Cellobiose Dehydrogenase from the Ligninolytic Basidiomycete *Ceriporiopsis subvermispora*[∇]

Wolfgang Harreither,¹ Christoph Sygmond,¹ Evelyn Dünhofen,^{1†} Rafael Vicuña,²
Dietmar Haltrich,¹ and Roland Ludwig^{1,3*}

Department of Food Sciences and Technology, Division of Food Biotechnology, BOKU University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria¹; Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, and Instituto Milenio de Biología Fundamental y Aplicada, Santiago, Chile²; and Research Centre Applied Biocatalysis, A-8010 Graz, Austria³

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Cellobiose dehydrogenase (CDH), an extracellular flavocytochrome produced by several wood-degrading fungi, was detected in cultures of the selective delignifier *Ceriporiopsis subvermispora* when grown on a cellulose- and yeast extract-based liquid medium. CDH amounted to up to 2.5% of total extracellular protein during latter phases of the cultivation and thus suggested an important function for the fungus under the given conditions. The enzyme was purified 44-fold to apparent homogeneity. It was found to be present in two glycoforms of 98 kDa and 87 kDa with carbohydrate contents of 16 and 4%, respectively. The isoelectric point of both glycoforms is around 3.0, differing by 0.1 units, which is the most acidic value so far reported for a CDH. By using degenerated primers of known CDH sequences, one *cdh* gene was found in the genomic DNA, cloned, and sequenced. Alignment of the 774-amino-acid protein sequence revealed a high similarity to CDH from other white rot fungi. One notable difference was found in the longer interdomain peptide linker, which might affect the interdomain electron transfer at higher temperatures. The preferred substrate of *C. subvermispora* CDH is cellobiose, while glucose conversion is strongly discriminated by a 155,000-fold-lower catalytic efficiency. This is a typical feature of a basidiomycete CDH, as are the acidic pH optima for all tested electron acceptors in the range from 2.5 to 4.5.

White rot fungi are the most efficient lignocellulose degraders in our ecosystem, and several species, e.g., *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Ceriporiopsis subvermispora*, have been studied in great detail as model organisms for this complex process. The ability to degrade phenolic and non-phenolic lignin structures in wood has made these strains attractive for biotechnological applications mainly in the pulp and paper industry, where *C. subvermispora* exhibits a substantial advantage over *P. chrysosporium* and *T. versicolor* through its ability for selective removal of a large fraction of lignin without attacking the valuable cellulose (16, 38). The lignin-degrading system of these fungi is composed of extracellular enzymes together with low-molecular-mass cofactors (21, 46). Typically found ligninolytic enzymes are lignin peroxidase, manganese peroxidase (MnP), and laccase. The secretion pattern of these enzymes varies greatly in white rot fungi (22) and is influenced by culture conditions and medium composition. Whereas *P. chrysosporium* secretes high lignin and manganese peroxidase activities but no laccase activity (32, 33), *C. subvermispora* produces several MnP and laccase isoforms but no lignin peroxidase. *T. versicolor* is the only one of these model organisms known so far to express all three of these ligninolytic enzymes efficiently (5). Together with the cellulolytic enzyme system, these patterns of enzyme activities cause varied de-

grees of lignin and cellulose breakdown at different cultivation stages. The simultaneous attack of cellulose and lignin is the preferred strategy of *T. versicolor*, whereas *C. subvermispora* is a selective delignifier in the first stages of biotreatment, secreting only low activities of cellulolytic enzymes at a late culture stage (12, 23), and apparently lacks cellobiohydrolase activity (23).

Cellobiose dehydrogenase (CDH; EC1.1.99.18; cellobiose (acceptor) 1-oxidoreductase) is an extracellular flavocytochrome secreted by some white rot and brown rot plant pathogenic and saprotrophic fungi from the dicaryotic phyla Basidiomycota and Ascomycota (50). It shows a strong preference for cellobiose and cello-oligosaccharides, which are oxidized to the corresponding lactones during the reductive half-reaction of the FAD cofactor, and further hydrolyze to aldonic acids in the bulk water. In the oxidative half-reaction FAD transfers two reduction equivalents to either one two-electron acceptor, e.g., various quinones, or to two one-electron acceptors, like complexed Fe(III) or Mn(II) ions. At low pH values (usually below 5.5), the heme cofactor can be involved in the electron transfer to one-electron acceptors. Even though CDH has been studied for a considerable time, the exact role and function of the two prosthetic groups are not fully understood. The pH optima with most electron acceptors are rather acidic, but oxygen, although a poor electron acceptor, is also reduced to H₂O₂ under neutral and alkaline conditions (30).

In recent years CDH was shown to participate in the ligninolytic or cellulolytic metabolism of white rot fungi (3, 10, 24, 26, 50). The currently favored mechanism is the production of hydroxyl radicals through Fenton reaction chemistry by the ability of CDH to reduce Fe³⁺ to Fe²⁺ and to produce H₂O₂

* Corresponding author. Mailing address: Department für Lebensmittelwissenschaften und Technologie, Universität für Bodenkultur, Muthgasse 18/2/71, A-1190 Vienna, Austria. Phone: 431 36006 6280. Fax: 431 36006 6251. E-mail: roland.ludwig@b-a.at.

