An NADH:Quinone Oxidoreductase Active during Biodegradation by the Brown-Rot Basidiomycete *Gloeophyllum trabeum*

Kenneth A. Jensen, Jr., Zachary C. Ryan, Amber Vanden Wymelenberg, Daniel Cullen, and Kenneth E. Hammel*

Institute for Microbial and Biochemical Technology, USDA Forest Products Laboratory, Madison, Wisconsin 53705

Received 8 January 2002/Accepted 5 March 2002

The brown-rot basidiomycete *Gloeophyllum trabeum* uses a quinone redox cycle to generate extracellular Fenton reagent, a key component of the biodegradative system expressed by this highly destructive wood decay fungus. The hitherto uncharacterized quinone reductase that drives this cycle is a potential target for inhibitors of wood decay. We have identified the major quinone reductase expressed by *G. trabeum* under conditions that elicit high levels of quinone redox cycling. The enzyme comprises two identical 22-kDa subunits, each with one molecule of flavin mononucleotide. It is specific for NADH as the reductant and uses the quinones produced by *G. trabeum* (2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone) as electron acceptors. The affinity of the reductase for these quinones is so high that precise kinetic parameters were not obtainable, but it is clear that $k_{cat}/K_m$ for the quinones is greater than $10^6$ M⁻¹ s⁻¹. The reductase is encoded by a gene with substantial similarity to NAD(P)H:quinone reductase genes from other fungi. The *G. trabeum* quinone reductase may function in quinone detoxification, a role often proposed for these enzymes, but we hypothesize that the fungus has recruited it to drive extracellular oxydrol radical production.

MATERIALS AND METHODS

Organism. Static cultures of *G. trabeum* ATCC 11539 were grown at 31°C in the medium previously described (12). For experiments on quinone production and reduction by the intact fungus, we used mycelial mats from 5-ml cultures that were grown in 125-ml Erlenmeyer flasks for 1 to 10 days. For experiments on quinone reductase fractionation and purification, we used 50-ml cultures, each containing a 7-day mycelium with a dry weight of $10^7$ ± 9 mg, that were grown in 2.8-liter Fernbach flasks.

Production of extracellular quinones by *G. trabeum*. Each assay was done in triplicate as follows. The extracellular medium from three cultures of the same age was pooled, oxidized by adding FeCl₃ to a final concentration of 0.1 mM, and at filtered. A portion of the sample was then assayed for 2,5-DMBQ and 4,5-DMBQ by high-performance liquid chromatography (HPLC) as described previously (12, 19).

Reagents. 4,5-DMBQ was prepared by oxidizing and methylating phenol as described previously (12, 19). 2,5-DMBQ was purchased from TCI America (Portland, Oreg.). Other chemicals, all reagent grade, were obtained from Sigma/Aldrich or Pharmacia.

Reduction of quinones by *G. trabeum*. Each assay was done in triplicate as follows. Three *G. trabeum* mycelial mats of the same age were placed in a glass vial that contained 2,6-dimethoxy-p-benzoquinone (2,6-DMBQ) or 2,5-DMBQ (250 µM) in 20 ml of 25 mM sodium oxalate (pH 4.0). The solution was recirculated through a 1-cm-path-length quartz flow cell at a rate of 0 ml/min and at the ambient temperature. The decrease in absorbance of the solution was monitored at 394 nm for 2,6-DMBQ ($\Delta$ 0.64 mM-1 cm⁻¹) and at 380 nm for 2,5-DMBQ ($\Delta$ 0.31 mM-1 cm⁻¹). Previous work has shown that very little of the quinone is taken up by the mycelium in such experiments and therefore that the decrease in visible absorbance serves as a reliable measure of quinone reduction (12, 14).

