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Homologous Expression of Recombinant Cellobiose Dehydrogenase in *Phanerochaete chrysosporium*

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Cellobiose dehydrogenase (CDH) is a novel extracellular hemoflavoenzyme from *Phanerochaete chrysosporium* and is produced only in cultures supplemented with cellulose. In this report, CDH from *P. chrysosporium* has been homologously expressed in cultures supplemented with glucose as the sole carbon source when no endogenous CDH is expressed. This was achieved by placing the *cdh-1* gene under the control of the D-glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter (1.1 kb) fused upstream of the ATG start codon of *cdh-1*. The *gpd* promoter-*chd-1* construct was inserted into the multiple cloning site of the expression vector pOGI18, which contained the *Schizophyllum commune ade5* as a selectable marker. The *P. chrysosporium ade1* auxotrophic strain OGC107-1 was transformed with the pAGC1 construct, and the prototrophic transformants were assayed for CDH activity. Approximately 50% of the Ade$^+$ transformants exhibited CDH activity in the extracellular medium of stationary cultures. At least one of the transformants produced high levels (500–600 U/liter) of recombinant CDH (rCDH). Purification by ammonium sulfate precipitation, Sephacryl S-200 chromatography, and FPLC using a Mono-Q 5/5 column yielded homogeneous rCDH. Physical, spectral, and kinetic characteristics of purified homologously expressed rCDH were similar to those of wild-type CDH. This expression system will enable site-directed mutagenesis studies to be carried out on CDH.

Cellobiose dehydrogenase (CDH) is a novel extracellular hemoflavoenzyme produced by cellulolytic fungi such as *Phanerochaete chrysosporium*, *Sporotrichum thermophile*, *Trametes versicolor*, and *Coniophora puteana* (1–5). All CDHs contain one FAD and one heme b prosthetic group per enzyme molecule. The cofactors are non-covalently bound to distinct domains, which are separable after proteolytic hydrolysis (6, 7). Cellobiose, which is produced from cellulose hydrolysis, is oxidized to cellobionolactone by all CDHs (1–5). Many compounds, including cytochrome *c*, quinones, Mn(III)-complexes, and Fe(III), can function as electron acceptors in this reaction (2, 8, 9). In the absence of these electron acceptors, oxygen functions as an electron acceptor, which itself is reduced to hydrogen peroxide (2, 10). Several findings suggest that CDH is part of the *P. chrysosporium* cellulose-degrading system (11–14). Like cellulases, CDH binds strongly to cellulose (15, 16), and most fungi produce CDH only when the medium is supplemented with cellulose. There is also evidence that CDH enhances crystalline cellulose hydrolysis, possibly by oxidizing cellobiose, which is a competitive inhibitor of cellobiohydrolase (2, 17).

CDH sequences are known for three basidiomycetes [*P. chrysosporium* 18, 19), *T. versicolor* (20), and *Pycnoporus cinnabarinus* (21)] and one thermophilic ascomycete [*S. thermophile* (22)]. All of the sequences show high sequence similarity. CDH from *P. chrysosporium* contains 755 amino acids and has a predicted molecular weight of 80, 115 (18, 19). cDNA sequences support a two-domain organization for CDH from white-rot fungi, consisting of an N-terminal heme domain and a C-terminal flavin domain (18–21).

Several predictions regarding CDH structure have been made based on sequence comparisons (21–23). The heme domain shows little sequence similarity to any known heme protein sequence. Earlier spectroscopic studies suggested that a methionine and a histidine form the fifth and the sixth ligands of the heme iron (24). Comparisons of the available CDH sequences indicate that two histidines (His114 and His163) and one methionine (Met65) are conserved in the heme

Abbreviations used: CDH, cellobiose dehydrogenase; DCPIP, 2,6-dichlorophenol-indophenol; FAD, flavin adenine dinucleotide; gpd, D-glyceraldehyde-3-phosphate dehydrogenase; IEF, isoelectric focusing; LiP, lignin peroxidase; MnP, manganese peroxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; wtCDH, wild-type CDH.