† Present address: AGES PharmMed, A-1030 Vienna, Austria.

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(28, 31, 36, 37). CDH is believed to be involved in early stages of cellulose breakdown: knocking out the *cdh* gene in *T. versicolor* did not considerably affect its ability to grow on amorphous cellulosic substrates, while it could not grow on crystalline cellulose or recalcitrant substrates such as birch wood (13).

Interestingly, no CDH activity has been reported so far from cultures of *C. subvermispora*, even though it is closely related to other white rotters producing this enzyme, e.g., *Trametes* spp. (35, 41) or *Pycnoporus cinnabarinus* (45). It has been speculated that the lack of CDH might contribute to the selectivity of *C. subvermispora* in degrading lignin while growing on wood. It was therefore the aim of our study to show unequivocally whether *C. subvermispora* carries a *cdh* gene and can produce the enzyme under certain growth conditions.

MATERIALS AND METHODS

Organism and culture conditions. Stock cultures of *Ceriporiopsis subvermispora* FP-90031 (Center for Mycology Research, Forest Products Laboratory, Madison, WI) were maintained at 30°C in the dark on potato dextrose agar plates and periodically subcultured. For the production of CDH, a medium containing 30 g liter⁻¹ yeast extract (Fluka), 30 g liter⁻¹ α-cellulose (Sigma), 1 g liter⁻¹ MgSO₄ · 7H₂O, and 0.3 ml liter⁻¹ trace element solution (42) was used. Alternative medium components were peptone from meat (VWR), soybean (Fluka), or casein (Fluka) as nitrogen sources and glucose as carbon source. Prior to autoclaving the pH of the medium was adjusted to 5.4 with phosphoric acid. Shaking flask cultures were performed in 1-liter baffled flasks containing 300 ml medium, inoculated with 3 cm² of finely cut agar plugs from 5-day-old cultures. These were cultivated at 30°C with continuous agitation (eccentricity, 2.5 cm) at 110 rpm for up to 30 days. CDH production was carried out in a 40-liter bioreactor (Applikon) filled with 30 liters of medium. The pH was titrated to 5.4 before sterilization by using phosphoric acid but was allowed to float freely during the cultivation. The cultivation was started by adding 1.5 liters (5%, vol/vol) preculture from 7-day-old shaking flasks. The cultivation temperature was 30°C, the airflow rate was 30 liter min⁻¹, and the agitation was 150 rpm (three Rushton turbines; tip speed, 0.79 m s⁻¹). Samples were taken regularly and clarified by centrifugation, and the CDH activity and extracellular protein concentration were assayed in the supernatant.

Enzyme purification. Mycelium and residual cellulose particles were removed from the fermentation broth by centrifugation (6,000 × g for 15 min). The clear supernatant was concentrated and diafiltered to remove low-molecular-weight medium components by using a polyethersulfone hollow fiber cross-flow module with a cutoff of 10 kDa (Microza UF module SLP-1053; Pall Corporation) until a conductivity of 2 mS cm⁻¹ was reached. The concentrated enzyme solution was applied to a DEAE-Sepharose fast flow column (XK50/70; 1-liter bed volume), equilibrated with 50 mM sodium acetate buffer, pH 5.5. Proteins were eluted with a linear salt gradient in the same acetate buffer (0 to 0.5 M NaCl in 5.4 column volumes [CV]). Fractions containing CDH activity were pooled, and ammonium sulfate was added to a 20% saturation. The sample was loaded onto a PHE Sepharose fast flow column (HR26/20) equilibrated with 100 mM sodium acetate buffer, pH 5.5, containing 0.2 M NaCl and ammonium sulfate (20% saturation). Proteins were eluted with a linear gradient of 20 to 0% ammonium sulfate saturation in two CV, and fractions with CDH activity were pooled. After diafiltration using a polyethersulfone flat stack cross-flow module with a cutoff of 10 kDa (Vivaflow 50; Vivascience AG) to a conductivity of 5 mS in 50 mM sodium acetate buffer, pH 5.5, the sample was purified in five consecutive runs on a Mono Q column (1-ml bed volume) equilibrated in the same buffer. CDH activity was eluted with an increasing salt gradient (0 to 1 M NaCl in 15 CV), and the CDH-containing fractions of all runs were pooled, aliquoted, and frozen. All chromatographic material and equipment was from GE Healthcare Biosciences.

Deglycosylation. Homogeneous CDH samples from *C. subvermispora* were treated with PNGase F (New England Biolabs) according to the manufacturer's instructions. Briefly, CDH (5 mg ml⁻¹; 20 μl) was mixed with 2 μl of 10× glycoprotein denaturing buffer (80.5% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol) and incubated at 97°C for 10 min. Then, 4 μl of 10× G7 reaction buffer (0.5 M sodium phosphate buffer, pH 7.5), 4 μl of nonionic nonylphenol detergent (NP-40; 10%), and 1 μl PNGase F were added and incubated at 37°C for 1 hour.

