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Purification and Characterization of Cellobiose Dehydrogenases from the White Rot Fungus *Trametes versicolor*

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The white rot fungus *Trametes versicolor* degrades lignocellulosic material at least in part by oxidizing the lignin via a number of secreted oxidative and peroxidative enzymes. An extracellular reductive enzyme, cellobiose dehydrogenase (CDH), oxidizes cellobiose and reduces insoluble $Mn(IV)O_2$, commonly found as dark deposits in decaying wood, to form Mn(III), a powerful lignin-oxidizing agent. CDH also reduces *ortho*-quinones and produces sugar acids which can promote manganese peroxidase and therefore ligninolytic activity. To better understand the role of CDH in lignin degradation, proteins exhibiting cellobiose-dependent quinone-reducing activity were isolated and purified from cultures of *T. versicolor*. Two distinct proteins were isolated; the proteins had apparent molecular weights of 97,000 and 81,000 and isoelectric points of 4.2 and 6.4, respectively. The larger CDH (CDH 4.2) contained both flavin and heme cofactors, whereas the smaller contained only a flavin (CDH 6.4). These CDH enzymes were rapidly reduced by cellobiose and lactose and somewhat more slowly by cellulose and certain cello-oligosaccharides. Both glycoproteins were able to reduce a very wide range of quinones and organic radical species but differed in their ability to reduce metal ion complexes. Temperature and pH optima for CDH 4.2 were affected by the reduced substrate. Although CDH 4.2 showed rather high substrate specificity among the *ortho*-quinones, it could also rapidly reduce a structurally very diverse collection of other species, from negatively charged triiodide ions to positively charged hexaquo ferric ions. CDH 6.4 showed a higher K_m and a lower V_{max} and turnover number than did CDH 4.2 for all substrates tested. Furthermore, CDH 6.4 did not reduce the transition metals Fe(III), Cu(II), and Mn(III) at concentrations likely to be physiologically relevant, while CDH 4.2 was able to rapidly reduce even very low concentrations of these ions. The reduction of Fe(III) and Cu(II) by CDH 4.2 may be important in sustaining a Fenton's-type reaction, which produces hydroxyl radicals that can cleave both lignin and cellulose. Unlike the CDH proteins from *Phanerochaete chrysosporium*, CDH 4.2 and CDH 6.4 are unable to produce hydrogen peroxide.

Over a period of several days, the white rot fungus *Trametes versicolor* 52J can bleach and delignify both hardwood and softwood kraft pulps (1, 7, 39, 43, 45). Intimate contact between the fungus and pulp fibers is not required for this to occur: the culture liquor contains everything necessary to bleach and degrade kraft lignin, although constant regeneration or renewal of liquor components is necessary for delignification to continue (6). During pulp biobleaching, *T. versicolor* secretes both low-molecular-weight metabolites and a number of lytic enzymes (45). Two of these enzymes, manganese-dependent peroxidase (MnP) (40) and laccase (12) can, in the presence of appropriate cofactors, oxidize, demethylate, and delignify kraft pulps, although not nearly as extensively as a complete *T. versicolor* culture can.

Cellobiose dehydrogenase (CDH) (sometimes called cellobiose:quinone oxidoreductase) has been proposed as an enzyme important in both lignin (19, 20, 55) and cellulose biodegradation (9, 32). CDH is produced by many white rot fungi (4), including *T. versicolor* (55). CDH is also secreted by a number of non-white rot fungi (15, 16, 48, 50). CDH has reportedly been isolated from the white rot fungi *Phanerochaete chrysosporium* (55) and *Fomes annosus* (31). It should be noted that CDH activity may easily be underestimated or missed, since laccases can mask its presence in culture supernatants by rapid reoxidation of the hydroquinones produced by CDHs from quinone assay substrates (46). To counter this, a new CDH assay has been developed (46). CDHs reportedly can use electrons from the oxidation of cello-oligosaccharides (9, 32, 47) to reduce free radicals to phenolics (5, 45, 49, 55), quinones (15, 16, 55), Fe(III) (15, 32, 33), Mn(III) (10), and Mn(IV) (44, 47). Thus, CDH-mediated reduction produces many lignin-based structures which are good substrates for the laccases and MnPs commonly secreted by white rot fungi during delignification. The reduction of O_2 to H_2O_2 via superoxide is also reportedly catalyzed by *P. chrysosporium* CDH in vitro (8, 15, 29, 37); however, O_2 reduction rates are low compared with those of many other CDH substrates (57). A further consequence of the wide range of reductive reactions catalyzed by this enzyme has been that the nomenclature for this enzyme is in a state of flux. The term cellobiose dehydrogenase (i.e., CDH) is used in this report to describe both flavin only and heme-flavin cofactored proteins isolated from *T. versicolor*. The heme-flavin cofactored enzyme with a similar activity from *P. chrysosporium*, formerly referred to as cellobiose oxidase, is also referred to as CDH (2). The term cellobiose:quinone oxidoreductase is used here only for the nonheme enzyme from *P. chrysosporium.*

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On the basis of its broad substrate range and interaction with laccases, peroxidases, cellulose, and lignins, a wide variety of roles for CDH in wood and industrial pulp delignification have been proposed (3, 7, 19, 47). CDH may be an essential component of the delignification system in white rot fungi. The objective of this study was to determine some of the structural and catalytic properties of the *T. versicolor* CDHs to better understand their role in *T. versicolor*-mediated kraft pulp biobleaching and delignification.

MATERIALS AND METHODS

Fungal cultures. *T. versicolor* 52J was stored at −80°C and cultured on mycological broth plates as described previously (1). For the production of CDH, the complex spore germination medium described by Canevascini (14) (medium A; 0.5 g of KCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, 1 g of KH_2PO_4, 0.4 g of NH_4Cl [each per liter]), and 1 ml of a trace metals solution (45) was inoculated with 4 agar plugs (1-cm diameter) taken from the periphery of a growing colony of *T. versicolor*. Trameters defined medium (TDM) (45) was used for demonstrating lignolytic enzyme induction. Inocula for production cultures were prepared in a 500-ml plastic Erlenmeyer flask containing 200 ml of medium A and 5 g of glucose per liter and incubated at 27°C for 48 h with shaking ($r = 4.5$ mm) at 200 rpm; the inocula were then transferred to 800 ml of medium A plus 5 g of glucose per liter in a 3.8-liter Fernbach flask that was in turn incubated for 48 h agitated at 100 rpm ($r = 4.5$). CDH production was done in 14 liters of medium A with 5 g of cellulose (Solka Floc) per liter in aerated 20-liter Nalgene carboys inoculated with 1 liter of a growing *T. versicolor* culture and shaken at 75 rpm ($r = 4.5$) at 27°C. The culture was sampled every 12 h and clarified by centrifugation (10,000 × *g*; 5 min), and CDH activity was assayed.

Assays. CDH activity was determined by the cellobiose-dependent reduction of 3,5-di-*tert*-butylbenzoquinone(1,2) (TBBQ) at 420 nm (55). The assay mixture contained Na acetate (100 mM; pH 4.5), ethanol (20%, vol/vol), cellobiose (2 mM), and TBBQ (0.33 mM) in a total volume of 1.5 ml. To prevent interference caused by laccase-mediated reoxidation of the hydroquinone formed from TBBQ by CDH, the reaction was made anoxic by bubbling N_2 through the mixture for 3 min. One unit of enzyme was defined as the amount that reduced 1 μmol of TBBQ min^{-1} ml^{-1}. Alternatively, CDH activity was measured by the chlorpromazine radical reduction assay, as described elsewhere (46). The reduction of other quinone substrates was measured in the Na acetate-ethanol buffer mixture and monitored at the λ_{max} of the substrate. The activity was calculated by use of experimentally determined extinction coefficients. Quinones synthesized as described by Teuber and Staiger (53) and used as CDH substrates were measured in 100 mM acetate buffer (pH 4.5) containing 20% (vol/vol) ethanol.