Subcellular fractionation of quinone reductase activity. The mycelial mats from 12 Fernbach cultures were harvested on day 7, collected by filtration through miracloth, and rinsed with ice-cold Milli-Q water. All subsequent steps were done at 0 to 4°C. The mycelium was combined with 150 ml of homogenization buffer, which consisted of 50 mM sodium citrate (pH 6.0), 300 mM sucrose, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. The mixture was disrupted three times for 1 min with a 10-min cooling period between bursts by using an ice-jacketed glass head ho-
mogenizer (Bead-Beater; Biospec Products, Bartlesville, Okla.). The homoge­
metized was centrifuged for 20 min at 10,000 × g to remove mitochondria and cell wall material, and the supernatant fraction was retained. A measured portion of the supernatant fraction was ultracentrifuged for 60 min at 100,000 × g to separate the soluble fraction from the crude microsomal fraction, and the mi-
crosomal pellet was resuspended in 50 mM sodium citrate (pH 6.0) that con­tained 300 mM sucrose and 5 mM KCl. The three fractions were assayed for

Enzyme purification. All enzyme purification steps were done at 0 to 5°C
unless stated otherwise. The mycelial mats from 25 Fernbach cultures were harvested on day 7 as described above and frozen for 1 h at −75°C. The frozen
mycelium was suspended in 150 ml of buffer that contained 100 mM sodium
bicarbonate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. The mixture was homogenized as described above, and the glass
beads were rinsed with additional buffer to give about 200 ml of crude extract.
The extract was centrifuged at 14,000 × g for 20 min, and the supernatant
fraction was loaded on a column of Phenyl Sepharose 6 fast flow grade; 18.5 by
2.5 cm; Sigma) that had been equilibrated beforehand with 100 mM sodium
citrate (pH 6.0). The column was washed with 200 ml of 100 mM sodium citrate
(pH 6.0) and then with a 600-ml linear gradient that began with 100 mM sodium citrate (pH 6.0) and ended with 70% ethylene glycol in 10 mM sodium citrate
(pH 6.0). The NADH:quinone reductase activity eluted in a single peak at an
ethylene glycol concentration of about 45%. The active fractions were concen-
trated by ultracentrifugation and dialyzed under a vacuum in a collodion bag appar-
atus (10-kDa cut-off) against 20 mM sodium citrate (pH 6.0) that contained 20% (w/v) ethylene glycol and then dialyzed against final volume of 100 µl. This
small sample was brought to a volume of 10 ml with 20 mM sodium phosphate
(pH 6.0) and was immediately applied to a preequilibrated 2-ml column of
DEAE Sepharose (9.0 by 1.0 cm; Pharmacia) that had been equilibrated before-
hand with the same buffer. The column was washed with 20 ml of this buffer and
then with a 100-ml linear gradient from 10 to 100 mM sodium phosphate (pH 6.0).
The NADH:quinone reductase activity eluted in a single peak at a phos-
phate concentration of about 40 mM. The recovered enzyme was immediately
concentrated and dialyzed against 20 mM citrate buffer–20% ethylene glycol and concentrated as described above. Any enzyme in the removal of phosphate resulted in an increased loss of enzyme activity.

Enzyme samples (approximately 300 µl) were further purified by gel per-
meation chromatography on a column of Superdex 200 30.0 by 1.0 cm; Phar-
macia), which was operated on a Pharmacia fast-performance liquid chroma-
tography apparatus in 100 mM sodium citrate (pH 6.0) at room temperature and
was calibrated with a protein molecular weight standard kit (Pharmacia). The
NADH:quinone reductase eluted in a single peak. It was concentrated and
dialyzed against 20 mM citrate buffer (pH 6.0) that contained 20% ethylene glycol and was stored at −20°C.

Enzyme and protein assays. For routine assays of quinone reduction, it was most convenient to use 2.6-DMBQ as the acceptor instead of 2.5-DMBQ or
4,5-DMBQ. 2.5-DMBQ dissolves slowly in water, which makes stock solutions
difficult to prepare, and 4,5-DMBQ is not commercially available.

NAD(P)H:quinone reductase activity was assayed spectrophotometrically by
monitoring the decrease in absorbance at 340 nm due to NAD(P)H oxidation
with purified G. trabeum quinone reductase, the amount of hydroquinone produced was determined by
HPLC as described previously (12). Protein was assayed with a Coomassie blue
dye binding assay (Bio-Rad), which was standardized with bovine serum albumin.

To determine the stoichiometry of quinone reduction by the G. trabeum
quinone reductase, the amount of hydroquinone produced was determined by
The enzyme sample was further purified by gel per-
mcano chromatography on a column of Superdex 200 (30.0 by 1.0 cm; Phar-
macia). The NADH:quinone reductase eluted in a single peak. It was concentrated
and dialyzed against 20 mM citrate buffer–20% ethylene glycol and was stored at −20°C.