Electrophoretic analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Hoefer Mighty Small SE 250 vertical electrophore-

sis unit. Gels (10.5 by 10 cm; 10% T, 2.7% C) were cast and run according to the manufacturer's modifications of the Laemmli procedure. Proteins were visualized by silver staining (4). Isoelectric focusing in the range of 2.5 to 10 was performed on a Multiphor II system using precast, dry gels (Clean gel) rehydrated with carrier ampholytes. The broad-range pI marker protein kit (pH 3 to 10) was used to determine the pI values. CDH bands were visualized by active staining. To this end, the gel was first soaked in 3 mM 2,6-dichloroindophenol (DCIP) solution, then in 200 mM sodium acetate buffer, pH 6.0, before adding 300 mM lactose solution onto the surface of the gel. Shortly after, colorless bands appeared in the purple-stained gel because of DCIP reduction. Gels were then transferred into 20% acetic acid, fixed overnight, and stained with Coomassie blue R-250 according to the manual. All electrophoretic equipment and consumables were from GE Healthcare Biosciences.

Enzyme assays and protein determination. The DCIP assay was performed by measuring the time-dependent reduction of 300 μM DCIP at a wavelength of 520 nm and 30°C in 86 mM sodium acetate buffer, pH 4.0, containing 30 mM lactose and 4 mM NaF to inhibit laccase activity (6). The absorption coefficient for DCIP is pH dependent; however, the absorbance differs only by about 3% at 520 nm in the pH range of 3.0 to 8.0 and was determined to be 6.8 mM⁻¹ cm⁻¹ (30). This assay measures the activity of intact CDH as well as of the catalytically active flavin domain (50). Alternatively, CDH activity was specifically determined by following the reduction of 20 μM cytochrome *c* (cyt *c*; ε₅₅₀, 19.6 mM⁻¹ cm⁻¹) (11) at 30°C in 86 mM sodium acetate buffer, pH 4, containing 30 mM lactose. This assay only determines the activity of the intact protein containing both the flavin and the heme domain. One unit of enzymatic activity was defined as the amount of enzyme that oxidizes 1 μmol of lactose per min under the assay conditions. The protein concentration was determined by the method of Bradford (9) using a prefabricated assay from Bio-Rad Laboratories (Hercules, CA) and bovine serum albumin as the standard.

Steady-state kinetics. Initial rates for the determination of pH profiles and kinetic constants were determined at 30°C in sodium citrate buffer in the pH range of 2.5 to 6.0 (pH values are indicated in the tables), with a final buffer concentration in the cuvette ranging from 26 to 86 mM. Stock solutions of carbohydrates used for kinetic measurements were prepared in the appropriate buffer and allowed to stand for at least 1 h to allow mutarotation, while stock solutions of electron acceptors were prepared in water and used immediately. Kinetic constants for electron acceptors were determined at their pH optimum using 10 mM cellobiose as electron donor. In addition to DCIP and cyt *c*, 1,4-benzoquinone (ε₂₉₀, 2.24 mM⁻¹ cm⁻¹), ferricenium hexafluorophosphate (ε₄₀₀, 19.6 mM⁻¹ cm⁻¹), and potassium ferricyanide (ε₄₂₀, 0.98 mM⁻¹ cm⁻¹) were used. The reaction stoichiometry is 1 for the two-electron acceptors DCIP and 1,4-benzoquinone but 2 for the one-electron acceptors cyt *c*, ferricenium, and ferricyanide. Initial reaction rates and *k*_{cat} values of electron acceptors represent the turnover of the respective electron acceptor, not of cellobiose. All kinetic constants were calculated using nonlinear least-squares regression by fitting the observed data to the Michaelis-Menten equation. Because of the presence of two CDH glycoforms, *k*_{cat} values were calculated using the molecular mass of the nonglycosylated protein. The temperature profiles were determined by measuring the average activity over 5 min in preheated DCIP or cyt *c* standard assays from 21 to 70°C.

Spectral characterization. The spectrum of homogeneous CDH was recorded from 250 to 650 nm in both the oxidized and reduced state using an Agilent 8453 diode array spectrophotometer. CDH was diluted in 50 mM sodium citrate buffer, pH 4.0, to an absorbance of ~1.6 at 280 nm, and the spectrum recorded before and shortly after the addition of an approximately 1,000-fold molar excess of lactose to the cuvette.

Isolation of CDH-encoding genomic DNA and cDNA. Mycelia for nucleic acid isolation were harvested from cellulose-induced growing cultures after 9 days, when CDH activity was detected for the first time. After filtration, mycelia were frozen in liquid nitrogen and homogenized using a mortar and pestle. Portions of 100 mg of mycelia were used for DNA extraction (34). PCRs were done either with GoTaq polymerase (Promega) or, for the amplification of the full-length cDNA, with the Phusion high-fidelity DNA polymerase (New England Biolabs), deoxynucleoside triphosphate mix (Fermentas), oligonucleotide primers (VBC Biotech), and a Biometra TRIO thermocycler (Biometra). PCR fragments were cloned into the pCR 2.1 TOPO or the pCR Blunt II TOPO vector (Invitrogen) according to the manufacturer's manual and sequenced by a commercial service provider (AGOWA).