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domain. Of those two histidine residues, only the sequence surrounding His114 of *P. chrysosporium* is conserved (21, 22). Based on this comparison, Met65 and His114 were proposed to function as the axial ligands for the heme iron (21, 22). The flavin domain of CDHs shows ~50% sequence similarity with a group of FAD-dependent enzymes belonging to the glucose-methanol-choline oxidoreductase family (25). Members of this family include glucose oxidase from *Aspergillus niger,* choline dehydrogenase from *Escherichia coli,* glucose dehydrogenase from *Drosophila melanogaster,* and methanol oxidase from *Pichia augusta.* Multiple sequence alignment of CDH with the oxidoreductases revealed a unique hydrophilic stretch (XRXPXTDX-PSXDGXRY) in the flavin domain of CDH (22). Glucose-methanol-choline oxidoreductases possess a conserved His and Asn near the C-terminal flavin domain, proposed to be involved in enzyme catalysis (23). CDH also contains the conserved His and Asn in the C-terminus of the flavin domain (22).

Confirmation of these structural predictions by site-directed mutagenesis will require an efficient expression system. The homologous expression of manganese peroxidase (MnP) and lignin peroxidase (LiP) from *P. chrysosporium* has been achieved (26, 27). Both peroxidases are involved in lignin degradation and are expressed only under secondary metabolic conditions triggered by the depletion of nutrient nitrogen (28). Homologous expression of MnP and LiP in the presence of high nitrogen concentrations was achieved by fusing the coding region of *mnp* and *lip* to the D-glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter in a plasmid containing a selectable auxotrophic marker such as adenine or uracil biosynthetic genes (26, 27). Several mutants of MnP have been expressed successfully using this system (29–32). Expression of catalytically active MnP and LiP in the extracellular fluid suggested that heme insertion, protein folding, and protein secretion proceed normally in the transformants. This report describes the successful homologous expression of *cdh* in *P. chrysosporium* accomplished by placing the *cdh-1* gene under the control of the *gpd* promoter.

EXPERIMENTAL PROCEDURES

Organism. Growth and maintenance of *P. chrysosporium* wild-type strain OGC101 and auxotrophic strain OGC107-1 (Ade1) were as described previously (33). *Escherichia coli* XL1-Blue, used in subcloning, was obtained from Stratagene (La Jolla, CA).

Construction and expression of vector pAGC1. The *cdh-1* PCR fragment was prepared using Vent DNA polymerase, *cdh-1* as template, a forward primer (5′-CGCGGCATGCTAGGTCGATCGT) starting at the ATG translation codon, and a reverse primer (5′-CGCGGAATTCGCGAGTGGTGGCG) 150 bp downstream of the TGA stop signal. The resulting fragment (3.15 kb) contained a unique *Sph*I site at the 5′ end, a unique *Eco*RI site at the 3′ end, the entire *cdh* coding region, and 150 bp of the 3′ non-coding region. The fragment was digested with *Sph*I and *Eco*RI. It was then fused in a three-way ligation to a 1.16-kb *Xba*I-*Nsp*I fragment of the *gpd* promoter containing the ATG translation codon at the 3′-end (26), and the transformation vector pOGI18, digested with *Eco*RI and *Xba*I (Fig. 1). The cohesive ends generated from *Nsp*I and *Sph*I restriction are compatible.

