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

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An intracellular, soluble 1,4-benzoquinone reductase was purified from agitated cultures of *Phanerochaete chrysosporium* and characterized. The quinone reductase was expressed in cultures grown under both nitrogen-sufficient and nitrogen-limiting (12 and 1.2 mM ammonium tartrate) conditions. The protein was purified to homogeneity by using ammonium sulfate fractionation, hydrophobic interaction, and ion-exchange and blue-agarose affinity chromatographies. The native flavin mononucleotide-containing protein, pI 4.3, has a molecular mass of 44 kDa as determined by gel filtration. The protein has a subunit molecular mass of ~22 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The quinone reductase exhibits a broad pH optimum between 5.0 and 6.5 and a temperature optimum of 30°C. The enzyme catalyzes the two-electron reduction of several quinones and other electron acceptors utilizing either NADH or NADPH as an electron donor. The apparent K_m for 2-methoxy-1,4-benzoquinone is 2.4 μM , and the apparent k_{cat} is $4.4 \times 10^5 \text{ s}^{-1}$. Enzyme activity is strongly inhibited by Cibacron blue 3GA and by dicumarol.

The wood-rotting basidiomycete *Phanerochaete chrysosporium* degrades lignin (8, 19, 30) and a variety of aromatic pollutants (3, 20, 23). During secondary metabolic (idiophasic) growth, *P. chrysosporium* secretes two extracellular heme peroxidases, lignin peroxidase and manganese peroxidase, which catalyze the initial steps in the depolymerization of lignin (18, 32, 42), generating a wide variety of metabolic intermediates, including substituted benzoquinones, benzaldehydes, aromatic acids, and ring-opened fragments (8, 13, 30, 39, 43, 47). Vanillate (4-hydroxy-3-methoxy-benzoic acid) is an abundant product of the fungal degradation of wood (2, 5, 10, 13). 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 (44), 2,4,5-trichlorophenol (29), and 2,7-dichlorodibenzo-*p*-dioxin (46). 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 (29, 38, 44–46). With regard to the latter activity, 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 (38).

Since quinones, which are generated by the extracellular peroxidase oxidation of both lignin and aromatic pollutants (29, 38, 39, 43–48), 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*.

MATERIALS AND METHODS

Culture conditions. Stock cultures of *P. chrysosporium* OGC101 (a derivative of strain BKM-F-1767 [unpublished observations]) were maintained on malt agar slants as described previously (1). The organism was grown from a conidial inoculum at 38°C in stationary culture (50 ml/1-liter flask) for 3 days (38). The medium was as described elsewhere (31) and supplemented with 2% glucose, 12 mM 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-liter flask containing 1 liter 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 was added to the cultures 72 h after inoculation.

Preparation of enzyme extracts. Cells (80 g) 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 and then transferred to a bead beater (BioSpec Inc.) and homogenized three times in 2-min bursts. Extraction buffer (700 ml) consisted of 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, and 0.004% phenylmethylsulfonyl fluoride. The homogenate was centrifuged at $17,300 \times g$ for 20 min, and the supernatant from the first spin was centrifuged at $105,000 \times g$ for 30 min.

Purification of the quinone reductase. The high-speed supernatant was fractionated by sequential additions of solid ammonium sulfate, with each addition being followed by centrifugation at $17,000 \times g$. The quinone reductase activity precipitated between 45 and 65% ammonium sulfate saturation and was redissolved in extraction buffer containing 20% glycerol.

(i) Phenyl Sepharose chromatography. A phenyl Sepharose column (6 by 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; this was followed by elution with a descending ammonium sulfate gradient (0.5 to 0 M in a total volume of 200 ml of 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 . After three to four uses, ethylene glycol was replaced with water. Active fractions were pooled and concentrated in an Amicon stirred cell by using a PM-10 membrane.

(ii) 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 to 125 mM in 25 min) at a flow rate of 1 ml/min. Active fractions were pooled and concentrated as described above.