The amino acid sequences of known basidiomycete CDHs were used to generate conserved sequence blocks with the program Block Maker (http://blocks.fhcr.org/blocks/make_blocks.html). These blocks provided a basis for the program CODEHOP (<http://blocks.fhcr.org/blocks/codehop.html>) to design degenerated primers. Primer CDHfw1 [5'-CGACCACCGTCACTCCAC(A

FIG. 1. Cultivation of *C. subvermispora* in a stirred bioreactor. The pH (black line) was allowed to float freely. Dissolved oxygen tension (gray line), protein concentration (black line, filled squares), DCIP activity (black line, filled circles) and cytochrome *c* activity (black line, empty circles) in the culture supernatant were measured.

GCT)A(CT)TGGA-3'] and CDHrv1 [5'-CGTAAGCGTCGATGGAAGG(A)TG(AGCT)GT(AG)AA-3'] were used to amplify a 1.6-kb-long fragment of genomic DNA. Sequencing of the fragment revealed high similarity to basidiomycete CDHs. For the amplification of the adjacent upstream region the DNA Walking SpeedUp premix kit (Seegene) was used. Three target-specific reverse primers (TSP1, 5'-GATATTCCTCCGCTTGCCAG-3'; TSP2, 5'-CCTGAGC GTGTATGTATCG-3'; TSP3, 5'-GGCAGCGGTATACATACTTCC-3') were designed and used together with the DNA walking-annealing control primer and a universal primer, which are part of the kit, to perform the PCRs according to the manufacturer's guidelines. For the amplification of the 3' region with the CERfw1 primer (5'-GATGTTCTTCGCACTCGACG-3') and universal primer (5'-GGCCACGCGTCTGACTAGTAC-3'), cDNA was used as a template. RNA was isolated using the Spectrum Plant total RNA kit (Sigma). cDNA first-strand synthesis was performed with the high-capacity cDNA reverse transcription kit and the anchor primer (5'-GGCCACGCGTCTGACTAGTACTTTTITTTTITTTT-3'). To obtain a full-length cDNA clone encoding the CDH protein, a nested PCR with two forward primers, CERfw2 (5'-CTTATCAGCTGGCGTTCACG-3') and CERfw3 (5'-TACTGGATGCCGAGCAGGATGC-3'), specific for a sequence upstream of the putative start codon, and two reverse primers, CERrv1 (5'-GCGAAACACGT ATTCCAATCG-3') and CERrv2 (5'-CAGGACTGCCGACAGACTT-3'), specific for a sequence shortly downstream of the stop codon, was done.

Analysis of gene and protein sequences. Analysis of the N-terminal signal sequence peptidase cleavage site was done with the programs SignalP and TargetP hosted at the ExPASy Proteomics server (www.expasy.ch) (7, 14, 39). The molecular mass and the isoelectric point of mature CDH were calculated with the program Compute pI/M_w hosted by the ExPASy Proteomics server. Potential N-glycosylation sites were determined with NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc) (8). Sequence similarity was determined using the BLAST algorithm (2).

Verification of strain identity. The identity of strain FP-90031 was checked by sequencing the internal transcribed spacer (ITS) region, a target to assess phylogenetic relationships of closely related taxa. Two primers, ITS1-F (5'-CTTG GTCATTTAGAGGAAGTAA-3') and ITS4-B (5'-CAGGAGACTTGTACAC GGTCCAG-3'), which are optimized for basidiomycetes (18), were used for the amplification of a 700-bp-long ITS region from fungal DNA. The obtained PCR

fragment was sequenced and compared to the known ITS sequence from *C. subvermispora* ATCC 90467 available at GenBank (accession number FJ545252) and revealed a 100% identity to the published sequence.

Nucleotide sequence accession numbers. The obtained PCR fragment of the ITS region was sequenced and submitted to GenBank (accession number FJ713106). The complete *C. subvermispora* *cdh* gene was also deposited at GenBank (accession number EU660051).

RESULTS

Cultivation. CDH production by *C. subvermispora* was first studied in shaking flask experiments using a medium based on cellulose and yeast extract. In these cultures a first CDH maximum was observed after 16 days of growth (130 U liter⁻¹; extracellular protein, 1.5 g liter⁻¹), and then a short phase of decrease (minimum 19th day, 100 U liter⁻¹, 0.7 g liter⁻¹) was followed by a second maximum (23rd day, 125 U liter⁻¹, 1 g liter⁻¹). This type of oscillation has also been observed in CDH-producing cultures of *Heterobasidium annosum* (27). It was found that yeast extract can be replaced by peptone from meat, which yields the same CDH activity, but not by peptone from soybean or casein, with which the enzyme yield was reduced 5- and 10-fold, respectively. Especially peptone from casein did not support good growth of the fungus. In shaking flask experiments using glucose as carbon source, growth was fast and low CDH activities (30 to 50 U liter⁻¹) were found after 10 to 11 days when the glucose was consumed.

In bioreactor cultivations, growth was fast after an initial lag phase of 12 h, which was deduced from the high oxygen consumption (Fig. 1). The oxygen transfer rate of the reactor was sufficient to hold 30% dissolved oxygen concentration until the third day, when the oxygen demand of the culture became too high and resulted in almost complete oxygen depletion. Similar to the shaking flask experiments, the pH decreased to 4.5 after 4 days and remained constant for 2 days until it increased again, as did the dissolved oxygen concentration. The cultivation was terminated at this point to prevent autolysis and proteolytic cleavage of CDH. CDH activity was detected after 5 days and reached a volumetric activity of 170 U liter⁻¹ after 8 days. DCIP and cytochrome *c* activity were in very good agreement during this cultivation, which indicates that mainly intact CDH carrying the heme domain is present and proteolytic cleavage to the DCIP-active flavin fragment is negligible.

