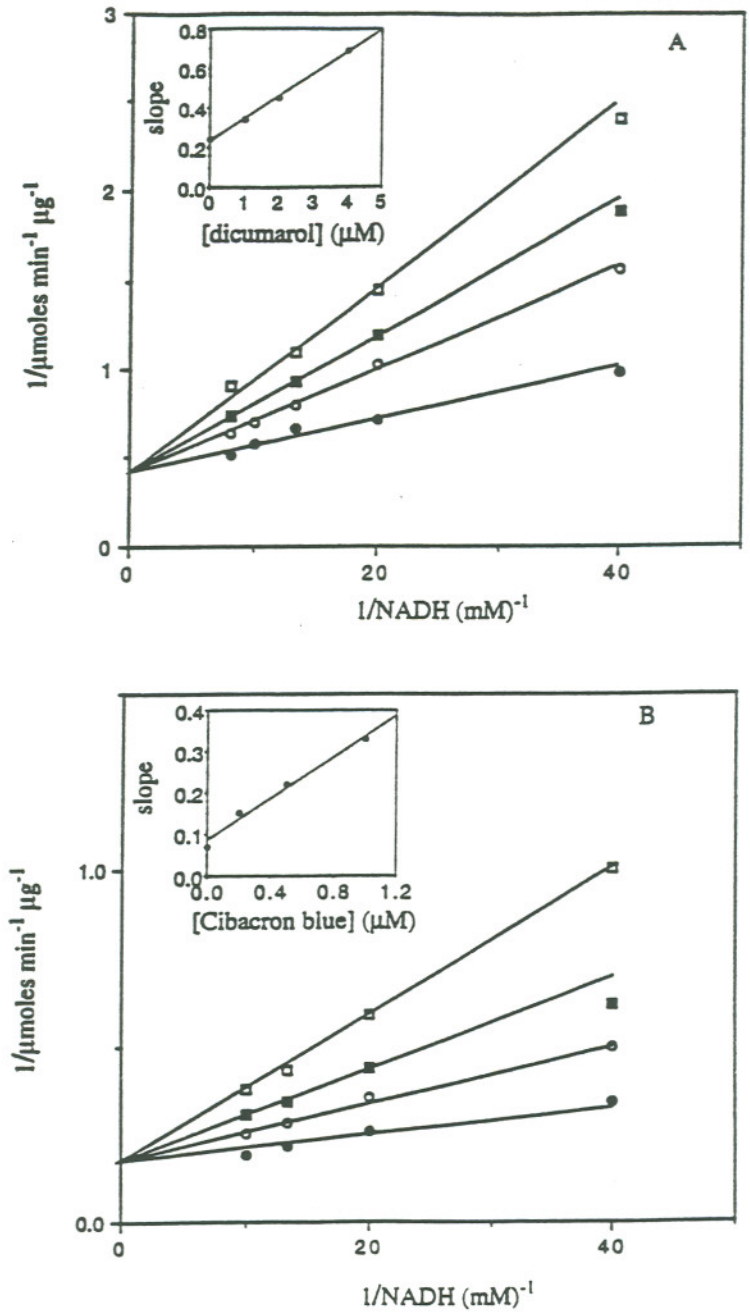

Figure 4.6 Inhibition of quinone reductase by dicumarol and Cibacron blue 3GA. (A) The oxidation of NADH (0.025-0.125 mM) was monitored in the presence of DMBQ (10 μM) and the following fixed concentrations of dicumarol: 0 μM (●), 1 μM (○), 2 μM (■), and 4 μM (□). (B) The oxidation of NADH was monitored in the presence of DMBQ (10 μM) and the following fixed concentrations of Cibacron blue: 0 μM (●), 0.25 μM (○), 0.50 μM (■), and 1 μM (□). Insets: replots of slope versus inhibitor concentration.

amount of protein using a millimolar extinction at 450 nm of 12.2. The FMN content of the enzyme has been reexamined by reconstituting the holoenzyme as described (211). As shown in Figure 4.7, enzyme activity increases with the stepwise addition of FMN up to a maximum of 2 equivalents of FMN per equivalent of dimer.

4.4 Discussion

Substituted quinones and their corresponding hydroquinones are key intermediates in the complete degradation of polymeric lignin, lignin model compounds, and aromatic pollutants (115, 183, 192, 215-217, 219, 228). These quinones are reduced to hydroquinones which undergo further metabolism (14, 57, 183). We previously described the purification and initial characterization of an intracellular quinone reductase from the wood-rotting fungus *P. chrysosporium* (14). Herein, the catalytic mechanism of this enzyme has been characterized in more detail.

The purified quinone reductase contains FMN. The oxidized, resting state of the enzyme is quantitatively reduced by the addition of one equivalent of NADH. The spectrum of the reduced enzyme intermediate is relatively stable under both aerobic and anaerobic conditions, suggesting that this enzyme reacts poorly or not at all with molecular oxygen. The oxidized enzyme is regenerated by the addition of one equivalent of DMBQ or other quinones to the two-electron reduced enzyme. As suggested by Massey (154), quantitative two-electron reduction by a donor with subsequent two-electron oxidation by an acceptor is indicative of a two-electron-transferring dehydrogenase. These results strongly suggest that the native enzyme is in the oxidized form. They also suggest an ordered, ping-pong catalytic mechanism, whereby the oxidized enzyme is first reduced by NAD(P)H and subsequently the reduced enzyme intermediate reduces the quinone acceptor. Our steady-state kinetic studies confirm that the enzyme obeys an ordered, ping-pong mechanism, whereby the native enzyme is first reduced by the electron donor and, subsequently, the reduced enzyme intermediate is reoxidized by the electron acceptor. The double reciprocal plot in Figure 4.4 exhibits parallel lines with NADH concentrations in the range of 50–100 μM. However, enzyme inhibition is observed at NADH concentrations below