(iii) Blue-agarose chromatography. The active fractions from the Mono Q

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TABLE 1. Constitutive and induced expression of quinone reductase activity during primary and secondary metabolic growth^a

Cells and culture conditions	Activity (U/ml) ^b	Protein concn (mg/ml)	Sp act (U/mg)	Induction (fold)
Primary metabolic cells				
Control	0.595	5.05	0.118	1.00
2-Methoxy- <i>p</i> -quinone	3.47	4.76	0.729	6.18
Vanillate	12.2	7.43	1.64	13.9
Secondary metabolic cells				
Control	0.600	3.39	0.177	1.00
2-Methoxy- <i>p</i> -quinone	0.600	1.93	0.311	1.76
Vanillate	1.00	4.02	0.249	1.41

^a Growth conditions are described in the text.^b 1 U = 1 μ mol of NAD(P)H oxidized min⁻¹.

column were concentrated and applied to a blue-agarose column (0.5 by 5 cm) equilibrated in buffer A containing 5 mM MgSO₄. 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.

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 μ M 2,6-dimethoxy-1,4-benzoquinone (2,6-DMBQ), and enzyme. Reactions were initiated by the addition of 200 μ M NADH unless otherwise indicated. Enzyme assays were carried out at room temperature with a Shimadzu UV-260 spectrophotometer.

Analytical methods. Protein concentration was measured by the bicinchoninic acid method (41), with bovine serum albumin as a standard. An extinction coefficient of 12.2 cm⁻¹ mM⁻¹ at 450 nm (16) 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 (11). Purity and subunit molecular weight were determined by analytical isoelectric focusing on a Pharmacia Phast system and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-PROTEAN; Bio Rad), respectively (33).

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 (16). 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% to 100% acetonitrile over 20 min with a flow rate of 1 ml/min (44).

Chemicals. Flavin standards, vanillic acid, 2-methyl-1,4-naphthoquinone (menadione), 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 (21). The purity of substrates was determined by silica gel thin-layer chromatography in CHCl₃-CH₃OH (99:1).

RESULTS

Expression of quinone reductase. As shown in Table 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 incu-

FIG. 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 (H₂O).

bation, respectively. The addition of vanillate or 2-MBQ 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 2-MBQ, 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.

Purification of quinone reductase. Table 2 summarizes the purification procedure from 80 g (wet weight) of HCHN, vanillate-induced, 92-h cells. The overall enzyme yield was 28%, with a concomitant 124-fold purification. The phenyl Sepharose chromatographic profile is shown in Fig. 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 (Fig. 2) and blue-agarose column (data not shown) chromatographies were performed immediately before the protein was required. Typically, 1 to 5 mg was purified in these steps. After blue-agarose chromatography the protein was more than 97% homogeneous by analytical isoelectric focusing (data not shown) and SDS-PAGE (see Fig. 4C, lane 2).

Characterization of quinone reductase. (i) Prosthetic group. The purified enzyme exhibited a typical oxidized flavin spectrum with visible maxima at 375 and 450 nm (Fig. 3). The flavin spectrum was sharply decreased upon reduction of the enzyme

TABLE 2. Purification of the quinone reductase

Step	Total vol (ml)	Protein concn (mg/ml)	Total protein (mg)	Total activity (U) ^a	Sp act (U/mg)	Yield (%)	Purification (fold)
Clarified extract	670	3.83	2,570	4,500	1.75	100	1
45-65% (NH ₄) ₂ SO ₄ fraction	48	28.2	1,350	4,340	3.22	96	1.8
Phenyl Sepharose	67.5	1.38	93.2	3,410	36.7	76	21
Ion exchange	10	2.63	26.3	1,600	60.9	35.6	35
Blue agarose	5.0	1.18	5.90	1,280	217	28.5	124

^a 1 U = 1 μ mol of NAD(P)H oxidized min⁻¹ ml⁻¹.

FIG. 2. Mono Q ion-exchange chromatography of the quinone reductase. The gradient was from 10 to 125 mM sodium phosphate, pH 7.0, in 25 min. —, A_{280} ; ●, enzyme activity; ---, phosphate gradient.

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 flavin mononucleotide (FMN) (6.8 min), whereas a flavin adenine dinucleotide (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).

(ii) Physical properties. The purified enzyme exhibited a pI value of 4.3 as determined by analytical isoelectric focusing. The enzyme exhibited a native molecular mass of approximately 44 kDa as determined by gel filtration on a Superose 12 column (Fig. 4A). The subunit molecular mass was estimated by SDS-PAGE to be 22 kDa (Fig. 4B).

Substrate specificity. Several different electron acceptors were tested as substrates for the purified enzyme (Table 4). When NADH was used as the electron donor, the k_{cat} ranged from $4.4 \times 10^5 \text{ s}^{-1}$ for 2-MBQ to $3.3 \times 10^2 \text{ s}^{-1}$ 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. Because of 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.