Purification. *C. subvermispora* CDH was purified by a three-step chromatographic procedure using a weak anion exchanger, followed by a hydrophobic interaction step and a strong anion exchanger (Table 1). The enzyme was purified 44.1-fold from the extracellular supernatant with a yield of 18.4%. The activity losses after the first chromatographic step

TABLE 1. Purification of CDH from *C. subvermispora*

Purification step	Total protein (mg)	Total activity ^a (μmol min ⁻¹)	Sp act (U mg ⁻¹)	A ₄₂₀ /A ₂₈₀ ^b	Yield (%)	Purification (fold)
Culture supernatant	12,170	5,640	0.46		100	1
DEAE-Sepharose	1,930	3,030	1.57	0.16	53.7	3.4
Phenyl source	135	1,625	12.0	0.43	28.8	26.1
Mono Q	51	1,035	20.3	0.52	18.4	44.1

^a Activity was measured using DCIP and lactose under standard assay conditions; this assay detects both intact CDH and the catalytically active flavin domain after proteolytic cleavage.

^b Absorbance ratio of pooled fractions; the highest measured ratio of a Mono Q fraction was 0.56.

FIG. 2. SDS-PAGE analysis (A) and isoelectric focusing (B) of CDH from *C. subvermispora* (CsCDH). (A) Lanes 1 and 6, molecular mass marker (GE Healthcare); lane 2, purified CsCDH; lane 3, partially purified CsCDH; lane 4, deglycosylated CsCDH; lane 5, purified CDH from *Sclerotium rolsii* (SrCDH); lane 7, deglycosylated SrCDH. (B) Lane 1, activity staining on purified CsCDH with lactose and DCIP; lane 2, same part of the gel after Coomassie 250 R staining; lane 3, pI marker proteins (GE Healthcare).

include also those during the first centrifugation and ultrafiltration steps, whereas losses in the other two steps result mainly from strictly pooling only fractions with the highest specific activities. This purification protocol resulted in a dark red protein solution, the purity of which was assessed by SDS-PAGE (Fig. 2A), which revealed the existence of two CDH isoforms with estimated molecular masses of 98 and 87 kDa. Isoelectric focusing also showed a CDH double band in the otherwise-homogeneous preparation (Fig. 2B). It should be noted that there were no indications of two CDH isoforms during the purification procedure.

Molecular properties. To elucidate the molecular mass and the nature of the CDH isoforms, a homogeneous CDH preparation was treated with PNGase F for the complete removal of glycosidic residues. After the treatment, both CDH bands (98 and 87 kDa) observed by SDS-PAGE (Fig. 2A) merged into a single band with an estimated molecular mass of 84 kDa. This indicates that *C. subvermispora* CDH exists as two glycoforms which are glycosylated to approximately 16% (98-kDa glycoform) and 4% (87-kDa glycoform) on a mass basis. *Sclerotium rolsii* CDH (SrCDH) was used as a positive control in this deglycosylation experiment. The molecular mass difference of glycosylated SrCDH (98 kDa) to the deglycosylated enzyme (84 kDa) results in 14% glycosylation, which nicely correlates with the published value of 15% (6). Isoelectric focusing revealed a very low isoelectric point for both glycoforms, which appeared as a double band with pI values of approximately 3.0 and 3.1 (Fig. 2B). This is the lowest value measured so far for a CDH. The next lowest known value is 3.8 from *Pycnoporus cinnabarinus* CDH (45), which is a phylogenetically close relative. The theoretically calculated (sequence-based) isoelectric points showed the same trend as the native proteins: *T. versicolor* and *P. chrysosporium* have calculated pI values of 4.9 and 5.0, respectively, and a measured value of 4.2. *P. cinnabarinus* has a calculated pI of 4.8. The calculated value for *C. subvermispora* CDH is 4.2 and the measured value is 3.0.

The absorption spectrum of oxidized CDH showed the characteristic peaks of the heme cofactor at 565, 532, and 421 nm (Fig. 3). Reduction with lactose increased the absorbance of

FIG. 3. Spectra of oxidized (black line, peaks at 421, 532, and 565 nm) and reduced (gray line, peaks at 429, 533, and 563 nm) CDH from *C. subvermispora* in 50 mM sodium acetate buffer, pH 5.0. Lactose was used in a 1,000-fold excess to reduce the enzyme. The upper right inset shows the differential spectrum (reduced - oxidized).

these three bands and the maxima of the α - and β -bands shifted slightly to 563 and 533 nm, whereas the Soret band showed the typical bathochromic shift to 429 nm. The FAD cofactor was visible as a shoulder of the Soret band (450 to 500 nm), which disappeared after reduction.