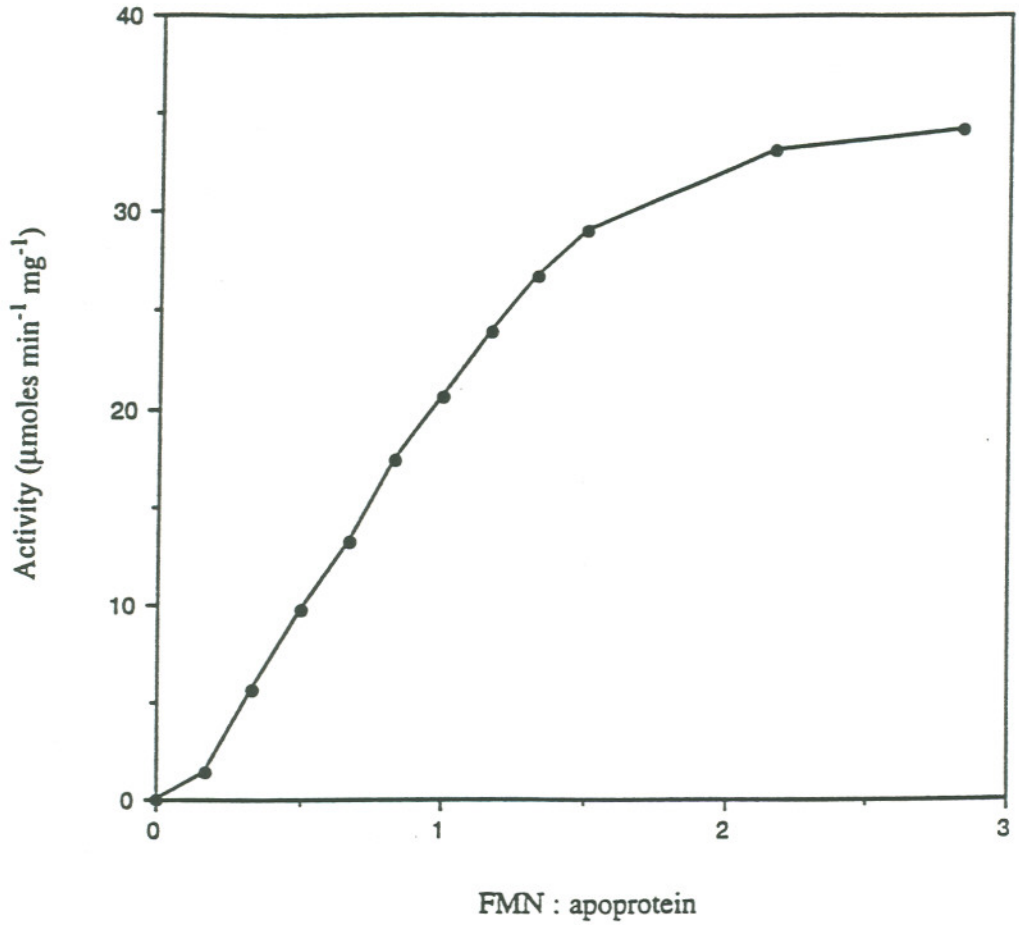

Figure 4.7 Reconstitution of apoenzyme with FMN. Apoenzyme (12 μM) was prepared and reconstituted with FMN as described in the text. After each addition of FMN, a 2-μl aliquot was removed and assayed for activity. Assay mixtures (1.0 ml) contained 50 mM sodium phosphate (pH 6.0), 100 μM DMBQ, 200 μM NADH, and enzyme.

50 μM. This type of inhibition also has been observed with mammalian DT-diaphorase (89). Since the *P. chrysosporium* quinone reductase is stabilized by the addition of exogenous NADH (data not shown), this apparent inhibition might be due to enzyme inactivation during turnover in the presence of low NADH concentrations.

Our spectral and HPLC analyses indicate that one equivalent of DMBQ is reduced to DMHQ for each equivalent of NADH oxidized. This strict adherence to a two-electron transfer mechanism suggests that semiquinones are not released from the enzyme active site. The one-electron reduction of quinones results in the formation of semiquinones (110), which are highly reactive species. Semiquinones contribute to oxidative stress (117, 197). Furthermore, quinones may behave as electrophiles, reacting with proteins and nucleic acids (34, 210) and contributing to the depletion of cellular glutathione (185). Thus, this enzyme may protect the cells from semiquinone- and quinone-associated toxicity. In mammalian systems, DT-diaphorase catalyzes the NAD(P)H-dependent two-electron reduction of a variety of quinones to hydroquinones, a reaction which apparently provides protection against quinone- and semiquinone-associated toxicity (147, 210).

The quinone reductase also catalyzes the one-electron reduction of ferricyanide. Addition of two equivalents of ferricyanide to the reduced enzyme regenerates the fully oxidized spectrum. In the presence of one equivalent of ferricyanide, transient enzyme species are not detected at long wavelengths, suggesting that the semiquinone form of the enzyme is not produced. Thus, the addition of one equivalent of ferricyanide to the reduced enzyme apparently results in the oxidation of 50% of the enzyme. The absence of a detectable semiquinone form of the enzyme recently has been reported for DT-diaphorase (212). The stoichiometry for ferricyanide reduction versus NADH oxidation is 2:1, and the ratio of the rate of ferricyanide reduction to NADH oxidation is $\sim$1.8:1. Under the conditions used, the enzyme does not reduce Fe^{3+}-EDTA, Fe^{3+}-citrate, or Fe^{3+}-ADP complexes (data not shown). In the presence of DMBQ and other quinones, the enzymatic reduction of ferricyanide is stimulated. In this case, the enzyme apparently produces the corresponding hydroquinone, which reduces the ferricyanide.

Recent reports on the response of prokaryotes to oxidative stress suggest that a flavoprotein diaphorase may catalyze the NADPH-dependent reduction of proteins involved in the regulation of gene expression (149). To determine if the *P. chrysosporium* quinone reductase can transfer electrons to proteins, we monitored the reduction of ferricytochrome *c* by this enzyme. Ferricytochrome *c* reduction by *P. chrysosporium* quinone reductase proceeds slowly; however, the reaction is stimulated by the addition of DMBQ. It has been reported that 1,4-dihydroxynapthalene, but not 1,4-benzoquinone, mediates ferricytochrome *c* reduction by DT-diaphorase (59). We confirm that 1,4-dihydroxynapthalene, but not 1,4-benzoquinone, can mediate the quinone reductase-catalyzed reduction of ferricytochrome *c*. In addition, our results show that methoxyquinones stimulate the reduction of ferricytochrome *c* by *P. chrysosporium* quinone reductase. Conducting the reactions under anaerobic conditions did not affect the quinone stimulation of this reaction, suggesting that superoxide is not involved. If quinone reduction is allowed to proceed to completion before ferricytochrome *c* is added, rapid reduction of ferricytochrome *c* still occurs, suggesting that the stimulation observed with quinones is due to the nonenzymatic reduction of ferricytochrome *c* by the enzyme-generated hydroquinone products. The results shown in Table 4.3 confirm that a variety of hydroquinones are able to reduce ferricyanide and ferricytochrome *c* in the absence of enzyme.

One possible physiological role for this *P. chrysosporium* quinone reductase is to maintain hydroxylated aromatics in their reduced (hydroquinone) form. Hydroxylated aromatics are substrates for aromatic ring-cleaving dioxygenases (31, 183). In addition, a variety of methoxylated aromatics are lignin peroxidase substrates (127, 160), and 1,2,4-trihydroxybenzene–the product of 2-methoxyhydroquinone demethylation–has been identified as a key metabolite in several degradative *P. chrysosporium* pathways (26, 183, 216, 217, 219). Thus, this quinone reductase may play an important role in the degradation of these compounds by maintaining intermediates in the reduced state.

The nonenzymatic reduction of chloroquinones by protein-free cell extracts raises the possibility that an enzyme may not be required to reduce these compounds

in vivo. In contrast, the reduction of 1,4-benzoquinone and methoxybenzoquinones requires an enzyme.

Dicumarol and Cibacron blue are efficient inhibitors of DMBQ reduction by quinone reductase. As has been observed for DT-diaphorase (60, 146, 175), inhibition is competitive with respect to NADH. Early reports suggested that oxidized DT-diaphorase possessed a separate dicumarol binding site (105) and that occupation of this site by dicumarol results in inhibition of NADPH binding. More recent kinetic evidence suggests that the binding of dicumarol results in a lowered redox potential for the oxidized enzyme rather than the inhibition of NADH binding (212). Inhibition of flavoproteins by Cibacron blue has been interpreted to indicate either the presence of an ATP binding site (12) or an NAD^+ binding site (204). The low K_i values for dicumarol and Cibacron blue suggest that these compounds might be useful as inhibitors *in vivo*.

We reinvestigated the FMN content of quinone reductase by using a recently published reconstitution protocol (211). Previously, we calculated a flavin content of one FMN per dimer using spectral methods. However, the results in Figure 4.7 suggest that the quinone reductase requires two molecules of FMN per dimer for complete reactivation of the apoenzyme. This suggests that the enzyme contains one tightly bound FMN and one more loosely bound FMN, and that only the apoenzyme can be fully reconstituted. The flavin content of DT-diaphorase is also two flavins/dimer (59).