The reductions of 2,6-dichlorophenolindophenol (DCIP), cytochrome *c*, ferricyanide ion, and triiodide ion were measured spectrophotometrically in a 1-ml assay volume, and enzyme activity was calculated with molar extinction coefficients of 7,100, 33,700, 1,040, and 26,200, respectively. The kinetics of cellobiose oxidation were measured with 0.2 mM DCIP in 50 mM Na acetate (pH 4.5). Fe(III) reduction was measured in the presence of 0.1% phenanthroline; the appearance of Fe(II)-phenanthroline was indicated by an increase in A_{510} and quantified with an experimentally determined molar extinction coefficient of 16,200. The reduction of Cu(II) to Cu(I) was measured with 1 mM 2,9-dimethyl-4,7-diphenyl-1,10 phenanthrolinedisulfonic acid (bathocuproinedisulfonic acid). Reduction to the Cu(I)-chelate was measured at 483 nm with a molar extinction coefficient of 12,250 (17). Mn(III)-malonate was prepared by mixing 100 mM Mn(III)-acetate with 100 mM Na malonate, stirring for 4 h at 23°C, and filtering (0.22-μm pore size). The concentration of Mn(III)-malonate obtained was determined at 270 nm (molar extinction coefficient, 8,000). Unless otherwise stated, all enzymatic assays were performed at 23°C in 50 mM Na acetate (pH 4.5)–2 mM cellobiose.

Cellobiose-dependent O_2 uptake by CDH was measured with a polarographic oxygen electrode (Rank Brothers, Cambridge, United Kingdom) at 25°C in 3 ml. The reaction mixture contained 10 U of CDH per ml and 2 mM cellobiose in 100 mM Na acetate (pH 4.5). H_2O_2 was determined by the addition of 600 U of catalase per ml to the reaction mixture and measuring the O_2 produced.

Purification procedure. When maximum CDH activity was obtained (72 to 96 h), the culture supernatant was separated from the residual cellulose and fungal biomass by filtration through cheesecloth and Whatman no. 4 filter paper (yielding >90% recovery of TBBQ-reducing activity). The clarified supernatant was concentrated 15-fold by ultrafiltration with 10,000-molecular-weight (10,000-MW)-cutoff polysulfone membranes. The concentrated supernatants were dialyzed first against distilled water (10 times, 1 liter) and then against buffer B (10 mM Bis-Tris, pH 6.5; four times, 1 liter) and then further concentrated to 200 ml again by using 10,000-MW-cutoff membranes. The concentrated crude enzyme was applied to a DEAE-Sephacryl gel column (5 by 30 cm; bed volume, 250 ml) previously equilibrated with buffer B. The column was washed with 500 ml of buffer B and then eluted with a linear salt gradient from 0 to 500 mM NaCl in a total volume of 2 liters at a flow rate of 80 ml/h. Fractions containing CDH activity were pooled, dialyzed (5× buffer B), and reconcentrated (10,000-MW cutoff) and then applied to a Sephacryl S-300 (Pharmacia) column (2.5 by 92 cm) equilibrated in 5× buffer B at a flow rate of 22 ml/h. Active fractions were pooled, dialyzed (10 mM Bis-Tris propane buffer [pH 6.4]), and concentrated (as described above). The final steps consisted of running the pooled active fractions on a Mono-Q HR 5/5 strong anion-exchange column equilibrated with Bis-Tris propane buffer (pH 6.4; 20 mM) twice, with an intermediate step of pooling and dialyzing the CDH-containing fractions. The column was washed with 2 ml of buffer, and retained proteins were eluted with a linear salt gradient (0 to 400 mM NaCl) in starting buffer in a total volume of 40 ml. Active fractions were pooled, dialyzed against water, concentrated, and stored at 4°C at 1 mg ml^{-1}.

Protein concentration and cofactor identification. Protein content was measured at 595 nm by use of Coomassie blue (13), with bovine serum albumin as the standard. The flavin coenzyme was released from homogeneous CDH by precipitating the protein with 5% (wt/vol) trichloroacetic acid (37). The protein was removed by centrifugation (10,000 × *g*, 10 min), the supernatant was extracted four times with diethyl ether (4°C), and the aqueous phase was lyophilized. The residue was reconstituted in H_2O (100 μl) and separated by thin-layer chromatography on silica gel plates with Na_2HPO_4 (5%, wt/vol) in water as the solvent (22), and the resolved flavin bands were observed with UV light. The R_f of the dissolved material resolved by thin-layer chromatography was obtained and compared with that of genuine flavin adenine dinucleotide (FAD). The amount of FAD released from CDH was quantified spectrophotometrically by use of a molar extinction coefficient of 11,300 at 450 nm for FAD (22). The nature of the heme group was identified by its Soret peak in the spectrum of the CDH with a pI of 4.2. The heme was extracted from CDH with acidified (0.1 M HCl) acetone and quantified by the pyridine-hemochromogen method (37).

Enzyme kinetics. The Michaelis-Menten K_m and V_{max} values were determined for a number of CDH substrates by use of the Eadie-Hofstee plots of kinetic data. TBBQ or DCIP was used as the electron acceptor with most electron donors, while cellobiose was used as the electron donor for most electron acceptor trials. All determinations were at pH 4.5 in 100 mM Na acetate. Temperature optima of the two CDH enzymes were determined with a water-jacketed cuvette holder and by use of TBBQ or DCIP and cellobiose as substrates at pH 5 in 100 mM Na acetate. The optimal pHs of the two CDH enzymes were determined by use of Na acetate (pH 4.0, 4.5, and 5.0), Na succinate (pH 5.5), K phosphate (pH 6.0, 6.5, and 7.0), and Tris-HCl (pH 7.5, 8.0, and 8.5).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Pharmacia Phast Gel system with 10 to 25% gradient acrylamide gels against MW standards. Isoelectric focusing (IEF) was performed with the Phast Gel (IEF) apparatus with pH ranges of 3 to 9 and 2.5 to 6 in conjunction with appropriate IEF isoelectric point (pI) standards (Pharmacia). Protein bands were visualized by staining with Coomassie blue, and CDH activity was detected with 2 mM DCIP (pH 6) and 2 mM cellobiose. Schiff staining was used to identify glycoproteins following SDS-PAGE (23).

Chemicals. Quinone substrates were synthesized by use of Fremy's salt (potassium nitrosodisulfonate) oxidation of the corresponding phenols as follows: 2-methoxy-4-methyl-1,2-benzoquinone from 2-methoxy-4-methylphenol, 4-*tert*-octyl-1,2-benzoquinone from 4-*tert*-octylphenol, 4-*tert*-butyl-1,2-benzoquinone from 4-*tert*-butylphenol, 4-methyl-1,2-benzoquinone from 4-cresol, 3,4,5-trimethyl-1,2-benzoquinone from 3,4,5-trimethylphenol, and 3,4-dimethyl-1,2-benzoquinone from 3,4-dimethylphenol (44, 53). Melting point and UV-visible absorbance spectra were compared with published standards to confirm the purity of the quinones (53). Other quinone substrates used to test CDH activity were from Aldrich Chemical Co. Cellobiose, lactose, glucose, galactose, xylose, and sorbose were from Sigma Chemical Co. Oligomers of glucose ($n = 3$ to 8) were synthesized as described previously (36). Kraft lignin (Indulin) was a commercial preparation of pine from Westvaco, Inc.

Spectroscopy. Absorption spectra were recorded on a Perkin-Elmer Lambda 3 spectrophotometer at room temperature in a cuvette with a 1-cm path length. Enzyme spectra were recorded in 20 mM Na acetate buffer (pH 4.5). Enzymes were reduced by using cellobiose (10-fold excess) or sodium dithionite (several grains).

The electron paramagnetic resonance (EPR) spectra of frozen CDH samples were measured with an X-band Bruker ER 200D-SRC spectrometer with 100-kHz modulation. The flavin semiquinone spectra were detected with the following conditions: temperature, 120 K; microwave power, 5 mW; microwave frequency, 9.3 GHz; modulation amplitude, 0.16 mT (37). Diphenyl-picryl-hydrazyl was used to measure the exact microwave frequency.

RESULTS

Purification of CDH. The production of CDHs by *T. versicolor* 52J was tried in several growth media. Although a kraft lignin (Indulin) greatly increased the yield of CDH (Table 1), its presence in the medium interfered with subsequent isolation and purification of the enzyme. As with the *P. chrysosporium* enzyme, CDH activity was also strongly induced by cellobiose and cellulose (data not shown), and the latter was used as a carbon source for the growth of *T. versicolor* for enzyme production. The induction by kraft lignin of high levels of

TABLE 1. Localization of total CDH activity[a]

Enzyme	Enzyme activity (U liter^{-1})[b]			
	Extracellular		Intracellular[c]	
	TDM	TDM + Indulin	TDM	TDM + Indulin
CDH	50 ± 10	270 ± 80	0	0
Laccase	3,710 ± 230	900 ± 50	75 ± 0	196 ± 10
Catalase	0	0	789 ± 73	2,300 ± 41

[a] Cultures were grown in TDM (45) with cellobiose as the carbon source and with or without Indulin (0.05 g of soluble kraft lignin ml^{-1}).
[b] Enzyme activity was calculated as the total units of enzyme recovered divided by the culture volume. CDH activity was measured by an assay based upon the reduction of a laccase-generated chlorpromazine radical (46). Values are the means of three determinations ± standard deviations.
[c] Intracellular enzymes were released at 4°C with a French pressure cell.

intracellular catalase (Table 1) suggests that H_2O_2 plays a role in delignification, either as a byproduct or as a substrate for peroxidases. At harvest, each 15-liter culture contained homogeneous 3- to 4-mm spherical white fungal colonies filling the lower third of the carboy. Nearly all of the CDH enzyme activity was found extracellularly (Table 1) as two proteins easily separable on nondenaturing polyacrylamide gels (Fig. 1, lane B). The time of harvest was critical since, after peaking, medium CDH activity would fall in a few hours to virtually zero. The CDHs secreted by *T. versicolor* 52J during growth on Solka Floc cellulose were isolated and purified from the culture medium in four steps. The initial separation on DEAE-Sephacryl gave three activity peaks (Fig. 2).