Kinetic properties. The pH dependence of CDH activity was measured for various electron acceptors, and all of them showed an acidic pH optimum. The two-electron acceptors DCIP and 1,4-benzoquinone exhibited rather broad ranges of activity from pH 2.5 to 6.5 with nearly identical turnover rates of the two substrates from pH 4.5 to 6.5 (Fig. 4A). The turnover rate of the anionic electron acceptor DCIP decreases below pH 4.5, whereas 1,4-benzoquinone is most efficiently reduced at pH 3.5. The one-electron acceptor ferricenium is also reduced up to pH 6.5 and has its optimum at 3.5. Cytochrome *c* has an identical pH optimum, but the reduction of this acceptor is limited to pH values below 4.5 (Fig. 4B). It is likely that higher pH values result in electrostatic repulsion of the heme domain and hence in limited interdomain electron transfer. The strong ferricyanide anion shows a monotonously decreasing activity profile, which is highest at 2.5 and ends at 4.5.

The catalytic constants for carbohydrates were measured at pH 4.0, which is a compromise to obtain almost-maximal turnover rates with DCIP (as a typical two-electron acceptor) and cyt *c* (as a strictly heme domain-dependent one-electron acceptor). The results indicate a clear preference of CDH for disaccharide substrates with a β -1,4 linkage (Table 2). Cellobiose, the supposed natural substrate, exhibited the lowest K_M value and the highest catalytic efficiency. Lactose, a structurally similar disaccharide except for the axial position of the C-4 hydroxyl function in the galactose moiety, showed a 30-fold higher K_M value and no substrate inhibition, the slightly higher k_{cat} value being probably a result of this. The structurally unfavorable α -1,4 linkage in maltose resulted in very high K_M values and very low catalytic efficiencies. Similar results were observed for glucose, which is not able to interact with both sugar binding sites (25). The efficient discrimination of glucose conversion is reflected by a 25,000-

FIG. 4. pH profiles of *C. subvermispura* CDH activity. Turnover numbers for the conversion of two-electron acceptors were measured for DCIP (filled circles) and 1,4-benzoquinone (filled squares) (A) and the one-electron acceptors cyt *c* (empty circles), ferricyanide (empty squares), and ferricenium (empty diamonds) using lactose as the electron donor (B).

times-higher K_M value over cellobiose and a 150,000-times-lower catalytic efficiency. Nearly identical kinetic constants were obtained with both electron acceptors used (DCIP and cyt *c*), and only in the case of cellobiose was a slight difference in the K_M and K_I values observed when using cyt *c*.

The reoxidation of CDH was investigated for five electron acceptors, and their catalytic constants were determined at their pH optima (Table 3). The almost-twofold-higher k_{cat} values of the one-electron acceptors over the two-electron acceptors indicate that the rate-limiting step is either the bind-

TABLE 3. Kinetic constants of cellobiose dehydrogenase for some of its electron acceptors

Electron acceptor	Stoichiometry ^a	Assay pH	K_M (μ M)	K_I (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_M (M ⁻¹ s ⁻¹)
DCIP	1	4.5	12		29	2.4×10^6
1,4-Benzoquinone	1	3.5	26		45	1.7×10^6
cyt <i>c</i>	2	3.5	0.94	195	60	64×10^6
Ferricenium	2	3.5	0.32		58	181×10^6
Ferricyanide	2	3.5	11		61	5.5×10^6

^a Moles of electron acceptor reduced per mole of cellobiose oxidized. Cellobiose was used as the second, saturating substrate at concentrations of 10 mM.

ing or oxidation of the carbohydrate and not the binding of the electron acceptor or subsequent electron transfer. The affinity of CDH for electron acceptors is generally high, resulting in low K_M values, especially for ferricenium and cyt *c*. These values yield catalytic efficiencies between 1.7×10^6 and 1.8×10^8 M⁻¹ s⁻¹, revealing a very efficient and almost diffusion-limited reoxidation process.

The interdomain electron transfer and the "temperature stability" of this transfer were investigated using the standard DCIP and cyt *c* assays at different temperatures, which were run for 5 min and the average activity calculated (Fig. 5). The activity of the flavin domain (DCIP activity) increased with temperature until reaching a temperature maximum at 60°C. The activation energy was 18.7 kJ mol⁻¹, which is low in comparison to other values published for CDHs, and was calculated from initial rates in the range of 21 to 50°C. For the heme domain-dependent reaction (cyt *c* activity), the activity increment with higher temperatures was much lower, leveled off at 30°C, and decreased rapidly above 40°C. From the linear increase of the absorbance in cyt *c* assays up to 50°C, which indicates a stable steady-state turnover, it can be concluded that denaturation of the heme domain is not the cause of the decrease in activity, but there is a less efficient interdomain electron transfer between both domains at higher temperatures.

Analysis of gene and protein sequences. The mRNA of *C. subvermispura* CDH contains an open reading frame of 2,325 bp translating into a polypeptide of 775 amino acids. The N-terminal sequence is immediately preceded by a putative

TABLE 2. Kinetic constants of cellobiose dehydrogenase for some carbohydrate substrates

Substrate	Electron acceptor ^a	K_M (mM)	K_I (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M (M ⁻¹ s ⁻¹)
Cellobiose	DCIP	0.14	125	25	179,000
	cyt <i>c</i>	0.082	150	26	325,000
Lactose	DCIP	4.4		28	6,360
	cyt <i>c</i>	4.3		27	6,280
Maltose	DCIP	300		1.03	3.43
	cyt <i>c</i>	280		0.91	3.25
Glucose	DCIP	3,300		3.8	1.15
	cyt <i>c</i>	3,800		3.4	0.89

^a DCIP and cyt *c* were used as second, saturating substrates at concentrations of 300 and 20 μ M, respectively.