A recent report claims that a membrane-bound enzyme plays a critical role in quinone reduction by *P. chrysosporium* (203). In that study, 2-methoxy-1,4-benzoquinone was reduced at a rate of 13 nmoles min^{-1} mg^{-1} by washed *P. chrysosporium* mycelia. In contrast, the rate of 2-methoxy-1,4-benzoquinone reduction by our purified quinone reductase is 2.17×10^5 nmoles min^{-1} mg^{-1}, and the rate with the crude extract from induced cells is 1750 nmoles min^{-1} mg^{-1} (14). These rates are 1.7×10^4 and 1.35×10^2 times faster, respectively, than that reported for the membrane-bound activity (203). It also was reported that a crude intracellular extract exhibited a K_m of 150 μM for 1,4-benzoquinone reduction (203). In contrast, using our purified enzyme, the K_m for the physiological substrate 2-

methoxy-1,4-benzoquinone was determined to be 2.4 μM. Thus, our results suggest that this intracellular enzyme is largely responsible for the reduction of 2-methoxy-1,4-benzoquinone and DMBQ. The rapid induction of intracellular quinone reductase activity in response to exogenous quinones and quinone precursors (14, 41) confirms the critical physiological role of this intracellular enzyme in methoxyquinone metabolism.

The recent report of Stahl et al. (203) also claims that 1,4-benzoquinone reduction by *P. chrysosporium* cells is inhibited competitively by ferricyanide. Since ferricyanide cannot enter the cells, this was used as the basis for the proposal that quinones are being reduced by a membrane-bound enzyme (203). In contrast, our results indicate that 1,4-hydroquinone and 2-methoxy-1,4-hydroquinone reduce ferricyanide in the absence of enzyme. This strongly suggests that ferricyanide does not competitively inhibit a membrane-bound enzyme as claimed (203), but rather the quinone is being converted to a hydroquinone which is reoxidized in the presence of ferricyanide. Finally, the chloroquinones reported to be reduced by the membrane-bound enzyme activity (203) are very strong oxidants. In Table 4.1, we show that they are readily reduced by NADH in the absence of enzyme. In addition, chloroquinones are reduced by protein-free filtrates of the cytosolic fraction. Thus, an enzyme-catalyzed reaction may not be required for their reduction *in vivo*.

This quinone reductase may have several metabolic and other physiological functions. One likely role is in the metabolism of vanillic acid which is an important metabolite extracted from *P. chrysosporium*-degraded wood (7, 39). Vanillic acid is oxidatively decarboxylated to methoxyhydroquinone by an intracellular vanillate hydroxylase (26, 231). The quinone reductase would maintain this 2-methoxyhydroquinone in the reduced form as has been previously suggested (14, 31). Methoxyhydroquinone is probably a substrate for an as-yet-unidentified demethylating enzyme. The latter enzyme would generate 1,2,4-trihydroxybenzene which is ring-cleaved by THB dioxygenase (183). The fact that vanillic acid is a strong inducer of quinone reductase activity (14, 31, 41) suggests that the quinone reductase plays an important role in its metabolism. The reduction of lignin- and pollutant-derived quinones such as 2-methoxy-1,4-benzoquinone and DMBQ to hydroquinones by this

enzyme strongly suggests that the quinone reductase also plays a role in lignin degradation. In addition, our results suggest the enzyme protects cells against quinone and semiquinone toxicity. The latter role is similar to that proposed for DT-diaphorase. Finally, its ability to reduce Cc^{3+}, albeit at slow *in vitro* rates, suggests other possible functions for this enzyme. In this regard, we are continuing to study the mechanism, regulation, and physiological role of this enzyme.

CHAPTER 5

Regulation of an Intracellular NAD(P)H-Dependent Quinone Reductase from the White-Rot Fungus *Phanerochaete chrysosporium*

5.1 Introduction

The wood-rotting basidiomycete *Phanerochaete chrysosporium* is capable of degrading polymeric lignin (33, 78, 131) and a variety of aromatic pollutants (24, 79, 94). During secondary metabolic (idiophasic) growth, *P. chrysosporium* secretes two families of extracellular peroxidases lignin peroxidase (LiP) and manganese peroxidase (MnP) (76, 130, 137, 142, 214). These enzymes along with an H_2O_2-generating system (120) catalyze the initial steps in the depolymerization of lignin, generating a wide variety of metabolic intermediates, including substituted quinones, hydroquinones, benzaldehydes, and ring-opened fragments (33, 57, 131, 192, 215, 229).

The recent elucidation of metabolic pathways for the degradation of aromatic pollutants has led to the identification of metabolites and has suggested roles for intracellular enzymes in these pathways (115, 216, 217, 219). Substituted quinones are key metabolites in these pathways. Quinones produced by the action of extracellular and intracellular enzymes are reduced by two electrons to hydroquinones by an intracellular quinone reductase. Hydroquinones are metabolized further by oxidation and subsequent aromatic ring cleavage. Both the dioxygenase and quinone reductase have been purified and characterized.

Expression of *P. chrysosporium* extracellular enzymes has been characterized in some detail. The expression of LiP is regulated by nutrient nitrogen, carbon, or sulfur depletion (8, 35, 145). To date, induction of LiP gene expression by exogenous agents or environmental stimuli has been not been observed. In contrast,

the regulation of expression of MnP has been characterized in some detail. Manganese peroxidase genes are expressed in response to nitrogen depletion (176). In addition, they are regulated by manganese (17, 18) chemical and oxidative stress (144), and heat shock (19, 77).

Little is known concerning the expression of intracellular enzymes in *P. chrysosporium*. Several intracellular enzymes have been isolated and characterized from *P. chrysosporium* (30, 42, 44, 164, 167, 182, 183). However, only quinone reductase has been studied with respect to the regulation of its expression. Quinone reductase activity is induced by vanillic acid (14, 28, 41, 194) and 2-methoxy,1-4-benzoquinone (14). This enzyme is produced under sufficient nutrient nitrogen and glucose conditions, suggesting that it is regulated independently of the secondary metabolic lignin-degrading system. Herein, we have studied in more detail the regulation of the expression of the intracellular quinone reductase.

5.2 Materials and Methods

5.2.1 Culture conditions

Stock cultures of *P. chrysosporium* strain OGC101 (3), *Trametes versicolor* (L.:Fr.) Pilat (obtained from Forest Products Laboratory, Madison, WI), *Ceriporiopsis subvermaspora* (obtained from R. Blanchette, University of Minnesota), *Letinus tigrinus* (Bull.:Fr.) Fr. (obtained from F. Nerud, Czechoslovak Academy of Sciences), and *Dichomitus squalens* (P. Karst) Reid (obtained from F. Zadrazil), were maintained on malt agar slants as described (3). The organisms were grown from a conidial inoculum in stationary culture (50 ml/1-L flask). *P. chrysosporium* was grown for 2 days at 38°C, while *T. versicolor, C. subvermaspora, L. tigrinus*, and *D. squalens* were grown for 7 days at 28°C. The medium was as described elsewhere (132) and supplemented with 2% glucose, 1.2 mM ammonium tartrate, and 10 mM dimethyl succinate (pH 4.5). Mycelial mats from two flasks were homogenized for 20 seconds in a Waring blender and 10 ml aliquots were used to inoculate 250-ml Erlenmeyer flasks containing 100 ml of 20 mM sodium acetate (pH 4.5),

supplemented with 2% glucose, 12 mM ammonium tartrate (sufficient nitrogen) or 1.2 mM ammonium tartrate (limiting nitrogen). Cultures were incubated at 28°C on a rotary shaker (150 rpm). Vanillic acid was added to *P. chrysosporium*, *T. versicolor*, and *C. subvermaspora* cultures 72 h after inoculation or as described. *D. squalens* and *L. tigrinus* cultures required 6 and 8 days growth, respectively, before addition of inducer to the cultures. Unless otherwise described, cells were harvested 20 h after addition of inducer.

5.2.2 Preparation of enzyme extracts

Cells were harvested by filtration, washed in ice-cold distilled water, vacuum filtered through Miracloth, and stored at -80°C. All subsequent steps were carried out at 4°C. Frozen cells (0.2 grams) were broken in 1.5 ml Sarstadt tubes containing 1.0 gram glass beads and 1.0 ml extraction buffer. Extraction buffer consisted of 50 mM sodium phosphate (pH 7.0), containing 1 mM EDTA, and 0.004% phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 17,300 $\times$ *g* for 20 min.