Fractions containing the first CDH activity peak (Fig. 2, peak A) were pooled and subsequently purified. Peak A contained 0 to 10% of the total TBBQ-reducing activity in concentrated unfractionated culture supernatants and coeluted with >90% of the laccase activity in the crude preparation. The fractions containing the second and third CDH activity peaks

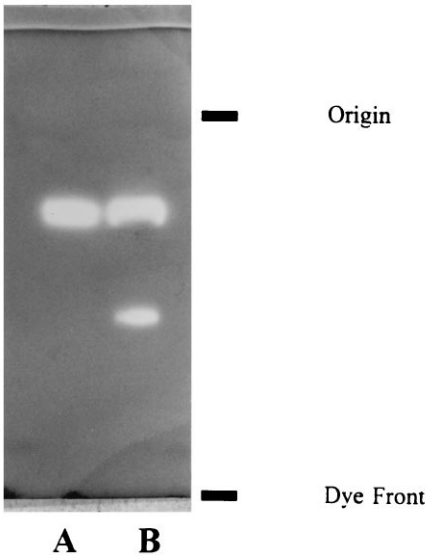


FIG. 1. Resolution of concentrated (MW, >10,000) supernatant proteins by nondenaturing PAGE. Proteins were separated (400 V · h) on 8 to 25% gradient Phast Gels. The gel was stained with 2 mM DCIP in 20 mM phosphate buffer (pH 6.5), and proteins with CDH activity were detected as clear bands appearing in the blue-stained gel. These bands appeared only after the addition of cellobiose (2 mM) to the staining solution. Lanes: A, purified preparation of CDH 4.2; B, concentrated extracellular supernatant from *T. versicolor.*

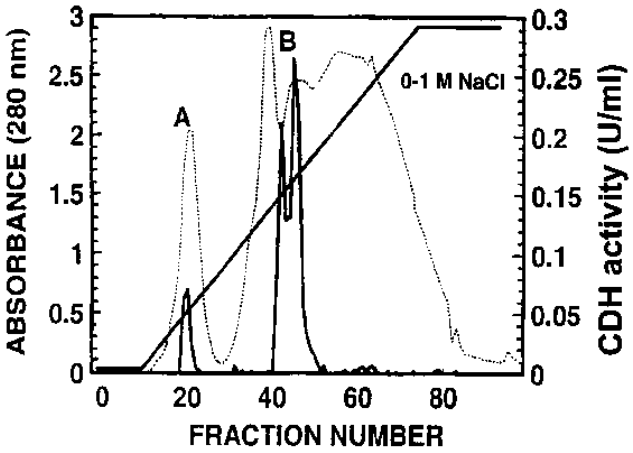


FIG. 2. Purification of CDH by ion-exchange chromatography. Concentrated and dialyzed culture supernatant was passed through DEAE-Sephacryl equilibrated with Bis-Tris buffer (20 mM, pH 6.5), washed with 1 liter of the same buffer, and eluted with a linear gradient of NaCl (0 to 1 M in 2 liters). The eluate was assayed for protein (– – –) and CDH (——) activity.

(Fig. 2, peak B) were pooled and further purified. Table 2 shows the results of a typical purification procedure.

Unfortunately, this procedure often gave very low yields and high losses, especially in the ion-exchange steps. More recently, both the yield and stability of CDH have been improved by harvesting early, when there is only about 5 chlorpromazine U/ml of CDH in the supernatant, and by changing the buffer used throughout to 10 mM ammonium acetate (pH 5.0) and using 10 to 500 mM gradients of the same buffer to elute the DEAE and Mono Q columns. Postharvest CDH activity losses appear to be due largely to proteolytic cleavage, with active CDH fragments with MWs of 47,000 and 57,000 often found.

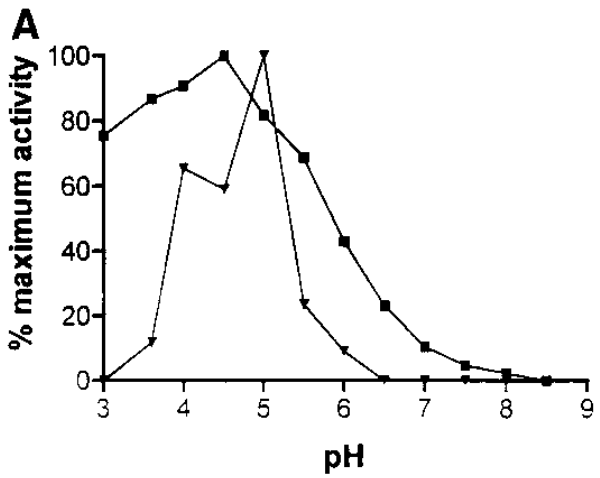
Purification of CDH peak A activity increased its specific activity fivefold and gave a pale yellow solution. The molecular mass of the protein was estimated to be 81 kDa by SDS-PAGE. Gel permeation chromatography gave a similar estimate of size, suggesting that the enzyme occurs as a monomer. By IEF, the pI of CDH peak A was determined to be 6.4. Therefore, CDH A was designated CDH 6.4.

Purification of CDH peak B yielded a red-brown protein producing a single band on SDS-PAGE gels with an estimated molecular mass of 97 kDa. When a nondenaturing PAGE gradient gel (10 to 25%) was stained for activity with DCIP or for protein with Coomassie blue, a single diffuse band was observed. The turnover number (k_{cat}) of the purified CDH

TABLE 2. Purification of *T. versicolor* CDH pI 4.2 and pI 6.4 proteins[a]

Purification step	Total protein[b] (mg)	Enzyme activity[c] (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Supernatant		2,000		100	
Ultrafiltration	2,544	1,774	0.70	88.7	1
DEAE-Sephacryl	141 (17)	900 (44)	6.38 (2.59)	45.0 (2.2)	9.1 (3.7)
Sephacryl S300	67 (11)	840 (32)	12.5 (2.91)	42.0 (1.6)	17.9 (4.2)
Mono-Q (FPLC)[d]	24 (5)	590 (17)	24.6 (3.4)	29.5 (0.9)	35.1 (4.9)

[a] Values in parentheses are for the flavin-cofactored CDH (pI = 6.4).
[b] Protein was determined by the Bio-Rad dye-binding protein assay.
[c] Activity was measured with TBBQ and cellobiose as the substrates and included both CDHs (pIs = 4.2 and 6.4).
[d] FPLC, fast protein liquid chromatography.



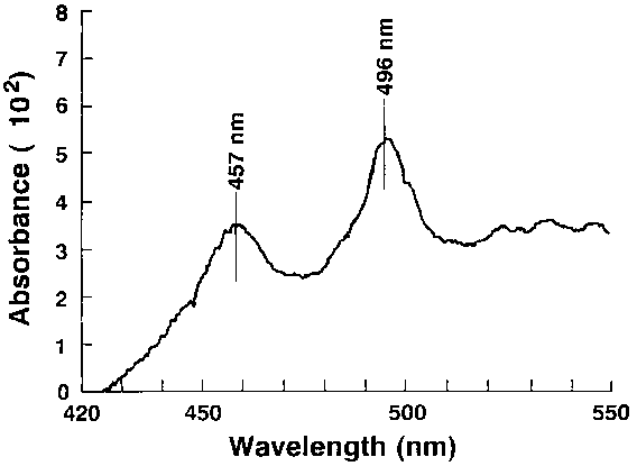


FIG. 4. Visible spectrum of a purified preparation of CDH 6.4 in 50 mM Na acetate buffer (pH 4.5). The enzyme was purified as described in Table 2, and the absorbance profile of the enzyme was recorded.