FIG. 5. Temperature profiles of *C. subvermispura* CDH activity using either DCIP (pH 4.5; filled circles) or cyt *c* (pH 3.5; empty circles) together with lactose as the substrates.

FIG. 6. Unrooted phylogenetic tree of basidiomycete CDHs. The numbers in the node represent the likelihood output, and the scale bar indicates the branch length corresponding to 0.05 amino acid substitutions per site.

signal peptidase cleavage site between amino acids 18 and 19. The protein is therefore classified with a high probability (0.924; determined with the programs Signal P and Target P) as a secretory protein. The calculated molecular mass of the mature protein is 80,671 Da with an isoelectric point of 4.2. The amino acid sequence of CDH contains nine potential N-glycosylation sites (N128, N140, N287, N400, N453, N463, N532, N612, and N618). However, positions N400, N453, and N463 show a low probability for being glycosylated. The alignment with basidiomycete CDH sequences shows a high similarity of *C. subvermispora* CDH (GenBank accession number EU660051) with other published white rot CDHs. Comparison using the BLAST algorithm showed the highest similarity to *Grifola frondosa* CDH (accession number AB083245; identities, 76%; positives, 85%; gaps, 1%) followed by *Trametes versicolor* (accession number AF029668; identities, 71%; positives, 82%; gaps, 0%), *Pycnoporus cinnabarinus* (accession number AF081574; identities, 71%; positives, 81%; gaps, 2%), and *Phanerochaete chrysosporium* (accession number U46081; identities, 68%; positives, 80%; gaps, 0%). The phylogenetic relationship (Fig. 6) shows a clustering of the white rot sequences, whereas CDH of *Sclerotium rolfsii* (accession number AY187232), a plant pathogen basidiomycete, is clearly separated.

DISCUSSION

Ceriporiopsis subvermispora has been studied in detail because of its remarkable selectivity, that is, lignin is preferentially utilized by this fungus when grown on lignocellulose whereas cellulose is hardly attacked during early phases of growth (16, 38). It has been speculated that *C. subvermispora* is not able to synthesize cellobiose dehydrogenase and that this lack of CDH activity contributes to the poor degradation of cellulose. Although *C. subvermispora* is known to produce some enzymes of the cellulolytic complex, it apparently lacks cellobiohydrolase activity. Furthermore, these cellulolytic ac-

tivities are only produced at later phases of growth and only at relatively low levels (17, 23).

In this study we were able to show unequivocally that *C. subvermispora* both carries a *cdh* gene and actively synthesizes the enzyme under certain growth conditions. CDH is formed in liquid culture on a cellulose-based medium during later phases of growth. Under these conditions the organism formed up to 170 U of CDH activity per liter of culture medium (corresponding to approximately 2.5% of the extracellular protein), which is in good agreement with yields reported for other basidiomycetes. *C. subvermispora* CDH was purified from the culture supernatant to apparent homogeneity. The spectral A_{420}/A_{280} ratio (RZ value) of 0.52 confirms this high purity of the enzyme preparation (6, 29, 35), which could be obtained by the three-step purification protocol established. CDH was rather stable during the purification even though there were losses, especially during cross-flow ultrafiltration, most probably due to high shear stress or binding on membranes. The low yield of only 18%, however, can be partially attributed to strict pooling of the fractions containing CDH activity, using only the fractions that contained the highest specific activities. Hence, the yield was a compromise for the high purity of the enzyme preparation desired. The spectra of the oxidized and reduced *C. subvermispora* CDH are in good agreement with those previously reported for other CDHs, indicating the presence of both the FAD and heme *b* prosthetic group. The preparation contained two different isoforms of CDH, which behaved identically during the chromatographic steps and coeluted and apparently only differ in their glycosylation, as established by enzymatic deglycosylation. There was no indication of a second *cdh* gene when using consensus CDH sequences for constructing the primers employed for isolation of either CDH-encoding genomic DNA or the corresponding mRNA.

The polypeptide encoded by the *cdh* gene showed high identity (68 to 76%) to other CDHs from white rot fungi. These sequences showed the highest amino acid similarity in the flavin domain, whereas most differences appeared in the heme domain and the linker sequence. The linker of *C. subvermispora* CDH is 3 amino acid residues longer (28 amino acids total) than other linker regions from basidiomycete fungi (25 amino acids) when applying the definition of the linker sequence from *P. chrysosporium* CDH (20) (Fig. 7). The high similarities in the N-terminal region before the linker (axial heme ligand) and the C-terminal region after the linker sequence (Rossman fold) emphasize this difference.