5.2.3 Activity and protein assays

Quinone reductase activity was determined by following the oxidation of NADH at 340 nm. Standard reaction mixtures (1 ml) contained 50 mM sodium citrate buffer (pH 6.0), 100 μM 2,6-dimethoxy-1,4-benzoquinone (DMBQ), and enzyme. Reactions were initiated by the addition of 200 μM NADH. Enzyme assays were carried out at room temperature using a Shimadzu UV-260 spectrophotometer. Protein concentration was measured using the BCA method (199), using bovine serum albumin as a standard.

5.2.4 Slab electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude cell extracts was carried out as described (138). Rabbit polyclonal antibody was raised against purified quinone reductase as described (14). Nitrocellulose transfers were carried out using a Bio-Rad transfer apparatus as described in (18), and bands were

detected by the 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium assay (141).

5.3 Results

5.3.1 Constitutive activity and induction window

A time course for constitutive quinone reductase activity (day 2 through day 6) in primary (HCHN) and secondary (HCLN) metabolic cells is shown in Figure 5.1. Cultures grown in the absence of inducer had low levels of activity throughout the course of the experiment. The addition of 2 mM vanillic acid to HCHN cultures results in a 35-fold increase in activity on days 2 and 4, while the increase in activity drops to 15-fold when cultures are induced on day 6. In HCLN cells, a 10-fold increase in activity is observed when cultures are induced on day 2, and then the increase falls to 5 and 2 fold when cells are induced on days 4 and 6, respectively. After 24 h of growth, very little mycelium is available for induction and addition of an inducer at that early stage of growth may be deleterious to the cells. Induction of 2-day-old HCHN cells yields almost twice as much quinone reductase as induction of 2-day-old HCLN cultures. At all times, activity was greater in HCHN cells.

5.3.2 Time course of induction with vanillic acid and MBQ

As shown in Figure 5.2, vanillic acid and MBQ rapidly induce quinone reductase activity in primary metabolic cultures. With both inducers, an increase in activity is observed after 2 h. The activity of vanillic acid-induced cells increased linearly up to 12 h, and remained constant between 12 and 16 h. A slight decline in activity was observed after 16 h. In contrast, maximum activity in MBQ induced cells was reached 4 h after addition of MBQ to the cultures. Activity decreased from 4-12 h and leveled off between 12-24 h after the addition of MBQ.

5.3.3 Dependence of induction on vanillic acid concentration

As shown in Figure 5.3, induction by vanillic acid is concentration dependent. A small increase in activity is observed in cultures induced with 0.1 mM vanillic acid.

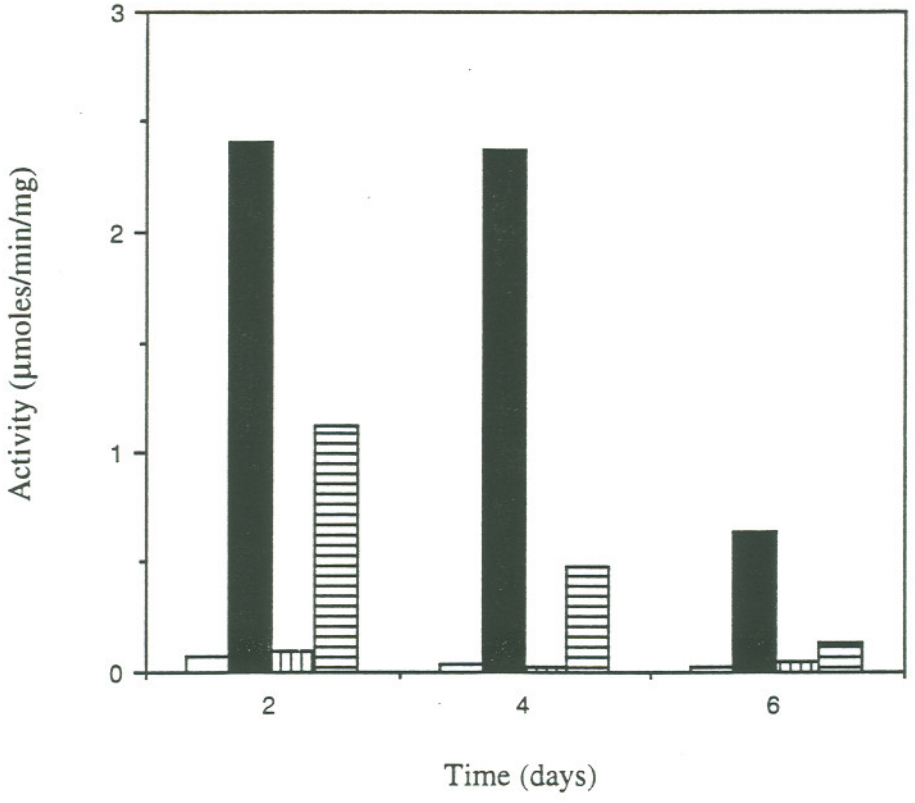

Figure 5.1 Constitutive activity and time course of inducibility for quinone reductase in primary (HCHN) and secondary (HCLN) metabolic cells. Inducers were added to HCHN and HCLN cultures on days 2, 4, and 6, and cells were harvested 20 h after addition of inducer. HCHN control (clear bars); HCHN induced (solid bars); HCLN control (vertical stripes); HCLN induced (horizontal stripes).

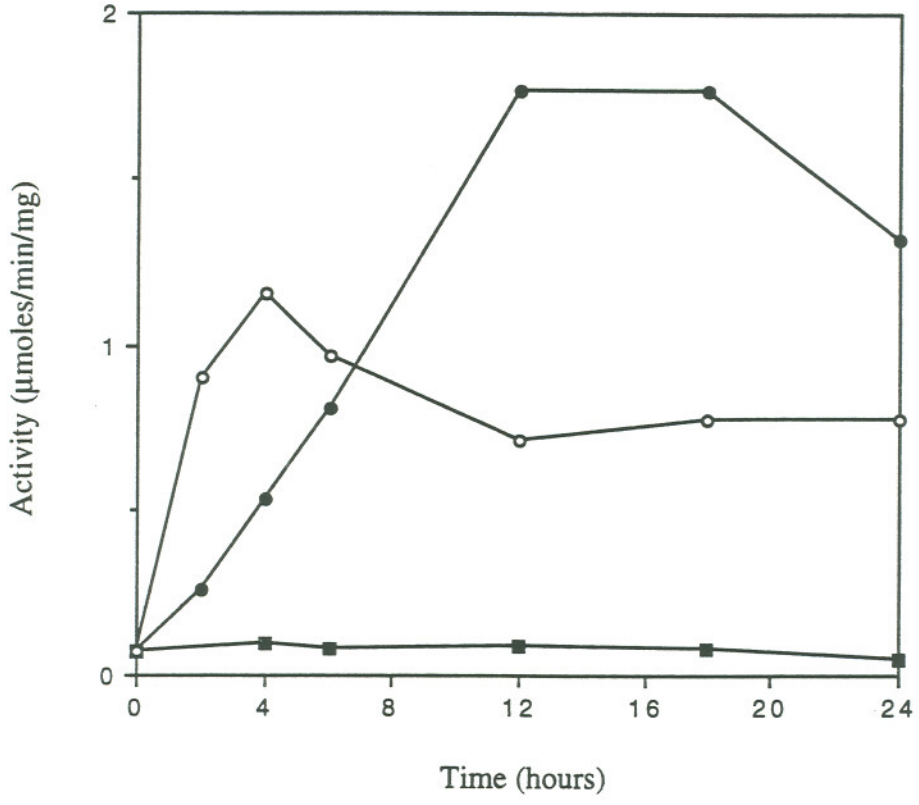

Figure 5.2 Time dependence of the induction of quinone reductase. Vanillic acid (●) or MBQ (○) were added to 2-day-old cells. Cells were harvested for activity assays at 0, 2, 4, 6, 12, 18, and 24 h after addition of inducer. Uninduced cells (■) were collected at 0, 4, 6, 12, 18 and 24 h.