FIG. 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. 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	0.52
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 by 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.

FIG. 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

TABLE 4. Steady-state kinetic parameters for the quinone reductase^a

Compound	Apparent K_m (μ M)	Apparent k_{cat} (s^{-1})	k_{cat}/K_m
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- <i>tert</i> -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 μ M was used in determining the K_m for the electron acceptors. A fixed 2,6-DMBQ concentration of 100 μ M was used in determining the K_m for the electron donors.

pH and temperature dependence of quinone reductase activity. The effect of pH on activity was measured with a citric acid-sodium phosphate buffer (35). The enzyme had a broad pH range with the optimum 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. When 2,6-DMBQ was used as the electron acceptor, the activation energy for the oxidation of NADH was 22.5 kJ mol⁻¹ as calculated from an Arrhenius plot.

Inhibitors. The enzyme was not inhibited by 1 mM concentrations of KCN, Na₃N, ZnSO₄, MgSO₄, or CuSO₄. MnSO₄ (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 other flavoproteins (12, 36). Dicumarol and Cibacron blue, both inhibitors of the mammalian NAD(P)H:(quinone acceptor) oxidoreductase (DT diaphorase) (14, 15, 37), were efficient inhibitors of this fungal enzyme. In the presence of 4 μ M dicumarol, the enzyme activity was lowered 37%. In the presence of 2 μ M Cibacron blue, enzyme activity was lowered 53%.

DISCUSSION

Various substituted quinones have been identified as intermediates in the degradation of lignin model compounds, polymeric lignin, and aromatic pollutants (29, 38, 39, 43–47); 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 (4, 7, 40), and one such enzyme has been purified (11) 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

kDa), and cytochrome *c* (12.4 kDa). ○, molecular mass of the quinone reductase. (B) Molecular mass determination by SDS-PAGE. Molecular mass markers were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). (C) Stained gel. Lane 1, phenyl Sepharose fraction; lane 2, blue-agarose fraction. Molecular mass markers are indicated. The gel, including molecular mass markers, was scanned and reproduced with Adobe Photoshop.

metabolic cells results in a pronounced 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 peroxidases (8, 19, 30). The latter enzymes only are expressed during the secondary metabolic phase of growth, and their expression is not induced by aromatic substrates (19). The regulation of this quinone reductase appears to be similar to that described for vanillate hydroxylase (6, 49), 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. Induction of quinone reductase activity by quinone strongly suggests that it is involved in quinone and lignin degradation. The significant 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, and ion-exchange and blue agarose affinity chromatographies. The homogeneous enzyme has a molecular mass of 44 kDa and is composed of two similar 22-kDa subunits as demonstrated by SDS-PAGE. This dimeric composition is similar to that reported for DT diaphorase (25, 26), a mammalian quinone reductase. In contrast, Constam et al. (11) 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 two FAD molecules per holoenzyme (25). The cofactor for the enzyme(s) isolated by Constam et al. (11) 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. (11) were 5.7 to 6.3. The purified quinone reductase utilizes either NADH or NADPH as the electron donor, whereas the purified enzyme of Constam et al. (11) utilized only NADH. The apparent K_m for NADH and NADPH are 55 and 48 μ M, 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 (43–45, 47). 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 (13, 43, 48). The high turnover numbers exhibited by the enzyme for 2-MBQ and 2,6-DMBQ suggest that the fungus has a very efficient mechanism for conversion of these metabolic intermediates, which are derived from vanillic acid, syringic acid (13, 38), and soft- and hardwood lignins (10, 43, 48). 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 (11), suggests that this organism does not produce multiple specific quinone reductases, as has been proposed previously (40).

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 (14, 15, 37), 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. (34) have characterized an aryl aldehyde reductase which can regenerate veratryl alcohol from veratraldehyde. Likewise, quinone reductases regenerate hydroxyquinones, which are substrates for extracellular peroxidases (20, 44, 45). 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 (9, 27, 28). Semiquinones then are oxidized by O_2 to generate superoxide anion (24). Superoxide may be converted to H_2O_2 , either enzymatically by superoxide dismutase or nonenzymatically (17). In the presence of suitable electron donors, H_2O_2 can give rise to the highly reactive hydroxyl radical (22). 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 elucidate in more detail the regulation and kinetic mechanism of this enzyme.

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