Enzyme and protein assays. For routine assays of quinone reduction, it was most convenient to use 2.6-DMBQ as the acceptor instead of 2.5-DMBQ or
4,5-DMBQ. 2.5-DMBQ dissolves slowly in water, which makes stock solutions
difficult to prepare, and 4,5-DMBQ is not commercially available.

NAD(P)H:quinone reductase activity was assayed spectrophotometrically by
monitoring the decrease in absorbance at 340 nm due to NAD(P)H oxidation
with purified G. trabeum quinone reductase, the amount of hydroquinone produced was determined by
HPLC as described previously (12). Protein was assayed with a Coomassie blue
dye binding assay (Bio-Rad), which was standardized with bovine serum albumin.

To determine the stoichiometry of quinone reduction by the G. trabeum
quinone reductase, the amount of hydroquinone produced was determined by
physically characterize the quinone reductase. The UV-visible absorp-
tion spectrum of the purified enzyme (0.2 ng/ml) was recorded in 20 mM sodium
acetate (pH 6.0) that contained 20% ethylene glycol. Flavin was eluted from G. trabeum quinone reductase by boiling a sample for 10 min. Because flavin adenine dinucleotide (FAD) can be hydrolyzed to
FMN, a control sample of the FAD-containing glutathione reductase from Sac-
charomyces cerevisiae was also boiled, as were authentic standards of FAD and
FMN. These controls established that FAD was not hydrolyzed under our ex-
traction conditions and that the recovery of both flavins for analysis was nearly
quantitative. The samples were analyzed by HPLC using external FAD and

FMN standards. The column (Phenylhexyl Luna; 150 by 4.6 mm; particle size, 5 µm; Phenomenex) was operated in water-acetonitrile-formic acid (87.5:12.5:0.1) at a rate of 1.0 ml/min and at the ambient temperature. FAD eluted at 6 min, and
FMN eluted at 10 min.

Sodium dodecyl sulfate (SDS) gel electrophoresis and analytical isoelectric focusing of the purified quinone reductase were done with precast polyacryl-
amide gels in a Pharmacia Phast System apparatus. The gels were calibrated with protein standard kits from Pharmacia and were stained with Coomassie blue
to 20°C.

Quinone reductase activity of G. trabeum in vivo. The spec-
ic activity of exogenous quinone reduction by intact myce-
lium remained roughly constant for the first 6 days of culture
and then increased (Fig. 1). A t test of the data indicated 98%
Confidence that the specific reduction rates on days 7 to 10 were significantly greater than those on days 1 to 6. The extracellular concentrations of 2,5-DMBQ and 4,5-DMBQ, the natural substrates of the reductase which we were looking for, declined slightly during the growth phase and then peaked on days 6 to 7 after growth ceased. These results led us to surmise that day 7 cultures would provide the best chance to identify the enzyme most likely to drive the G. trabeum quinone redox cycle is a soluble NADH:quinone oxidoreductase that exhibits low specificity for benzoquinones.

**Purification and physical characterization of the reductase.**

We purified the soluble activity by conventional chromatographic methods (Table 2). A single peak of activity appeared in all chromatographic steps, and the results of the final gel permeation chromatography step indicated that the holoenzyme's molecular weight was about 50,000. Typical preparations were purified 250- to 330-fold, had specific activities between 2,500 and 3,000 U/mg, and produced a single 22-kDa band during SDS-polyacrylamide gel electrophoresis (Fig. 2).

Analytical isoelectric focusing of the native protein confirmed its purity and gave an isoelectric point of 3.3. The UV-visible absorption spectrum of the purified native enzyme (Fig. 3) was typical of a flavoprotein, with an absorption spectrum of the purified native enzyme (Fig. 3) was typical of a flavoprotein, with a peak at 450 nm. The enzyme's redox state was determined by the quantum yield of the enzyme (Fig. 3) was typical of a flavoprotein, with a peak at 450 nm. The enzyme's redox state was determined by the quantum yield of the enzyme (Fig. 3) was typical of a flavoprotein, with a peak at 450 nm. The enzyme's redox state was determined by the quantum yield of the enzyme (Fig. 3) was typical of a flavoprotein, with a peak at 450 nm. The enzyme's redox state was determined by the quantum yield of the enzyme (Fig. 3) was typical of a flavoprotein, with a peak at 450 nm. The enzyme's redox state was determined by the quantum yield of the enzyme.