Accordingly, these proteins have comparable properties, and CDH from *C. subvermispora* behaves like a typical basidiomycete CDH. This is evident, for example, from the determined catalytic properties. The kinetic constants for carbohydrates correlate well with published values of other white rot CDHs (50). The highest catalytic efficiency was observed for

FIG. 7. Alignment of the linker region of CDH from different white rot fungi.

cellobiose, and glucose was strongly discriminated. Also, the typical substrate inhibition for cellobiose, with a K_I/K_M ratio of $\sim 1,000$, was found. Compared to other basidiomycetes, *C. subvermispora*'s CDH exhibits more acidic pH optima for some electron acceptors, but the K_M values again match very well with the published data. In conclusion, CDH from *C. subvermispora* is a typical basidiomycete CDH with respect to its catalytic properties, which are well-adapted to the acidic pH conditions found in cultures of this fungus.

While the temperature profile of CDH activity with DCIP as the electron acceptor is in general agreement with other CDHs, the profile for cyt *c* activity shows an unusual behavior. At temperatures ranging from 20 to 45°C the turnover rate was hardly affected. However, above 50°C a dramatic decrease of the turnover rate was observed and cyt *c* activity was almost completely abolished at 60°C, while this temperature was the optimum for DCIP for the 5-min assay. This phenomenon has not been observed for any other CDH. Two explanations might be considered for this behavior: instability of the heme domain or an increased mobility of the heme domain at higher temperatures hampering the interdomain electron transfer. Since cyt *c* reduction proceeded linearly during the assay even at elevated temperatures, instability and local unfolding of the heme domain seem unlikely. Indeed, alignment of five different basidiomycete CDH linker regions shows that *C. subvermispora* CDH has a longer interdomain linker, presumably resulting in an increased flexibility and concomitant impaired interdomain interactions. The role of the linker has not been studied in any detail for CDH, but it could be shown for yeast flavocytochromes that the linker (hinge) region is important for interdomain electron transfer (43).

Phylogenetic analysis of several white rot CDHs showed an unexpected clading. CDH from *G. frondosa*, which is from the order of Agaricales rather than the order of Polyporales, like most of the other white rot sources of CDH, showed the highest sequence homology to *C. subvermispora* CDH. Overall, *C. subvermispora* CDH is closely related to CDHs from basidiomycetes and shows typical properties of these class I CDHs, which are characterized by shorter sequences lacking a C-terminal carbohydrate binding module and a more conserved linker region compared to ascomycete class II CDHs (49).

The biological function of CDH has not been elucidated unequivocally, but strong support for engagement in the degradation of polymers by a radical mechanism exists (13, 28). In *C. subvermispora*, CDH could interact beneficially with the wood-degrading system in several ways.

First, it can produce H_2O_2 by the oxidation of cellobiose or cello-oligosaccharides with the concomitant, partial reduction of oxygen. This would increase the activity of peroxidases and enhance ligninolysis (3). However, also other ways for the production of H_2O_2 have been suggested in *C. subvermispora*, like the MnP/Mn^{3+} -dependent extracellular oxidation of glyoxylic and oxalic acids (47) or the action of a periplasmic oxalate oxidase (1). Also, the production of MnP in shaking flask cultures starts during the earliest culture stage and gradually decreases thereafter (15), similar to the stability and reactivity of the manganese ion during cultivations (44), whereas CDH was found at a later stage.

Second, CDH could help to attack lignin in early phases by reducing Fe^{3+} (chelated by, for example, oxalate) to Fe^{2+} ,

which together with H_2O_2 forms the highly reactive hydroxyl radical. This mechanism seems unlikely for *C. subvermispora*, as the production of alkylitaconic (cereporic) acids in the early stages of wood degradation was reported. These cereporic acids inhibit hydroxyl radical production by forming complexes with Fe^{2+} or Fe^{3+} ions (40, 48). However, in later culture stages alkylitaconic acids are not formed anymore and CDH could then support Fenton's chemistry (17, 19). The activity of CDH in the early phases of wood attack by *C. subvermispora* would be additionally hampered by the availability of cello-oligosaccharides, which are supposedly formed at late culture stages when cellulases are secreted. This suggests that in *C. subvermispora* cultures, CDH does not participate in lignin degradation but could help its weak cellulolytic system, lacking cellobiohydrolase, to degrade cellulose at a late culture stage. CDH and reactive radicals formed by the mechanism outlined above could thus have a role in loosening highly crystalline regions of cellulose, which cannot be attacked by endoglucanases and β -glucosidases, the components of the cellulolytic system present in *C. subvermispora*. This attack could explain cellulose depolymerization observed in biopulping experiments despite only very low cellulase activities measured (17). This proposed role in cellulose degradation is supported by the late appearance of CDH activity. Furthermore, CDH activity is formed by *C. subvermispora* on glucose-based media, under derepressed conditions when glucose is exhausted in the culture. This lack of an available carbon source could thus be a signal to synthesize CDH and start the attack on cellulose.

The detection of CDH in *C. subvermispora* leads to new perspectives for the optimization of this organism for biopulping applications. Although a CDH knockout mutant of *T. versicolor* showed a greatly reduced ability to invade wood (13), the situation of a *C. subvermispora* CDH knockout mutant might be different due to the supposedly later expression of its CDH and its proposed role in late phases of growth. Knocking out the *cdh* gene from *C. subvermispora* might thus improve its selectivity for lignin degradation and its applicability for biopulping.

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