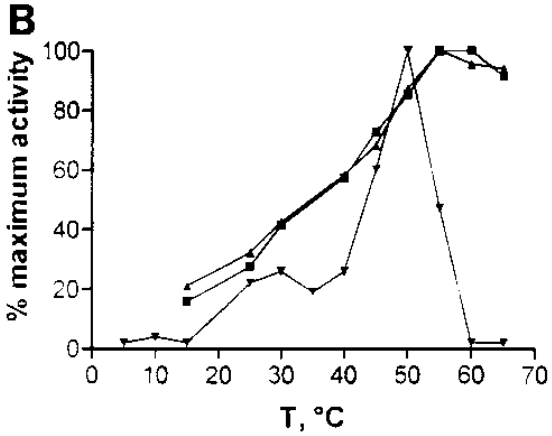


FIG. 3. pH and temperature profiles of CDH 4.2 and 6.4. The CDH 4.2 pH range (A) was determined with 0.33 mM TBBQ (▼) or 0.2 mM DCIP (■) as the substrate and cellobiose (2 mM) in Na citrate (pH 3.0 and 3.5), Na acetate (pH 4.0, 4.5, and 5.0), Na succinate (pH 5.5), K phosphate (pH 6.0, 6.5, 7.0, and 7.5), and Tris-HCl (pH 8.0 and 8.5) buffers as described in Materials and Methods. The values shown are the means of triplicate determinations. (B) Reducing activity of CDH 4.2 and CDH 6.4 at various temperatures. The activity of CDH 4.2 was measured in 50 mM Na acetate buffer (pH 5.0 for TBBQ; pH 4.5 with DCIP) with 0.33 mM TBBQ (▼) or 0.2 mM DCIP (■) and 2 mM cellobiose as substrates. The temperature dependence of CDH 6.4 (▲) was measured with 0.2 mM DCIP as the reduced substrate under the same conditions as CDH 4.2. The values shown are the means of duplicate determinations.

preparation was 25 s^{-1}, and its specific activity was 25 μmol min^{-1} mg of $protein^{-1}$, with TBBQ as the substrate for the enzyme. IEF gave a pI of CDH peak B of 4.2; therefore, it was designated CDH 4.2.

CDH 4.2 showed maximal activity with TBBQ in acetate buffer at pH 5.0 (Fig. 3). However, when DCIP is used as the substrate, the pH optimum shifts to 4.5 and the relative activity is higher at most pHs assayed. With TBBQ as the substrate, enzyme activity is optimal at 50°C and is rapidly lost at temperatures of >60°C (50% inactivation in 30 s at 60°C). However, with DCIP as the substrate, a shift in the temperature optimum to 55°C is observed. Furthermore, CDH activity is greatly enhanced at the higher temperatures assayed (55 to 65°C) with DCIP as the substrate compared with that with TBBQ as the substrate. DCIP reductive activity was completely lost at 70°C. The temperature profiles of CDH 4.2 and CDH 6.4 were identical with DCIP as the substrate (Fig. 3). Thus, the nature of the reduced CDH substrate appears to have a large effect on the useful pH and temperature ranges of the enzyme.

The purified CDHs (3 mg ml^{-1}) could be stored at 4°C in sterile buffer for several months without loss of enzymatic activity but lost 30 to 50% of their initial activity when frozen and thawed. Lyophilization resulted in complete inactivation of the CDH proteins. Full activity was maintained by storage of the enzymes in 50% glycerol at −20°C.

Cofactors of CDH 4.2 and CDH 6.4. Trichloroacetic acid (5%) applied to CDH 4.2 and CDH 6.4 released a fluorescent material having an R_f value identical to that of purified FAD on silica gel thin-layer chromatography plates eluted with Na phosphate buffer (22). Furthermore, the spectral properties of the eluted material were identical to those of genuine FAD (Fig. 4). These observations confirmed FAD as a cofactor of CDH 4.2 and CDH 6.4. The cofactor contents were approximately 0.8 and 0.9 FAD molecules per molecule of CDH 4.2 and CDH 6.4, respectively. The ratio of less than 1:1 may reflect the presence of modified flavins in the enzyme preparation (38), less than quantitative flavin recovery, or the presence of deflavinated protein in the enzyme preparations. Subsequent extraction of CDH 4.2 with acidified acetone and preparation of a pyridine hemochromogen extract yielded a solution with a strong A_{555}, demonstrating that the enzyme's heme was of the cytochrome *b* type, similar to that of *P. chrysosporium* CDH (10, 37).

Spectrophotometric analysis of CDH reduction. CDH 6.4 (Fig. 4) had absorbance peaks at 457 and 496 nm which are similar to those of the CDH from *P. chrysosporium* (38, 56). Addition of Na_2SO_3 or cellobiose eliminated these peaks, presumably by reduction of the FAD. Unlike the *P. chrysosporium* CDH (38), only a single isozyme of CDH 6.4 was obtained from cultures of *T. versicolor* 52J, as determined by IEF and nondenaturing polyacrylamide gels and protein separations on Mono Q columns. Homogeneous oxidized CDH 6.4 preparations had an A_{457}/A_{276} ratio of approximately 0.09, while CDH 4.2 had an A_{420}/A_{280} ratio of 0.53 to 0.59. The latter is similar to values reported for the *P. chrysosporium* heme CDH (28).

Oxidized CDH 4.2 differed from oxidized CDH 6.4 in having

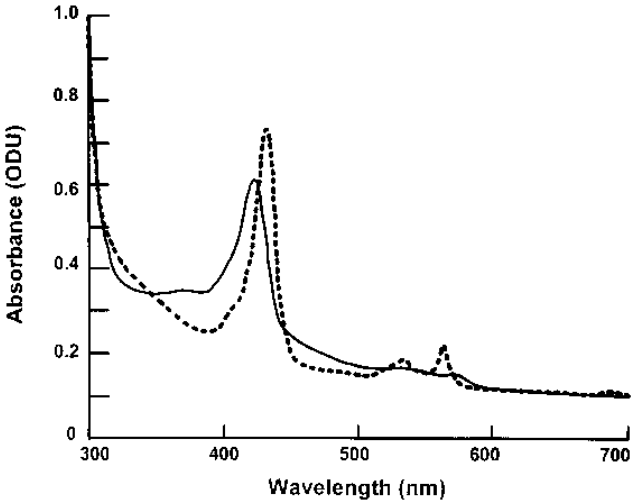


FIG. 5. Visible spectrum of oxidized (——) and reduced (– – –) CDH 4.2 (8 μM) in 50 mM Na acetate buffer (pH 4.5). The enzyme was purified as described in Table 2, and the absorbance profile was recorded. The enzyme was reduced by adding 2 mM cellobiose to the enzyme preparation. ODU, optical density units.

absorbance peaks at 419 nm (Σ mM = 56.2), 535 nm (Σ mM = 5.2), and 564 nm (Σ mM = 4.7) (Fig. 5). When CDH 4.2 was reduced to the ferrous form with cellobiose, the absorbance maxima were shifted to 425, 538, and 570 nm and the molar absorbancies of the peaks at 538 nm (Σ mM = 6.7) and 570 nm (Σ mM = 7.2) were increased (Fig. 5). The observed decrease between A_{500} and A_{450} upon reduction by cellobiose (Fig. 5) has been associated with the reduction of the flavin cofactor in *P. chrysosporium* CDH (10, 37).

When CDH 4.2 (8 μM) was reduced with 50 μM cellobiose, an EPR signal (120 K) not seen with the oxidized form of the enzyme appeared. The EPR signal was centered on a field strength of 357.9 mT and had a calculated *g* value of 1.947 with a line width of approximately 6 mT, similar to that reported for the red (anionic) flavin semiquinone (35). At 120 K, no EPR signal was detected in preparations of oxidized CDH 6.4; however, reduction of the enzyme with cellobiose produced a signal. This EPR signal (data not shown) is similar to that of reduced *P. chrysosporium* CDH (37).

Catalytic properties of *T. versicolor* CDHs. With TBBQ as the electron acceptor, both CDHs oxidized cellobiose as their preferred substrate (Table 3), although cellotriose and lactose were also used at lower but very significant rates. The enzyme will not significantly reduce glucose or any other of the monosaccharides tested at realistic concentrations, although very slow oxidation was observed in 1.5 M glucose (data not shown). Both CDH isoforms are quite specific for the β-1,4 glycosidic bond, since of the disaccharides tested, only cellobiose (glc-β-1,4-glc) and lactose (gal-β-1,4-glc) were measurably oxidized (Table 3). The specificity of CDH for glucose-containing saccharides is revealed by the fact that lactose, which differs from cellobiose only at the epimeric C-4 of the galactose residue, is oxidized at a much lower rate than cellobiose is (Table 3). Although the cello-oligosaccharides did not reduce detectable amounts of TBBQ in a short (5- to 10-min) assay, TBBQ reduction by CDH was detectable with longer incubation periods (6 to 24 h). CDH 4.2-mediated oxidation of cellulose was also detected by a more sensitive assay which showed a marked CDH-dependent increase in the number of carboxylic acid groups on insoluble cellulose (46).