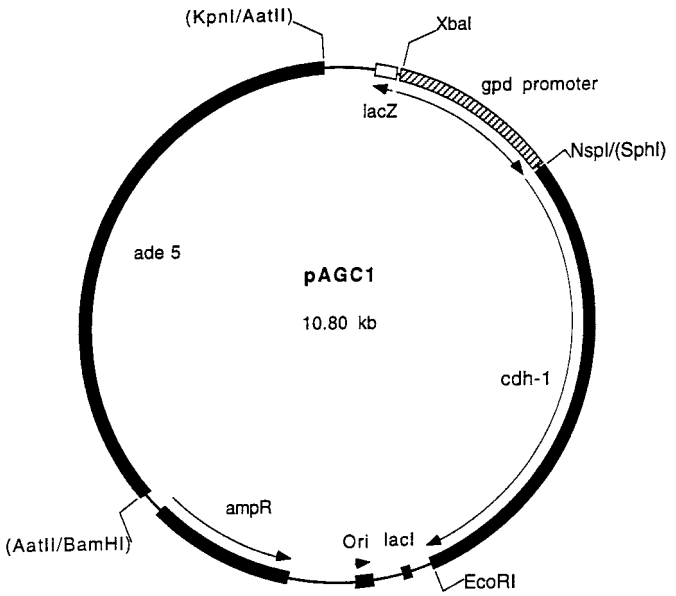

FIG. 1. Restriction map of the pAGC1 expression vector, containing the *ade5* gene and the *gpd* promoter fused to *cdh-1* at the ATG translation initiation site. Only selected restriction sites are presented.

P. chrysosporium transformations. Protocols for the preparation and transformation of protoplasts of *P. chrysosporium* auxotrophic strain OGC107-1 (Ade1) were as described (33, 34). The adenine auxotroph was induced to fruit on one-sixth-strength modified Vogel's medium, supplemented with 4.5% acid-treated cellulose and 0.01% adenine (1). The spores were washed from the lids of fruiting plates with modified Vogel's medium (pH 4.8), supplemented with 3% malt extract and 0.15% yeast extract. Spores were swollen at 36°C for 4 h with shaking (150 rpm) and the protoplasted using Novozyme 234 (10 mg/ml) and Cellulase P (10 mg/ml) as described (26, 33, 34). Protoplasts (10^6 cells) were transformed with 5 μg of *Eco*RI-linearized pOGI18 or pAGC1 as described (26, 35). Prototrophic transformants were grown on minimal medium containing asparagine and glucose and were purified by isolating single basidiospores (33–35).

Screening for rCDH expression. Conidiospores of isolated pAGC1 transformants were inoculated into 250-ml Erlenmeyer flasks containing 20 ml of a medium as described (36), supplemented with 2% glucose, 12 mM ammonium tartrate, 0.2% tryptone, and 20 mM sodium-2,2-dimethyl succinate (pH 6.0). The cultures were incubated without shaking at 37°C, and the extracellular medium was monitored periodically for CDH activity using the cytochrome *c* assay (2). The assay mixture contained extracellular medium (0.2 ml), cellobiose (400 μM), and cytochrome *c* (12.5 μM) in 20 mM succinate buffer (pH 4.5) in a total volume of 1 ml.

Production of rCDH in stationary and shaking cultures. A pAGC1 transformant, which produced high levels of CDH in glucose cultures, was selected for the production of rCDH. The transformant was grown from conidial inocula at 37°C in 20-ml stationary cultures in 250-ml Erlenmeyer flasks. The medium composition was as described (36), except that it contained 2% glucose, 12 mM ammonium

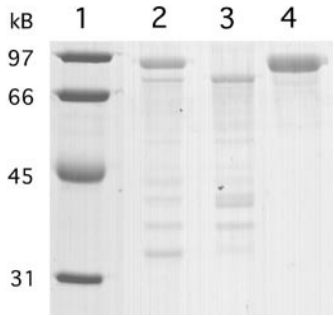

FIG. 2. SDS-PAGE of extracellular proteins (3 mg) from 9-day-old cultures of wild-type and pAGC1 transformed strains of *P. chrysosporium*. Lane 1, molecular weight markers; lane 2, pAGC1 transformant; lane 3, wild-type strain; and lane 4, purified CDH (90,000 D). Strains were cultured in 2% glucose-supplemented cultures, and on day 9 the extracellular medium was filtered, dialyzed, and concentrated prior to gel loading.

tartrate, 20 mM sodium 2,2-dimethyl succinate (pH 6), and 0.2% tryptone.

The medium composition of the shaking cultures was the same as for the stationary cultures, described above. The mycelial mats from four 2-day-old stationary cultures were homogenized for 15 sec and added to a 2-liter flask containing 1 liter of medium. The cultures were incubated at 37°C with shaking (150 rpm). CDH expression was monitored using the cytochrome *c* assay (2).