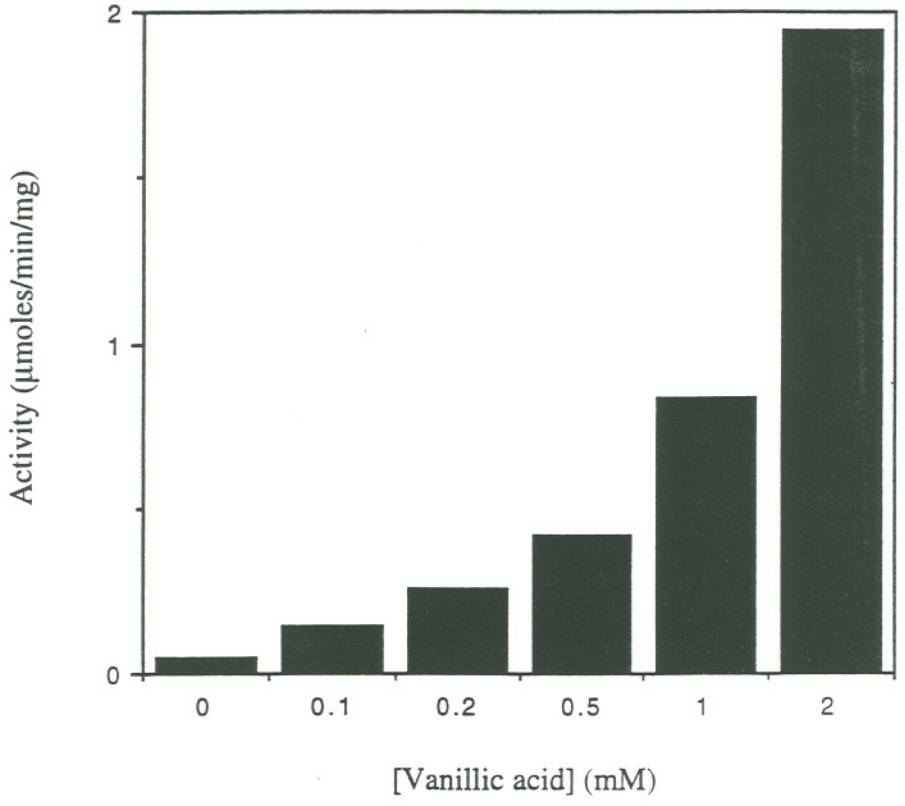

Figure 5.3 Dependence of induction ratio on the concentration of vanillic acid. The indicated concentrations of vanillic acid were added to 2-day-old cells, and the cells were harvested and assayed as described in Materials and Methods.

Induction of expression increases with increasing vanillic acid concentration up to a maximum of 2 mM. Vanillic acid concentrations above 2 mM were toxic to cells as determined by visual observation of the cultures. Induction by MBQ is also concentration dependent in the range of 0.01 to 0.2 mM; concentrations above 0.2 mM were toxic to the cells (data not shown).

5.3.4 Immumoblot analysis of induction

Immunoblot analysis of control and induced cell extracts was performed to confirm that we were observing an increase in quinone reductase protein rather than stabilization or activation of enzyme. Figure 5.4 demonstrates that the increase in activity is associated with an increase in quinone reductase protein. Both time- and concentration-dependent increases in quinone reductase protein were detected in induced cultures. The addition of vanillic acid to purified quinone reductase has no effect on its activity.

5.3.5 Compounds that induce quinone reductase

Table 5.1 shows the induction of enzyme activity by a wide variety of compounds. Because of the variability in induction experiments, the fold induction values in parentheses are only meant to represent general trends and not absolute values. In general, the most effective inducers are the lignin-related compounds vanillic acid and ferulic acid. A trend is observed with vanillic acid-related compounds. The two-electron reduced form of vanillic acid, vanillyl aldehyde, is a much weaker inducer than the acid, and no induction is observed with vanillyl alcohol. Veratric acid, which differs from vanillic acid by a methylation of the 4-hydroxy group, is a weak inducer. Several quinones and their respective hydroquinones are equally efficient as inducers. A large number of chlorinated aromatic and nitroaromatic compounds were screened for their ability to induce activity. Only the simple compounds, 4-chlorophenol and 2-nitrophenol, caused any significant induction. The oxidative stress-inducing agents paraquat and H_2O_2 did not induce quinone reductase activity. Shifting primary metabolic cells from 28°C to 45°C for 20 h resulted in relatively weak (2 fold) induction (data not shown).

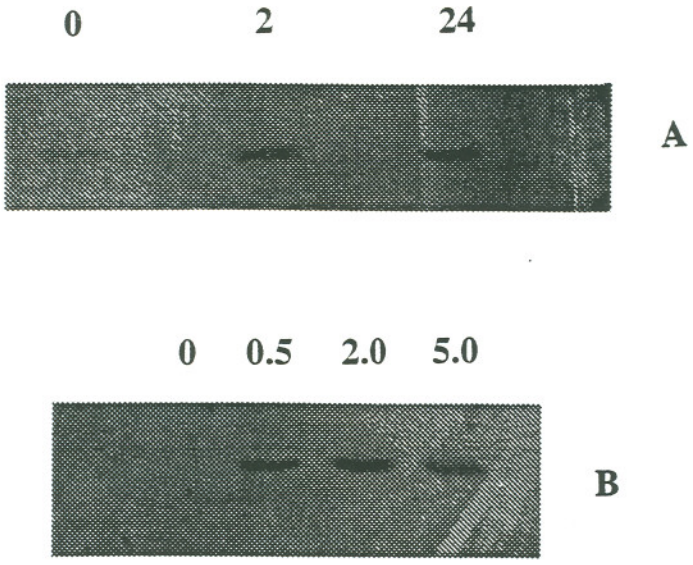

Figure 5.4 Immunoblot analysis of vanillic acid-induced cells. (A) 2-day-old cells were induced with 2 mM vanillic acid and harvested 0, 2, and 24 hours after vanillic acid addition. (B) 2-day-old cells were induced with 0, 0.5, 2.0, and 5.0 mM vanillic acid and harvested 20 h after the addition. Crude cell extracts were separated on SDS-PAGE, transferred to nitrocellulose, and immunodetected as described in Materials and Methods.