Three general types of electron-accepting CDH substrates were evaluated: metal ion complexes (Fig. 6, group IV), oxidized phenthiazoline-type cation radicals (Fig. 6, group II), and a variety of *ortho*- and *para*-quinones (Fig. 6, groups I and III; see also reference 44). Consistent with the low specificity of similar enzymes isolated from *P. chrysosporium*, CDH 4.2 from *T. versicolor* 52J reduced a wide range of substrates (Tables 4 to 6). Kinetic parameters for the reduction of various quinones showed that functional group substitution of hydrogens at positions adjacent to the ring oxygens decreased the V_{max} of CDH 4.2 catalysis significantly (Table 5); however, the enzyme reduced most of the quinones tested. The larger *para*-quinone substrates (such as the acid dyes shown in Table 4, group III) were not reduced at a detectable rate in a 5- to 30-min cuvette assay. In contrast, cation radicals with similar conjugated ring structures (e.g., ·ABTS) were readily reduced by CDH (Fig. 6, group II). A comparison of the turnover numbers (Table 4) for CDH with various substrates (Fig. 6) shows that overall, CDH was most effective at reducing Mn(III) complexes, followed by the free radicals of chlorpromazine and ABTS, and then, finally, by quinones. For *P. chrysosporium*, it was suggested that the heme-cofactored CDH (which is similar in its behavior with quinones to the *T. versicolor* CDH 4.2) functions as a peroxide-generating enzyme (8). The *T. versicolor* CDHs reduce quinones much more rapidly than they form H_2O_2 (Table 6). Even in pure oxygen, CDH 4.2 was ineffective as a peroxide generator.

A comparison of the reductive activities of CDH 4.2 and CDH 6.4 with various substrates reveals that the two enzymes possess similar substrate specificities among nonmetallic substrates, but CDH 6.4 has a higher K_m and a lower turnover number (k_{cat}) and maximum velocity for all substrates tested (Table 6). Among transition metals, the differences were very pronounced. Oxidized forms of the three transition metals (Fe, Mn, and Cu) suspected of involvement in fungal delignification were far more readily reduced by CDH 4.2 than by CDH 6.4. In fact, the latter enzyme was entirely unable to reduce cytochrome *c* or ferricyanide ions, substrates rapidly reduced by CDH 4.2 (Table 6; Fig. 7).

Stability of CDH. It was observed during production of

TABLE 3. CDH activity with various sugar substrates[a]

Substrate[b]	Activity (U mg^{-1})	
	CDH 6.4	CDH 4.2
Cellobiose (glcβ1,4-glc)	3.6 ± 0.51	25 ± 0.4
Cellotriose	1.6 ± 0.14	17 ± 0.4
Lactose (glcβ1,4-gal)	1.8 ± 0.17	3.1 ± 0.0
Mannitol	0.34 ± 0.03	0
Sorbose	0.36 ± 0.06	0.1 ± 0.004
Raffinose	0.49 ± 0.11	0
Cellulose oligomers	0	ND[c]
Bacterial cellulose	0	0
Whatman cellulose	0	0
Solka Floc cellulose	0	0

[a] The electron acceptor was TBBQ in 100 mM Na acetate (pH 4.5). The results are the means of duplicate determinations ± standard deviations. In addition to the substrates shown, D-glucose, L-arabinose, D-fructose, D-galactose, D-gentobiose, D-gluconate, glycerol, glucuronate, sorbitol, D-xylose, sucrose, maltose, and D-mannose were tried and could not serve as electron donors for CDH in this short-term assay.

[b] The cellulose preparations (Solka floc, bacterial cellulose, Whatman cellulose) and cellulo-oligosaccharides (cellotetraose, -pentaose, -hexaose, and -heptaose) were tested for their ability to reduce CDH 6.4. Although none of these polymers gave significant amounts of TBBQ reduction in a 5- to 10-min assay, TBBQ was significantly reduced by CDH 6.4 incubated with cellulose over longer time periods (6 to 24 h).

[c] ND, not determined.

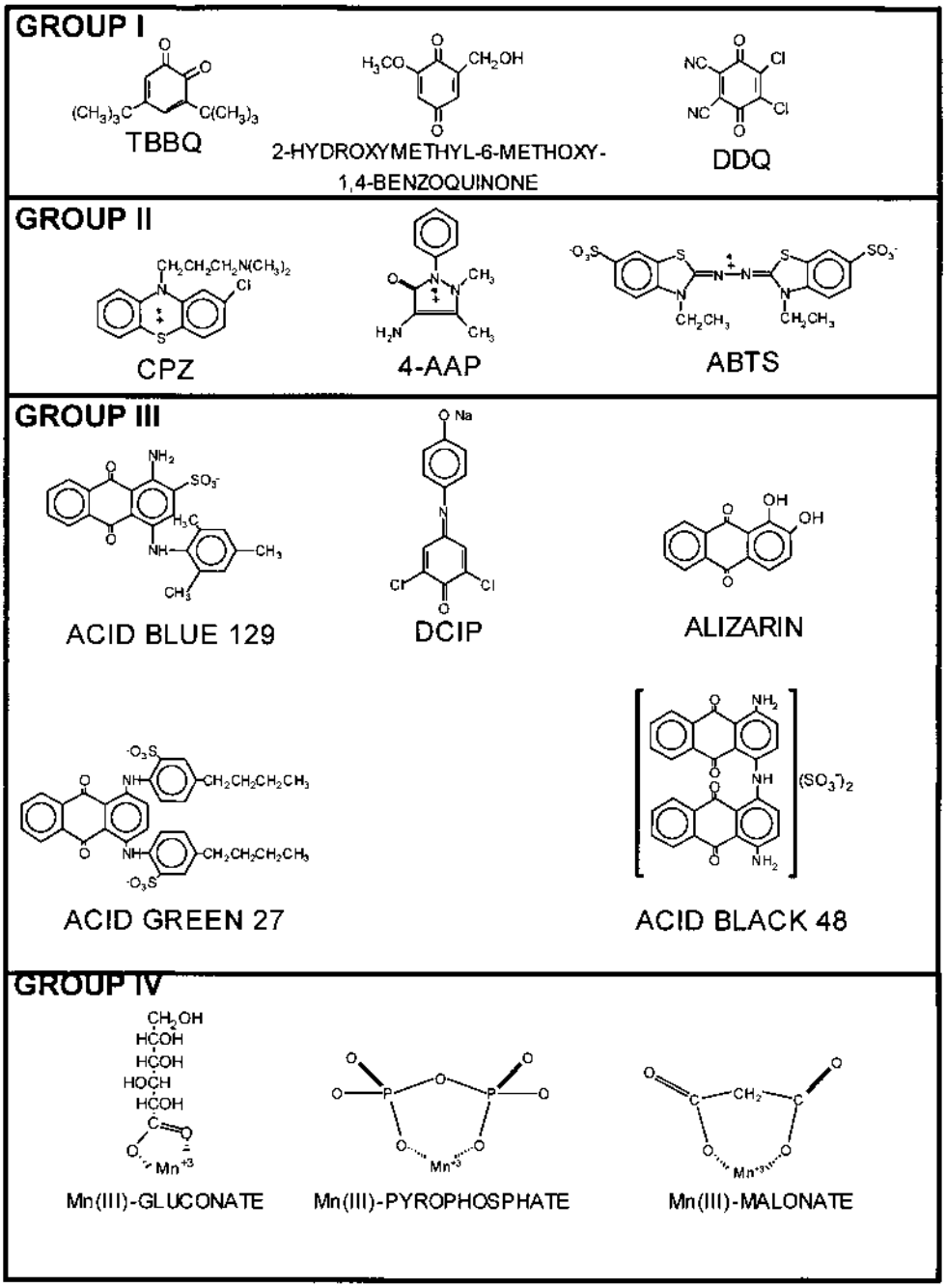


FIG. 6. Structures of compounds used to evaluate the substrate specificity of CDH. Structures are classified as simple single aromatic ring quinones (group I), phenthiazoline radicals (group II), multiple aromatic ring quinones (group III), and metal-organic acid complexes (group IV). The free radical forms of the group II compounds are the substrates for CDH. See Table 4 for the complete names that are abbreviated here.