**Subcellular location of the principal quinone reductase.**

We fractionated a day 7 mycelial extract by differential centrifugation and assayed the fractions for 2,6-DMBQ reduction (Table 1). The extract contained a high level of NADH-dependent quinone reductase activity, more than 95% of which was soluble rather than membrane associated. The rate of NADH-dependent quinone reduction by the soluble fraction (0.19 U/mg [dry weight]) was more than sufficient to account for the rate of quinone reduction by intact G. trabeum mycelium on day 7 (0.0027 U/mg [dry weight]). A lower level of NADPH-dependent activity, comprising less than 10% of the total, was also detected. Glucose oxidase activity was undetectable, and no quinone reduction occurred when glucose rather than NADH was used as the electron donor; that is, the extract lacked sugar oxidases that can use quinones as alternative electron acceptors (15). Repetitions of these assays with 2,5-DMBQ or 4,5-DMBQ as the electron acceptor gave results little different from those obtained with 2,6-DMBQ. We concluded that the enzyme most likely to drive the G. trabeum quinone redox cycle is a soluble NADH:quinone oxidoreductase that exhibits low specificity for benzoquinones.

---

**Table 1. Subcellular fractionation of G. trabeum quinone reductase**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Reductase activity</th>
<th>Sp act (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000-x-g supernatant</td>
<td>54</td>
<td>233</td>
<td>4.3</td>
<td>22</td>
<td>0.41</td>
</tr>
<tr>
<td>100,000-x-g supernatant</td>
<td>40</td>
<td>235</td>
<td>5.8</td>
<td>14</td>
<td>0.35</td>
</tr>
<tr>
<td>100,000-x-g pellet</td>
<td>13</td>
<td>12</td>
<td>0.92</td>
<td>2</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Data for a quantity of mycelium equivalent to 1,285 mg (dry weight).
sence of ethylene glycol or in the presence of phosphate buffer. It was about twofold more active in pH 3.0 citrate buffer than in pH 6.0 citrate buffer, but we routinely assayed it at the higher pH because it lost all activity within a few hours at pH 3. These assays showed that the reductase exhibited low specificity for quinone electron acceptors; 1,4-benzoquinone, 2-methoxy-1,4-benzoquinone, 2,6-DMBQ, 2,5-DMBQ, and 4,5-DMBQ were all similarly reactive. The enzyme was active with NADH as the donor but showed no activity with NADPH. HPLC analysis of the hydroquinone reaction products showed that the enzyme reduced 1.1 mol of quinone per mol of NADH supplied; i.e., the reaction proceeds with a 1:1 stoichiometry between reductant and oxidant.

**Kinetic parameters of the reductase.** We did Michaelis-Menten experiments at pH 6.0 with the two physiological quinones, 2,5-DMBQ and 4,5-DMBQ. Each experiment was done two or three times, and all of the results yielded linear Lineweaver-Burk plots, which showed that the $K_m$ for both 2,5-DMBQ and 4,5-DMBQ was 5 to 7 µM, whereas the $K_m$ for NADH was 85 to 90 µM. Estimation of the enzyme's $k_{cat}$ gave values between 1,100 and 1,600 s⁻¹. These $K_m$ and $k_{cat}$ values are subject to considerable error, because the enzyme's extremely high affinity for quinones required us to measure small absorbance changes during the kinetics experiments. Nevertheless, the results show that the catalytic efficiency ($k_{cat}/K_m$) of the enzyme for 2,5-DMBQ and 4,5-DMBQ is very high, greater than $10^8$ M⁻¹ s⁻¹.

**Inferred amino acid sequence of the reductase.** Attempted N-terminal analyses of the reductase yielded no data, and therefore the N terminus of the mature 22-kDa protein is probably blocked. However, tryptic digestion followed by HPLC separation and Edman degradation of selected peptides yielded three internal sequences: NYDGFLFPIPTR, GGSPWGAGTFANSDGSR, and SFYEYVAR.