Table 5.1

Inducers of Quinone Reductase

Inducer	Concentration (mM)	Induction (fold)
quinones		
p-benzoquinone	0.2	12
2-methoxybenzoquinone	0.2	12
2,6-dimethoxybenzoquinone	0.2	12
1,4-Napthaquinone	0.2	10
3,5-di-tert-butyl-1,2-orthoquinone	0.2	1.3
2-chloroquinone	0.2	2.2
hydroquinones		
p-hydroquinone	0.2	6.8
2-methoxyhydroquinone	0.2	11
2,6-dimethoxyhydroquinone	0.2	11
aromatic acids		
benzoic acid	2.0	0
p-hydroxybenzoic acid	2.0	8.8
vanillic acid	2.0	35
3,4-dihydroxybenzoic acid	2.0	1.4
3,5-dihydroxybenzoic acid	2.0	0
syringic acid	2.0	1.3
ferulic acid	2.0	28
veratric acid	2.0	4.0
aromatic aldehyde		
vanillyl aldehyde	2.0	15
aromatic alcohols		
veratry alcohol	2.0	0
vanillyl alcohol	2.0	0
phenols		
phenol	0.2	0
4-chlorophenol	0.2	7.3
2,4-dichlorophenol	0.2	0
2,6-dichlorophenol	0.2	2.2
2,3-dichlorophenol	0.2	2.0
3,4-dichlorophenol	0.2	1.9
3,5-dichlorophenol	0.2	0
2,4,5-trichlorophenol	0.2	1.9
2,4,6-trichlorophenol	0.2	0
pentachlorophenol	0.2	0
nitroaromatics		
nitrobenzene	0.2	0
2-nitrophenol	0.2	5.7
4-nitrophenol	0.2	1.8
2,4-dinitrobenzoic acid	0.2	0
3,5-dinitrobenzoic acid	0.2	0
2,4-dinitrotoluene	0.2	0
2,6-dinitrotoluene	0.2	0
4-nitrocatechol	0.2	0
2,4,6-trinitrotoluene	0.2	2.2

5.3.6 Inhibition of induction by benzenesulfinic acid

Induction by the diverse group of compounds listed in Table 5.1 suggests that induction is nonspecific, or these compounds are undergoing metabolic conversion to a common inducer. Many of these compounds can undergo metabolic conversion to a quinone, suggesting that quinones may be the inducing species. This was tested by carrying out the induction in the presence of a quinone-complexing agent. Benzenesulfinic acid has been reported to complex quinones (223). As demonstrated in Table 5.2, induction by both vanillic acid and MBQ is reduced 81 and 75%, respectively, in the presence of benzenesulfinic acid. The addition of benzenesulfinic acid to cultures had no effect on constitutive activity (data not shown).

5.3.7 Induction of quinone reductase in other white-rot fungi

As shown in Table 5.3, several species of white-rot fungi exhibited vanillic acid-inducible quinone reductase activity. *L. tigrinus* and *D. squalens* have relatively high levels of constitutive quinone reductase activity. The lowest constitutive activity as well as the most dramatic induction was observed with *P. chrysosporium*.

5.4 Discussion

Lignin and pollutant degradation by the white-rot fungus *P. chrysosporium* is a secondary metabolic process that requires both extracellular and intracellular enzymes. The extracellular peroxidases that catalyze the initial oxidative steps in biodegradation

are produced only under nutrient nitrogen-deficient conditions (145). However, the expression of quinone reductase does not appear to be restricted to secondary metabolic growth. The results in Figure 5.1 show that low levels of quinone reductase are produced constitutively during primary and secondary growth. The low levels of quinone reductase produced during secondary metabolic growth may be sufficient to reduce any quinones derived from lignin metabolism. In early reports, it was claimed that intracellular quinone reductase activity is induced by vanillic acid in

Table 5.2

Effect of Bezenesulfinic Acid on the Induction of Quinone Reductase Activity

Sample	Activity[a]	Induction (fold)
control	0.34	1.0
vanillic acid	12	35
2-methoxy-1,4 benzoquinone	4.1	12
vanillic acid + benzenesulfinic acid	2.3	6.8
2-methoxy-1,4-benzoquinone + benzenesulfinic acid	1.0	2.9

[a] μmoles NADH min^{-1} mg^{-1}. HCHN, 2-day-old cultures were induced with 2 mM vanillic acid or 0.2 mM MBQ in the presence and absence of 1 mM benzenesulfinic acid. Cells were harvested 20 h after addition of inducer, and crude cell extracts were assayed as described in the text.

Table 5.3

Induction of Quinone Reductase in Several White-Rot Fungi

	μmoles/min/mg		
	control	induced	fold induction
Phanerochaete chrysosporium	0.08	2.1	26
Ceriporiopsis subvermaspora	0.16	3.0	18
Coriolus versicolor	0.10	1.5	15
Letinus tigrinus	0.77	4.6	6.0
Dichomitus squalens	0.53	3.5	6.6

HCHN, 100-ml agitated cultures were grown as described in Materials and Methods. Cultures were induced with 2 mM vanillic acid and harvested 20 h after addition of inducer. Cells were homogenized and extracts were assayed as described in the text.

secondary metabolic cultures (31, 41). However, the results in Figure 5.1 suggest that induction is favored during primary metabolic growth.

Rapid induction by vanillic acid and MBQ suggests a role for quinone reductase in the metabolism of these two compounds. The first step in the catabolism of vanillate is an oxidative decarboxylation catalyzed by an intracellular vanillate hydroxylase (26, 30, 231) to generate 2-methoxyhydroquinone. 2-Methoxyhydroquinone undergoes autooxidation or possibly oxidation by an intracellular phenol oxidase to yield the MBQ. As shown in Table 5.1, vanillic acid induces quinone reductase activity. MBQ also has been identified as a product of the oxidation of veratryl alcohol (192) and the degradation of aromatic pollutants by *P. chrysosporium* (216, 217). Therefore, quinone reductase activity could be induced during lignin and aromatic pollutant degradation owing to the formation of MBQ.

Ferulic acid (3-methoxy,4-hydroxycinnamic acid) also is a main product from the degradation of guaiacyl lignin by white rot fungi (108), and degradation of ferulic acid by *P. chrysosporium* has been studied (52-54, 86). The main products identified were coniferyl alcohol, coniferyl aldehyde, dihydroferulic acid, and dihydroconiferyl alcohol. Small amounts of vanillyl alcohol, vanillic acid, and methoxyhydroquinone also were formed. The latter two metabolites can induce quinone reductase activity. The induction of quinone reductase activity by ferulic and vanillic acid suggests that metabolism of these two lignin-derived compounds is occurring rapidly in primary metabolic cells. Therefore, the expression of enzymes involved in the intracellular metabolism of low molecular weight, lignin-derived fragments may not be restricted to secondary metabolic growth.

The developmental state of the cells is important for maximal induction. The highest induction was observed in 48-hour-old HCHN and HCLN cultures, and the level of induction decreased dramatically in older cells. This may be a characteristic of the cell membrane, rather than an intracellular metabolic process. Younger cells may be more permeable to inducers.

An increase in quinone reductase activity is associated with an increase in expression of quinone reductase protein. With vanillic acid, a time- and concentration-dependent increase in quinone reductase protein is observed. The

results presented in Figure 5.4 suggests that the induction of *P. chrysosporium* quinone reductase is regulated at the level of protein expression and that an increase in activity probably is not due to stabilization or activation of preformed enzyme. Thus, the mechanism of induction of quinone reductase in *P. chrysosporium* may be similar to that of DT-diaphorase in mammalian cells (46, 64, 65, 111).

The variety of inducers seen in Table 5.1 suggests that structural and redox properties of inducers may be important. The oxidized and reduced form of the various quinoid compounds are equally effective as inducers. This suggests that quinones and hydroquinones are rapidly interconverted or that both the oxidized and reduced forms are recognized by the regulatory system. All compounds tested that contained a 3-methoxy-4-hydroxy substitution pattern, with the exception of vanillyl alcohol, are good inducers. This suggests that the substitution pattern on the benzene ring and the oxidation state of the benzylic carbon may be important for induction. The 3,4-dimethoxy-substituted compound, veratryl alcohol did not induce, and veratric acid was a weak inducer. Therefore, regardless of the oxidation state of the benzylic carbon on 3,4-dimethoxy-substituted compounds, these lignin-derived compounds are not effective inducers. This suggests that the immediate product of veratryl alcohol oxidation, veratryl aldehyde (214), does not induce quinone reductase activity.