CDH in the carboys that early CDH production was largely CDH 4.2 (as determined by cytochrome *c* reduction) but that by day 6 or 7 it was mostly CDH 6.4 and that the latter was much less stable through the purification procedure. From days 3 to 7 or 8 of the carboy cultures of 52J, CDH activity rose at an increasing rate, peaking at 13 to 17 chlorpromazine U/ml, followed by a "crash" in which virtually all CDH activity disappeared within 8 to 10 h. All of this is consistent with proteolytic degradation of CDH 4.2 to CDH 6.4 and finally to inactivity. The studies of Habu et al. (25), Wood and Wood (58), and Eggert et al. (18) have shown that in the *P. chrysosporium* system, secreted proteases cleave the heme-flavin CDH (analogous to *T. versicolor* CDH 4.2) to the flavin-only CBQ (analogous to *T. versicolor* CDH 6.4) and an inactive heme domain. The heme domain of purified CDH 4.2 from *T. versicolor* 52J was separated from the enzyme by binding the protein to a Mono Q column by use of a pH 7.5 K phosphate buffer (20 mM). The proteins were then eluted with a linear gradient of the same buffer (0 to 0.5 M), which resulted in the elution of two protein peaks. Approximately 1/10 of the total

TABLE 4. Reduction of various substrates by CDH 4.2

Substrate (abbreviation)	Substrate concn (mM)	Wavelength measured (nm)[a]	Extinction coefficient (mM^{-1} cm^{-1})	No. of turnovers (s^{-1})
3,5-Di-*tert*-butyl-1,2-benzoquinone (TBBQ)	0.33	420	1.4	25
4-Aminoantipyrine (4-AAP)	2.0	520	6.4	22
ABTS (ox)	1.0	420	36	54
Acid green 27	0.05–0.10[b]	631		0[c]
Acid blue 129	0.15–0.03[b]	629		0
Acid black 48	0.03–0.06[b]	666		0
2-Hydroxymethyl-6-dimethoxy-1,4-benzoquinone	0.33	430	1	8.1
2,3-Dichloro-5,6-dicyano-1,4 benzoquinone (DDQ)	0.33	440	1.1	0.2
Mn(III)-Pp_i	2.0	478	0.104	53
Mn(III)-malonate[d]	0.3	270	9.0[e]	87
MnO_2[f]	2.0	478	0.104	11
Mn(III)-gluconate	0.4	265	8.4	80
Chlorpromazine (CPZ)	0.1	520	11.8	48
Alizarin	0.2	420	0.8	0.7

[a] Wavelength at which reduction was measured with the experimentally determined extinction coefficients to calculate CDH activity.
[b] Extinction coefficients for the acid green, acid blue, and acid black dyes were not determined since these dyes are not of uniform composition nor do they completely dissolve in ethanol. Estimates of their concentrations in the assay were based on manufacturer's claimed dye contents of 40% (acid black 48), 25% (acid blue 129), and 65% (acid green 27). Each was added to give a final dye concentration of 150 μM. The ranges shown assume 50 to 100% dye solubilization.
[c] 0, no activity detected during a standard 15-min cuvette assay.
[d] This extinction coefficient is from reference 11.
[e] Some difficulty was encountered in reproducing this extinction coefficient, probably as a result of the formation of mono-, di-, and tri-malonate–Mn(III) complexes (6a). Wariishi et al. reported a value of 11.59 (54).
[f] Data are taken from reference 47.

TBBQ-reducing activity of the native enzyme was recovered in the peak which eluted first from the ion-exchange column. When the absorbance profile of each peak was recorded, the later-eluting peak had a visible absorbance profile very similar to that of the heme peak of the native enzyme with a much smaller A_{280} (A_{418}/A_{280} ratio of about 6.49 compared with 0.53 to 0.59 for the intact enzyme). The rest of the enzymatic activity was not recovered in the eluate. The earlier-eluting nonheme protein peak had a specific CDH activity of 4.2 U mg of $protein^{-1}$ with TBBQ as the substrate. This was very similar to the specific activity of CDH 6.4 recovered from the *T. versicolor* culture supernatant. Recombining the two eluted protein peaks, both with and without dialysis, did not restore or increase the specific activity of the dissociated CDH 4.2 protein.

Molecular modeling of quinone selectivity. To better understand the reactions of CDH with *ortho*-quinones, molecular modeling was used to predict the reactivities of the ring oxygens as the functional groups were changed. The effect of these substituents on the predicted reactivity of each of the *ortho*-quinones and their observed reactivity with CDH 4.2 were compared. First, the minimum energy conformations of each of these quinones was determined. Then, by use of this conformation, the lowest unoccupied molecular orbital and highest occupied molecular orbital for each of the quinone substrates were calculated. This information was used to determine the following properties of each of the *ortho*-quinones: (i) the band gap of the ring oxygens; (ii) an electron density map of each quinone; and (iii) an estimation of the electrophilicity of each ring oxygen (Fig. 8).

No large differences in the electrophilicity and electron density maps of these *ortho*-quinones were predicted by these modeling studies. However, a comparison of the experimentally determined reactivities of these substrates with CDH (measured as the V_{max} values) with the ring oxygen band gap values (calculated from the predicted lowest unoccupied molecular orbital values and highest occupied molecular orbital

TABLE 5. Determination of kinetic parameters for a number of CDH 4.2 substrates

Substrate[a]	Wavelength monitored (nm)	Extinction coefficient (mM^{-1} cm^{-1})[b]	Apparent K_m (μM)	V_{max} (mol min^{-1} mg^{-1})	No. of turnovers (s^{-1})[c]
TBBQ[d]	418	1.36	26	25[d]	25
3,4-Dimethyl-1,2-benzoquinone	416	0.42	20	26.2	20
3-Methoxy-5-methyl-1,2-benzoquinone	460	0.50	16,980	2,540	111
4-Methyl-1,2-benzoquinone	380	0.57	NR[e]	NR	0
4-*tert*-Octyl-1,2-benzoquinone	400	1.10	80.7	6.5	13
4-*tert*-Butyl-1,2-benzoquinone	400	0.35	2,370	66.9	41
3,4,5-Trimethyl-1,2-benzoquinone	430	0.16	NR	NR	0
DCIP	520	2.50	11	36	37
2,3-Dimethoxy-1,4-benzoquinone	410	0.73	1,103	9.1	4.1

[a] Substrates were in 100 mM Na acetate buffer (pH 4.5).
[b] Extinction coefficients were determined in the assay buffer system (50 mM Na acetate [pH 4.5] containing 20% [vol/vol] ethanol).
[c] Determined at a substrate concentration equal to the K_m for that substrate.
[d] In comparison to "good" CDH 4.2 substrates such as TBBQ, a rate of 0.0019 mol of O_2 reduced per min per mg was measured at a concentration of 210 μM O_2 (air saturation) at 25°C.
[e] NR, no reaction detectable over 30 min.

TABLE 6. Comparison of the reductive activities of CDH 4.2 and CDH 6.4[a]

Substrate	λ (nm)	CDH 4.2			CDH 6.4		
		K_m (μM)	V_{max} (μmol/min/ml)	k_{cat} (s^{-1})	K_m (μM)	V_{max} (μmol/min/ml)	k_{cat} (s^{-1})
Cellobiose[b]	520	120	6.3	6.1	220	1.9	1.5
DCIP	520	7.8	5.0	4.8	30.8	1.8	1.4
Cytochrome *c*	415	7.8	10.5	10.2		0	0
$Fe(CN)_6^{3-}$	420	110	60	5.3		0	0
Fe(III)	510	2.4	2.3	2.2	480	0.18	0.2
Cu(II)	483	0.89	2.6	2.5	123	0.81	0.7
O_2			<0.08	<0.07		<0.08	<0.07

[a] Reaction mixtures contained 2 mM cellobiose–50 mM Na acetate (pH 4.5) to which various concentrations of substrate were added.
[b] DCIP (0.2 mM) was used as the reduced substrate.

values) were somewhat correlated (Fig. 8). The correlation between band gap and reactivity was improved when the data for the *para*-quinones and the two *ortho*-quinones which did not react with CDH were excluded from the data set (Fig. 8). There is a trend that suggests that the reactivity of these quinones with CDH is dependent upon the band gap of the ring oxygens. The large differences between the *para*- and *ortho*-quinones can be explained by the large differences in their molecular geometry. The nonreactive *ortho*-quinones may have geometries which restricted the access of these substrates to the active site of CDH 4.2. Molecular modeling and kinetic studies with model quinones may thus prove to be a useful tool for predicting the range of *ortho*-quinone substrates that can be reduced by a CDH protein. They will not, however, explain why the enzyme exhibits such high selectivity among *ortho*-quinones and such low selectivity among other electron acceptors, readily reducing negatively charged, positively charged, and neutral species (Fig. 6).