Purification of rCDH. Extracellular medium (480 ml) from 7-day-old stationary cultures (30 flasks) was filtered through Miracloth (Calbiochem, La Jolla, CA). The medium was treated with protease inhibitors, 5 mM EDTA, and 0.5 mM phenylmethyl sulfonyl fluoride. The filtrate was concentrated and dialyzed against 50 mM potassium phosphate (pH 7) using an Amicon Diaflo ultrafiltration system (Millipore Corp., Bedford, MA). Proteins were precipitated with ammonium sulfate (410 g/liter), and the precipitation was allowed to proceed overnight at 4°C. The resulting precipitate was separated by centrifugation and dialyzed against 50 mM potassium phosphate (pH 6) and concentrated using an Amicon ultrafiltration unit with a PM-10 membrane. The concentrate was then applied to a Sephacryl S-200 column (65 × 2.5 cm, Pharmacia LKB Biotechnology, Alameda, CA) equilibrated with 50 mM potassium phosphate (pH 6). Fractions containing rCDH activity were pooled, dialyzed against 10 mM Tris-HCl (pH 8), concentrated, and further purified by FPLC using a Mono-Q HR 5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ). FPLC separations were performed in 10 mM Tris-HCl (pH 8), and rCDH was eluted with a 0–1 M NaCl gradient (20 ml).

Spectroscopic and kinetic procedures. The UV-visible absorption spectra and assays were determined using a Shimadzu Model 260 spectrophotometer. The kinetics of cellobiose oxidation were determined by monitoring ferrocytochrome *c* formation at 550 nm (ϵ = 28 mM^{-1} cm^{-1}). The assays contained a fixed level of ferricytochrome *c* (12.5 μM) and varying levels of cellobiose (5–400 μM) in 20 mM succinate (pH 4.5). The kinetics of cytochrome *c* and DCPIP reduction were determined in a similar fashion, with a fixed cellobiose concentration (400 μM) and variable cytochrome *c* and DCPIP concentrations (0.2–20 μM). Cytochrome *c* and DCPIP reductions were monitored at 550 nm (ϵ = 28 mM^{-1} cm^{-1}) and 515 nm (ϵ = 6.8 mM^{-1} cm^{-1}), respectively.

SDS-PAGE and isoelectric focusing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% or 12.5% Tris-glycine system in a Miniprotean II apparatus (Bio-Rad, Hercules, CA). The gels were stained with 0.1% Coomassie blue in 40% methanol-10% acetic acid solution. Isoelectric focusing (IEF) was performed using the Pharmacia Phastsystem with IEF Phastgels (pH 3–9) (Sigma Chemical Company, St. Louis, MO), and the gel was stained with Gelcode blue stain reagent (Pierce, Rockford, IL). The protein standards (IEF Mix 3.6-6.6) were obtained from Sigma.

Estimation of protein, heme, and flavin. Protein concentrations were determined by the bicinchoninic acid method (37). Heme was estimated by the pyridine hemochromogen method (38). For estimation of flavin content, homogeneous CDH was treated with trichloroacetic acid; denatured protein was removed by centrifugation and the supernatant was estimated for flavin at 450 nm. The amount of flavin released was calculated from a standard curve for FAD (2).

Chemicals. Glucose and potassium phosphate were obtained from J. T. Baker (Phillipsburg, NJ). Molecular Biology reagents were obtained from New England Biolabs (Beverly, MA). Novozyme 234 and cellulase CP were obtained from Interspec Products, Inc. (Foster City, CA). All other biochemicals were purchased from Sigma.