Induction by the compounds listed in Table 5.1 might indicate the ability of the fungus to convert these compounds to paraquinones during primary metabolism. Inhibition of induction by benzenesulfinic acid suggests that quinones are the actual inducers. Weak induction by the xenobiotics 2-nitrophenol and 4-chlorophenol suggests metabolic conversion of these compounds to quinones. Induction with 2-nitrophenol and 4-chlorophenol was relatively higher than that observed with the more highly substituted phenols, suggesting that the degree of substitution may affect the inducing properties of these classes of compounds. 2-Nitrophenol and 4-chlorophenol may act as weak substrates for vanillate hydroxylase which could convert these compounds to substituted hydroquinones or quinones. 1,4-Benzoquinone is produced from 4-nitrophenol metabolism in *Moraxella* sp., which contains an inducible quinone reductase (202).

Induction reached a maximum 4 h and 12 h after addition of MBQ and vanillic acid, respectively (Figure 5.2). This difference between peak induction could represent the time required to convert vanillic acid to the quinone, i.e., compounds that do not require metabolic conversion induce more rapidly. A pathway for ferulic and vanillic acid induction is proposed in Figure 5.5. Ferulic and vanillic acid are taken up by the cell where they are converted to methoxyhydroquinone. The reactions involved in the conversion of ferulic to vanillic acid have been described (54). Methoxyhydroquinone might either induce by itself or undergo oxidation to the quinone which is an inducing species. Although the results presented in Table 5.1 demonstrate that the methoxyhydroquinone can induce, the inhibition of induction by benzenesulfinic acid suggests that the methoxyquinone is responsible for induction *in vivo*.

Induction of enzymes by quinones has been suggested to indicate a toxic response (46, 208, 209). It is clear that quinones are efficient inducers of quinone reductase activity in *P. chrysosporium*, but evidence for toxicity or oxidative stress in this organism is not available. The oxidative stress agents paraquat and H_2O_2 did not induce quinone reductase activity (data not shown). Quinone-related oxidative stress could presumably occur in secondary metabolic cells during lignin degradation. If the lignin-derived quinones are not rapidly metabolized, they could accumulate to toxic levels in the cells. The results of Figure 5.1 suggest that quinone reductase activity is weakly induced in secondary metabolic cells.

The existence of inducible quinone reductase activity in other white-rot fungi suggests a conserved pathway of vanillic acid and quinone metabolism. Vanillic acid is a key intermediate in the degradation of lignin by white-rot fungi (39). White-rot fungi produce unique combinations of extracellular enzymes, yet these organisms degrade lignin and produce vanillic acid as an intermediate. For example, *D. squalens* produces laccase and MnP, but no LiP. The *P. chrysosporium* extracellular enzymes do not appear to be required for the catabolism of vanillic acid. It is therefore possible that the intracellular metabolic pathways for the degradation of lignin, including reduction of quinones by an intracellular quinone reductase, are similar among different species of white-rot fungi. This conservation of metabolism of low molecular weight lignin degradation products among white-rot fungi suggests

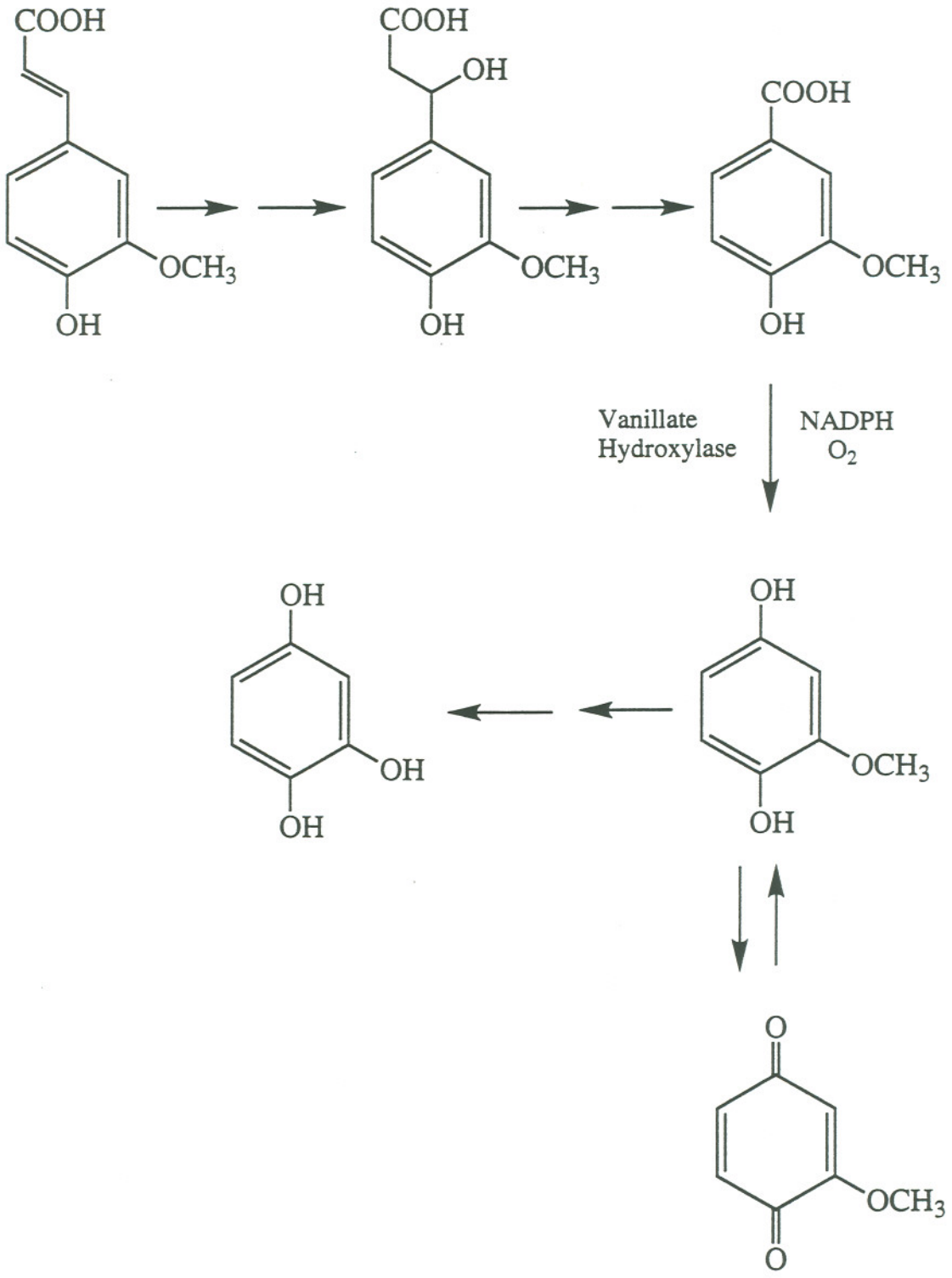

Figure 5.5 A proposed pathway for ferulic and vanillic acid metabolism in *P. chrysosporium*.

that other white-rot fungi may also have the capability to degrade environmental pollutants.

CHAPTER 6
Final Comments

6.1 Summary

Under ligninolytic conditions the white-rot basidiomycete *Phanerochaete chrysosporium* mineralized 2,4-dinitrotoluene (DNT). The DNT degradative pathway was elucidated by the characterization of fungal metabolites and oxidation products generated by lignin peroxidase (LiP), manganese peroxidase (MnP), and crude intracellular cell-free extracts.

One of the intracellular enzymes involved in the pathway, a 1,4-benzoquinone reductase, was purified from agitated cultures of *P. chrysosporium* and characterized. The mechanism of the quinone reductase was investigated by spectral and kinetic methods. The reduction of a variety of substituted quinones, THB autooxidation products, ferricyanide, and ferricytochrome *c* suggests that the enzyme is nonspecific and could be involved in a variety of physiological reactions.

The regulation of quinone reductase expression also was investigated. The enzyme is constitutively expressed under both primary and secondary metabolic conditions. Under primary metabolic growth conditions, activity is induced by a variety of substituted benzoic acids, quinones, and hydroquinones. This increase in activity is associated with an increase in quinone reductase protein, suggesting that these inducers are regulating gene expression.