Pulp binding of CDH. Both CDH 4.2 and CDH 6.4 bound readily to cellulosic substrates, including hardwood and softwood kraft pulps and purified cellulose in 10 mM Na acetate buffer (pH 4.5). This binding was reduced by the presence of 0.1 M NaCl from 80 to 90% to 10 to 30% (data not shown). Thus, it is likely that a significant fraction of the CDH activity in *T. versicolor* cultures becomes associated with pulp fibers and is not present in the culture supernatant; i.e., measured supernatant CDH levels in biobleaching cultures may greatly underestimate the amount of CDH actually secreted and functioning.

DISCUSSION

Archibald has demonstrated that all of the essential components of the *T. versicolor* biobleaching system are present in the extracellular supernatant of actively bleaching cultures (6). Although oxidative enzymes such as MnP and laccase do carry out a useful level of hardwood and softwood kraft pulp delignification, their effect is not as extensive as that observed when whole fungal cultures are used (12, 40). One hypothesis posits that a catalytic oxidative and reductive cycling of lignin substrates which favors a net depolymerization of lignin is established by ligninolytic cultures of *T. versicolor*. Similar catalytic schemes have been proposed by other investigators for the in vitro depolymerization of lignosulfonates by peroxidases and glucose oxidase (24, 41).

CDH is the only extracellular enzyme known in *T. versicolor* biobleaching cultures that can reduce aromatic substrates and organic free radicals. *T. versicolor* 52J secreted CDH isozymes into the culture medium under nitrogen-sufficient conditions when either cellulose or cellobiose was the carbon source, conditions similar to those under which biobleaching is observed (1, 39, 42, 45).

The reductive activities of these two enzymes are very similar to those of the equivalent enzymes isolated from *P. chrysosporium* (Table 7) (8, 10, 37, 38, 49, 55). A notable difference of the *T. versicolor* heme-flavin enzyme from those of other fungi is its inability to reduce oxygen to hydrogen peroxide. In addition, there is some difference in the apparent MW of the CDH proteins isolated from *T. versicolor* and *P. chrysosporium*. CDH 4.2 from *T. versicolor* gives a molecular mass of 97 kDa on SDS-PAGE gels (data not shown), somewhat larger than most reports of the equivalent enzyme from *P. chrysosporium*, which generally give an apparent MW of around 90,000 (21, 30, 58). However, other groups have reported the MW of *P. chrysosporium* CDH to be 98,000 (49) and as high as 102,000 by SDS-PAGE (8). Recently, Li et al. (34) have reported a calculated molecular mass of 80,115 Da for *P. chrysosporium* CDH on the basis of a cDNA sequence of 2,319 bp (773 amino acids, including an 18-amino-acid signal peptide). The discrepancy between the apparent MW of the CDH enzyme of *P. chrysosporium* as measured by SDS-PAGE and as calculated from the cDNA sequence is probably due to glycosylation. A range of MW values for the cellobiose:quinone oxidoreductase (flavin only) enzyme of *P. chrysosporium* has also been reported, from about 60,000 (21, 58) to 75,000 (49). The equivalent enzyme from *T. versicolor*, the flavin-only CDH 6.4, migrates on SDS-PAGE gels with an apparent MW of 81,000 (data not shown).

Purified preparations of the two quinone-reducing enzymes (Table 2) yielded two proteins with a 10-fold difference in their specific activities when TBBQ was used as the quinone substrate. The smaller, flavin-only enzyme, CDH 6.4, showed lower activity toward all substrates tested compared with the larger, heme-flavin CDH 4.2. Moreover, CDH 6.4 was unable to reduce cytochrome *c* or ferricyanide ions under the conditions tested and reduced Cu(II), Fe(III), or Mn(III) only very slowly, while CDH 4.2 rapidly and efficiently reduced all of them. When CDH 6.4 reduced Fe(III) and Cu(II), the K_m values were in the millimolar range and rates of catalysis were low, compared with the micromolar K_m values and rapid catalysis by CDH 4.2 (Table 6). The concentrations of these ions are likely to be in the micromolar range under physiological conditions. Thus, the presence of CDH 4.2 may render all three metals more bioavailable, produce lignin-oxidizing Mn(III) directly, and promote Fe(II) and Cu(I)-driven Fenton's chemistry. In a Fenton's reaction, Fe(II) or Cu(I) (26) reacts rapidly with H_2O_2 to form Fe(III) or Cu(II) and the hydroxyl free radical (·OH). This radical has been implicated in cleavage reactions of both cellulose (27, 51) and lignin (52). Although CDH from *T. versicolor* has been shown to not generate hydrogen peroxide, even under extended exposure to pure O_2, some peroxide is certainly present under delignifying conditions since it is required to sustain MnP activity. The reduction of Mn(III)-malonate by CDH may be important in dampening Mn(III) oxidative activity on lignin; thus, in addition to supporting MnP activity by providing Mn(II) from insoluble Mn(IV) (47), CDH may dampen the effects of MnP activity.

The lower specific activity, yield, and molecular weight of CDH 6.4 compared with that of CDH 4.2 may be because CDH 6.4, like the *P. chrysosporium* flavin-only cellobiose:quinone oxidoreductase (25), is formed from the larger heme-flavin protein by proteolysis and heme loss. CDH 4.2 may fragment because (i) the heme domain of the protein is not

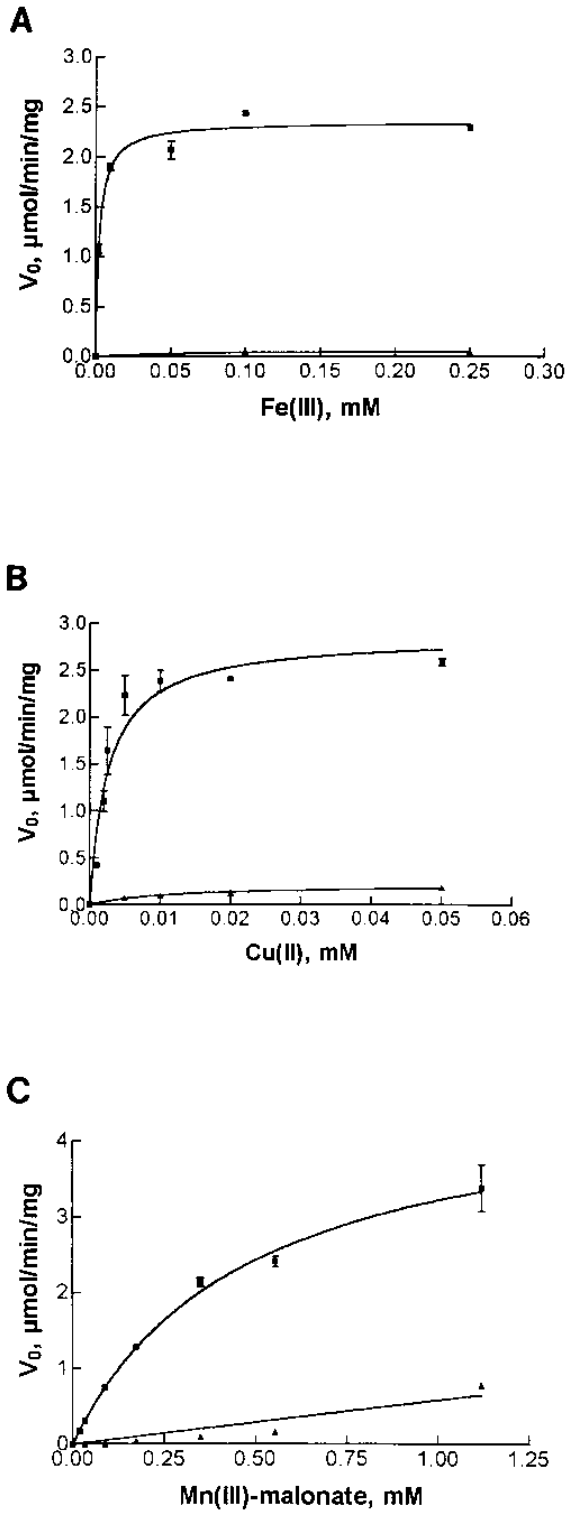


FIG. 7. Reduction of ferric, cupric, and manganic ions by CDH 4.2 and CDH 6.4. (A) Reduction of Fe(III) by CDH 4.2 and CDH 6.4. Assays were performed as described in Materials and Methods. To each mixture was added 10 μg of CDH 4.2 (■) or 30 μg of CDH 6.4 (▲), and the appearance of Fe(II) was monitored at 510 nm. Results were calculated by use of an experimentally determined extinction coefficient of 16.2 mM^{-1}. (B) Reduction of Cu(II) by CDH 4.2 and CDH 6.4. Assays were performed as described in Materials and Methods. To each mixture was added 5 μg of CDH 4.2 (■) or 10 μg of CDH 6.4 (▲), and the appearance of Cu(I) was monitored at 483 nm. Results were calculated by use of an extinction coefficient of 12.25 mM^{-1}. (C) Reduction of Mn(III)-malonate by CDH 4.2 and CDH 6.4. Each reaction mixture contained 50 mM Na acetate (pH 4.5), 2 mM cellobiose, and Mn(III)-malonate (prepared as described in Materials and Methods). To each mixture was added 10 μg of CDH 4.2 (■) or 20 μg of CDH 6.4 (▲). The reduction of Mn(III)-malonate was monitored at 270 nm.