We used degenerate primers based on portions of these peptide sequences to amplify cDNA from *G. trabeum* cultures that expressed high levels of quinone reductase activity. We sequenced the resulting cDNA clone and found that it contained nucleotide sequences that exactly matched the three experimentally determined peptide sequences. A comparison of the *G. trabeum* cDNA sequence with related sequences in the GenBank database showed that the SFYEYVAR peptide, in particular, is located in a highly variable region near the C terminus of the quinone reductase. Therefore, the probability is very high that the cDNA which we isolated corresponds to the enzyme which we purified.

The *G. trabeum* quinone reductase (accession number AAL67860; see the GenBank database) is similar to a protein expressed by *Paracoccidioides brasiliensis* (AAL50803; 66% identical) (5), to a protein encoded by the *S. cerevisiae* PST2 gene (CAA98854; 64% identical), and to a previously reported quinone reductase from the white-rot basidiomycete *Phanerochaete chrysosporium* (AAD21025; 56% identical) (1). The *G. trabeum* reductase also exhibited lower levels of similarity with some other known and putative quinone reductases from fungal and plant sources (16), including one encoded by a minor allergen gene of *Arabidopsis thaliana* (CAB16805; 42% identical).

Although we were unable to identify the N terminus of the *G. trabeum* reductase, the data suggest two possibilities. One is that translation of the mRNA starts with Met-55 of the sequence which we submitted (AAL67860), thus yielding a 21.9-kDa mature protein without posttranslational processing. However, it seems more likely that translation starts with Met-1 to give a 27.7-kDa precursor and that a leader sequence is then cleaved from the mature protein at an unknown location near Met-55. We draw this conclusion because putative leader sequences also occur in the mRNAs for the *P. chrysosporium* quinone reductase (1) and the *A. thaliana* allergen. The function of these leaders is unclear, but all three of them are hydrophilic, and the inferred N termini of the two fungal sequences exhibit substantial similarity.
DISCUSSION

Most of the *G. trabeum* Fenton system involves extracellular, nonenzymatic reactions in which electrons are transferred from hydroquinones or semiquinones to Fe$^{3+}$ or O$_2$. The fungus itself does not participate directly in these reactions, but it drives them by continuously reducing quinones back to hydroquinones so that the redox cycle can continue (12, 14). The quinone reductase that catalyzes this step is, accordingly, a critical component of the *G. trabeum* biodegradative arsenal. We found one major quinone reductase in cultures of *G. trabeum* grown under conditions that elicit high levels of extracellular quinone redox cycling. This result and the high catalytic efficiency of the reductase led us to hypothesize that *G. trabeum* uses this enzyme to drive Fenton chemistry.

However, an alternative (or perhaps additional) function for the *G. trabeum* reductase cannot be ruled out yet. This enzyme belongs to a widely distributed family of flavoprotein quinone reductases that are generally thought to detoxify intracellular quinones by maintaining them in the reduced form (16). Quinones are cytotoxic in part because they readily undergo intracellular one-electron reduction to semiquinones, which react rapidly with O$_2$ to produce superoxide. By contrast, hydroquinones are relatively nontoxic because they undergo rapid one-electron oxidation to semiquinones only in the presence of transition metal oxidants, such as Fe$^{3+}$, which are generally unavailable inside cells because they are sequestered in redox-inactive complexes (10). Since *G. trabeum* produces large amounts of quinones as natural metabolites (12, 14, 18), it may have an unusually high requirement for a quinone detoxification system.

Much of the current impetus for a better understanding of brown-rot mechanisms is aimed at devising better ways to inhibit wood decay. Approximately 10% of all trees cut in the United States go to replace decayed wood, and to minimize the losses, wood rot fungi are currently controlled with toxic, environmentally deleterious biocides, such as creosote, pentachlorophenol, and chromated copper arsenate (22). It would be advantageous to target these fungi with compounds that interfere more specifically with wood decay mechanisms.

Given the importance of quinone metabolism in brown rot (8, 12, 14, 18) and white rot (1, 3, 4, 9), fungal quinone reductases that drive Fenton chemistry or prevent toxicity from quinones are potential targets that merit further investigation.

ACKNOWLEDGMENTS

We are grateful to C. J. Houtman for a statistical analysis of the Fig. 1 data. This work was supported by U.S. Department of Energy grant DE-FG02-94ER20140 to K.E.H.