6.2 Future Directions

6.2.1 Nitroaromatic degradation by *P. chrysosporium*

Secondary metabolic cultures of *P. chrysosporium* degrade 2,4-dinitrotoluene to CO_2 and H_2O. This strongly suggests a role for the lignin degrading system, including LiP, MnP, and the H_2O_2-generating system in the degradation of nitroaromatics. The results from this thesis suggest that intracellular enzymes are required for the complete degradation of these compounds. Future research should include characterization of the intracellular enzymes, including the nitroreductase, quinone reductase, dioxygenase, and a possible demethylase. Furthermore, quinone and aromatic acid uptake systems should be identified.

The first step in degradation of nitroaromatic compounds by *P. chrysosporium* apparently is reduction of the nitro group to the corresponding amine. The intracellular location of this enzyme is in dispute. Both a solubilized enzyme and a membrane potential have been implicated in this reaction. Further work needs to be done to clarify the reductive mechanism. The solubilized enzyme should be purified in sufficient quantities for biochemical analysis. A purification protocol could be developed based on previous purifications of *P. chrysosporium* intracellular enzymes. Optimization of growth conditions that promote the production of high yields of enzyme should be established. A variety of compounds could be screened for their ability to induce nitroreductase activity in both primary and secondary metabolic cultures.

6.2.2 Quinone reductase mechanism and structure activity studies

Fundamental, steady-state mechanistic studies on the *P. chrysosporium* quinone reductase have been performed as reported in Chapters 3 and 4 of this thesis. However, more detailed studies are required to understand the role of this enzyme in lignin and pollutant degradation by *P. chrysosporium*. Measuring activity in the presence of FMN and NAD(P)H analogs would be a simple method to probe the active site of the enzyme. Sufficient quantities of enzyme can now be prepared to perform more sophisticated experiments. The enzyme is an excellent candidate for

transient-state kinetic studies. Stop flow, rapid kinetic experiments performed in combination with quenching techniques would aid in the identification of catalytic intermediates.

Another important area for future study is the elucidation of the enzyme structure by crystallographic and solution methods. A crystal structure of quinone reductase is essential for the identification of important amino residues in the active site. Subsequent to elucidation of the crystal structure, site-directed mutagenesis could be used to modify or improve the substrate specificity and catalytic efficiency of the enzyme. These techniques are currently being carried out with recombinant DT-diaphorase to determine what features allows this enzyme to utilize both NADH and NADPH equally efficiently as electron donors. Using site-directed mutagenesis, the substrate specificity of *P. chrysosporium* quinone reductase might be broadened to include other electron acceptors such as nitroaromatic compounds. Solution NMR studies of the enzyme could be used to analyze the active site environment.

6.2.3 Elucidation of the physiological role of *P. chrysosporium* quinone reductase

Much of the previous research concerning lignin and pollutant degradation by *P. chrysosporium* has focused on the extracellular enzymes LiP and MnP. Indeed, the discovery of these enzymes in the early 1980s represented the most significant advance in understanding how *P. chrysosporium* degrades lignin, and the characterization of these enzymes continues to be a critical area of research. Intracellular enzymes, such as the quinone reductase, may also catalyze essential steps in the degradation of recalcitrant substances by *P. chrysosporium*. Determining the physiological role of this enzyme will require the selective inhibition of the enzyme or the creation of null mutants by reverse genetic techniques. As described in Chapters 3 and 4, the inhibitors dicumarol and Cibacron blue could be used as possible *in vivo* inhibitors. Biodegradation experiments in the presence and absence of the quinone reductase inhibitors could be performed to monitor the rate of substrate disappearance and mineralization, or accumulation of quinone metabolites. These experiments could be carried out with synthetic lignin, lignin model compounds, and aromatic pollutants.

Null mutants created by reverse genetic techniques would also aid in determining the physiological role of the enzyme. This technique will, however, depend on the successful cloning of the quinone reductase gene. Disruption of a single copy gene in *P. chrysosporium* has been demonstrated in our laboratory. However, inhibition of expression of the extracellular peroxidases has proved difficult due to the presence of multiple related genes. The results presented in Chapter 3 suggest that only one predominant quinone reductase is produced in vanillic acid-induced, primary metabolic cultures. Therefore, it is possible that quinone reductase is encoded by a single gene, making it amenable to gene disruption experiments.

6.2.4 Regulation of expression of quinone reductase

As demonstrated in Chapter 5, quinone reductase activity is induced to a high level. The mechanism of induction should be examined. Are the inducing compounds acting extracellularly or intracellularly? Inducers containing charged groups which might retard their entry into the cell could be screened for their ability to induce activity. Induction with compounds that do not enter the cell would suggest the involvement of a membrane-bound receptor and a transducing cascade.

The polyclonal antibodies that were used in Chapter 5 to observe expression of the quinone reductase protein, should be used to screen existing *P. chrysosporium* expression libraries for the quinone reductase mRNA. In addition, an expression library from primary metabolic, vanillic acid-induced cells could be constructed. The isolated message could be used as a probe in northern blotting experiments to determine if expression is regulated at the level of gene expression.

Isolation and characterization of the quinone reductase gene will provide important information for future experiments. cDNA probes constructed from the mRNA could be used to isolate the gene from a *P. chrysosporium* genomic library. Characterization of the isolated genomic DNA will allow for the identification of regulatory elements and lead to a better understanding of how the expression of the gene is regulated. One interesting question is how similar are the regulatory elements in the *P. chrysosporium* quinone reductase gene to those in the DT-diaphorase gene? If they are similar, this might indicate conserved mechanisms of quinone metabolism

in mammals and fungi. The promoter region also could be used to isolate intracellular transacting factors by gel-shift mobility assays. If found, transacting factors could be compared to the factor involved in transcriptional regulation of the DT-diaphorase gene. This regulatory system also could be responsible for the regulation of expression of other intracellular enzymes such as vanillate hydroxylase and methyl transferase.

Once characterized, the promoter region of the quinone reductase gene might be exploited to drive the expression of other proteins in *P. chrysosporium*. This would allow for the production of enzymes in primary metabolic cells. Thus, the expression of other *P. chrysosporium* enzymes such as LiP and MnP could be induced in primary metabolic cells by the addition of vanillic acid or an appropriate inducer. If levels of recombinant protein are as high as quinone reductase (10% total cellular protein), then large quantities of important enzymes could be produced for biochemical studies. Use of the quinone reductase promoter may have advantages over the glyceraldehyde-3-phosphate dehydrogenase promoter, which is currently being used in our laboratory, because the quinone reductase gene is specifically induced in the presence of vanillic acid. Furthermore, induction by vanillic acid or quinones in primary metabolic cells has a built-in control, i.e., the expression of the endogenous quinone reductase. Many of these ideas are being pursued in our laboratory, and other experiments are in the planning stages.

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BIOGRAPHICAL SKETCH

Barry J. Brock was born in Lawton, Oklahoma, on November 15, 1960. He attended Western State College of Colorado in Gunnison, CO, and in 1983 earned a Bachelor of Arts in Biology with a minor in Chemistry. In 1985, he earned a Master of Arts in Biology. Barry worked as a Research Associate in the Biochemistry Department of Michigan State University for two years and as a Senior Research Technician at the Biotechnology Center of Utah State University for two years. In 1989, Barry joined the laboratory of Professor Michael H. Gold in the Department of Chemistry, Biochemistry, and Molecular Biology at the Oregon Graduate Institute of Science & Technology from which he received his Ph.D. in Biochemistry. He then accepted a postdoctoral research position with Professor Michael R. Waterman, Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, TN. From 1992-1994, Barry served as an Adjunct Assistant Professor at the University of Portland.

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