covalently linked to the rest of the protein and spontaneously dissociates, (ii) the protein has an autoproteolytic activity, or (iii) CDH 4.2 is partially degraded by a protease. The first two possibilities are not likely, since purified CDH 4.2 is quite stable at 4°C in dilute buffer. In *P. chrysosporium*, the heme and flavin domains are reportedly joined by a protease-sensitive region since not only proteases from this fungus (25) but also staphylococcal V8 protease (58) and papain (30) cleave the protein to yield heme- and flavin-containing fragments. Recently, Eggert et al. (18) have shown that at least three proteases from *P. chrysosporium* can cleave cellulose-bound CDH. Two of these proteases (designated II and III) cleave CDH in the presence of cellobiose alone, which suggests that the enzyme is susceptible to proteolysis only in its reduced form when its quinone or free-radical substrates are absent. If in the *T. versicolor* system CDH 6.4 is indeed a proteolysis product of CDH 4.2, then this cleavage could serve to modulate the activity of CDH 4.2. This could be especially relevant in terms of the Fenton's reaction; the ability of CDH to sustain a Fenton's reaction by reducing ferric and cupric ions to ferrous and cuprous ions would be greatly decreased under physiological conditions by the cleavage of CDH 4.2 to CDH 6.4. If, however, CDH 6.4 is transcribed from a separate gene, then the smaller enzyme may be playing another role in lignocellulose degradation. The possible proteolytic cleavage of CDH 4.2 to CDH 6.4 is currently under investigation.

The heme-containing CDH of *P. chrysosporium* has been thought to serve primarily as a generator of H_2O_2 (8); however, our results (Table 6) for the *T. versicolor* enzyme and those of others for *P. chrysosporium* (57) are not consistent with this hypothesis. Both CDH 4.2 and CDH 6.4 reduce quinones, organic radicals, and Fe, Cu, and Mn complexes, but neither reduces O_2 to O_2^- or to H_2O_2 (Tables 4 and 5). Although the list of quinones reduced by *T. versicolor* CDH is extensive, many *para*-quinones are only very slowly reduced, unlike most *ortho*-quinones, cation radicals, and Fe(III), Cu(II), and Mn(III) complexes, which were readily attacked (Tables 4 and 5; Fig. 8).

Conclusions. The following conclusions can be drawn. (i) *T. versicolor* CDH 4.2 is similar to the reported CDH proteins from *P. chrysosporium* in having FAD and heme cofactors, being of similar size, binding to cellulose, and being able to reduce many quinones, organic radicals, and metal ions. It differs at least in its inability to reduce O_2 to H_2O_2 in the presence of a suitable electron donor.

(ii) CDH 4.2 and CDH 6.4 specifically oxidize β1-4-linked glucose polymers. By using one of these as an electron source, CDH can reduce a broad range of quinone and nonquinone substrates. These CDH proteins are, however, unable to rapidly reduce some bulky *para*-quinones.

(iii) CDH 6.4 has a FAD cofactor only and is less efficient at catalyzing all substrates tested than is CDH 4.2. Compared with CDH 4.2, CDH 6.4 is almost inactive in reducing Fe(III), Cu(II), and Mn(III) complexes. As appears to be the case with the *P. chrysosporium* flavin-only CDH, CDH 6.4 may be a proteolysis product of CDH 4.2.

(iv) Neither protein is a useful generator of hydrogen peroxide, but both bind to, oxidize, and are induced by cellulose and kraft pulp.

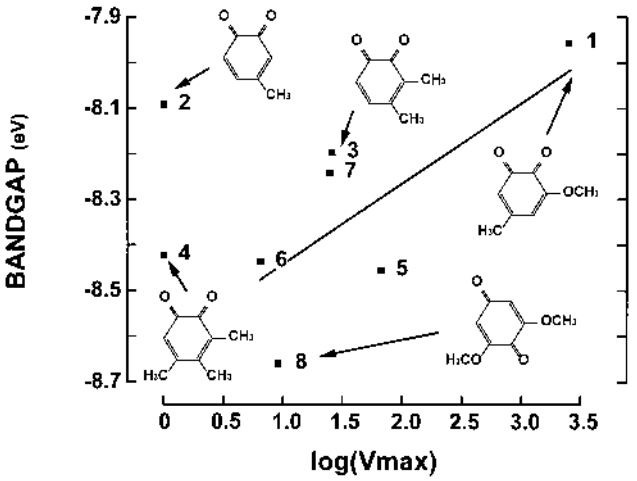


FIG. 8. Modeling of synthesis of *ortho*-quinones and estimation of band gap. The procedure was as follows. (i) Begin with simplest precursor (e.g., *ortho*-benzoquinone). (ii) Minimize total energy with molecular mechanics (MM+) by the conjugate gradient method (Fletcher-Reeves). (iii) Refine geometry with by the conjugate gradient method to obtain the lowest energy conformer. (iv) Add substituent. (v) Repeat steps ii and iii. (vi) Repeat steps iv and v until molecule is completed. (vii) Repeat, by the conjugant gradient method, for stereocenters (if present).

(v) *T. versicolor* CDH 4.2 may complement MnP activities in delignification by (a) oxidizing cellobiose, cello-oligomers, and cellulose to effective Mn-complexing agents; (b) reducing MnO_2 to Mn(II) and Mn(III), thus rendering Mn available to MnP as well as inducing MnP; (c) producing Mn(III) complexes directly, and; (d) producing phenolic substrates for MnP. Moreover, CDH 4.2 may dampen Mn(III) oxidative activity toward lignin by reducing the ion to Mn(II).

(vi) By reducing Mn(IV) and Mn(III) to Mn(II) and by reducing Cu(II) and Fe(III) to Cu(I) and Fe(II), CDH 4.2 can effectively solubilize and render more available these essential metals in the extracellular milieu.

(vii) CDH 4.2 may promote cellulose and hemicellulose polymer degradation by (a) reducing and oxidation and (b) by promotion of ·OH-mediated polymer cleavage via Fe(III) and Cu(II) reduction in the presence of hydrogen peroxide.

(viii) The temperature of usable pH ranges of CDH 4.2 are dependent to a significant extent on the nature of the reduced substrate.

TABLE 7. Comparison of *T. versicolor* CDH to the analogous *P. chrysosporium* proteins

Characteristic	*T. versicolor*[a]		*P. chrysosporium*[b]	
	CDH 4.2 (heme-FAD)	CDH 6.4 (FAD)	CDH or CBO (heme-FAD)	CBQ (FAD)
Molecular mass (kDa)	97[c]	81[c]	90	55
A_{420}/A_{280}	0.57		0.63	
pI	4.2	6.4	4.18	5.45
Temp (°C optimum)	50–60	50–60	50	NR[d]
pH (optimum)	4.5–5.0	ND[e]	5	NR
Reduction of:				
I_3^-				
K_m (μM)	850	ND	0.2	0.3
k_{cat} (s^{-1})	2.2	ND	17	14
Cytochrome *c*				
K_m (μM)	7.8	0	0.3	0.3?
k_{cat} (s^{-1})	10.2	0	13	0.07
$Fe(CN)_6^{3-}$				
K_m (μM)	110	0	7,000	4,000
k_{cat} (s^{-1})	5.2	0	5.5	1.0

[a] All assays were done at 23°C in 50 mM Na acetate buffer (pH 4.5) in a volume of 1 ml.
[b] From Henriksson et al. (29). CBO, cellobiose oxidase; CBQ, cellobiose: quinone oxidoreductase.
[c] MW measured by SDS-PAGE.
[d] NR, not reported.
[e] ND, not determined.

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