RESULTS AND DISCUSSION

CDH is a novel hemoflavoenzyme produced by cellulolytic fungi (1–5). Little is known about the structure and underlying mechanisms of this unique enzyme. Development of an efficient expression system would permit further examination of structural features such as heme ligands, the conserved CDH-specific motif in the flavin domain, and the putative cellulose-binding site (21, 22). Homologous expression of CDH in *P. chrysosporium* was considered because of our recent successes in the expression of rLiP, rMnP, and several

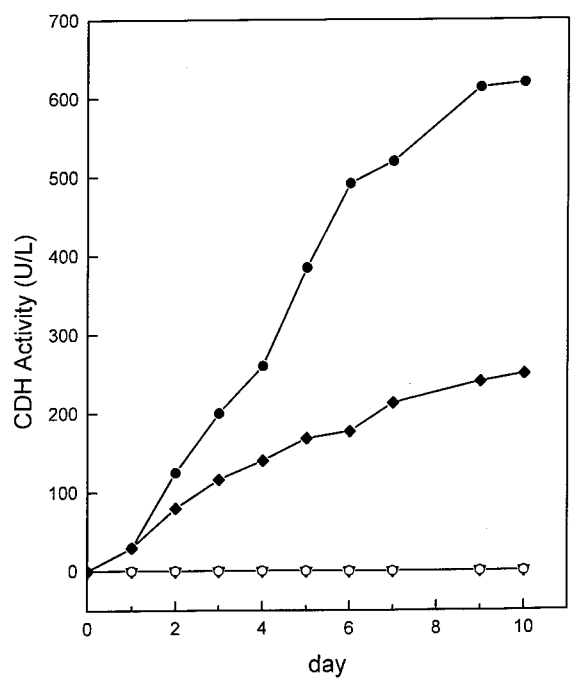

FIG. 3. Production of CDH in primary metabolic cultures of *P. chrysosporium*. Stationary cultures: pAGC1 transformant (●), pOGI18 transformant (▼), wild-type strain (○). Shaking cultures; pAGC1 (◆). Stationary and shaking cultures were grown with 2% glucose as carbon source. CDH activity was monitored using cytochrome *c* in 20 mM succinate (pH 4.5).

TABLE 1

Purification of Recombinant CDH from *P. chrysosporium*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Extracellular medium	143	285	2.0	100	1.0
$(NH_4)_2SO_4$	73	251	3.4	88	1.7
Sephacryl S-200	30	208	6.5	73	3.3
Mono-Q (FPLC)	11	91	8.0	32	4.0

mutants of rMnP (26, 27, 29–32). Wild-type LiP and MnP are expressed only under secondary metabolic conditions, triggered by the depletion of nutrient nitrogen. By placing *lip* or *mnp* genes under the control of a *gpd* promoter. LiP and MnP expression was achieved under primary metabolic conditions when the wild-type enzymes were not expressed. Previous whole culture experiments and Northern blot analyses suggested that *P. chrysosporium* produces CDH only in cultures that are supplemented with cellulose as an inducer (18). Thus, placement of the *cdh* gene under the control of a *gpd* promoter was expected to result in rCDH production in glucose-supplemented cultures when wild-type CDH (wtCDH) expression does not occur. Furthermore, by including the native signal peptide sequence in the expression vector, secretion of rCDH was expected (26, 27). CDH has two cofactors, a heme and a flavin (1–5); proper insertion of stoichiometric amounts of these cofactors is necessary to obtain active CDH. Since the heme proteins MnP and LiP are secreted in active form (26, 27), we anticipated the CDH would also be secreted in active form. CDH from *P. chrysosporium* is encoded by two alleles (*cdh-1* and *cdh-2*) of a single gene (39). Both alleles have 14 exons, and the 13 introns are located at the same positions in the two alleles. The alleles show 97% sequence similarity and differ at 86 locations. However, the translation products of the two alleles are identical, because the changes are found only in the third base of a codon (39). Sequence analysis of several CDH cDNA clones suggested that both alleles are expressed (39). In this report, *cdh-1* was selected for homologous expression.

Transformation of the *P. chrysosporium* Ade1 auxotroph with the *Eco*RI linearized *cdh* expression vector, pAGC1, produced 17 Ade^+ transformants. These were screened for extracellular CDH activity by growing the transformants individually in 20-ml stationary cultures, supplemented with glucose. Seven of the cultures exhibited significant CDH activity, and one of the transformants, which expressed CDH at high levels, was studied further. Comparison of the SDS-PAGE profiles of the extracellular proteins from the high-yielding pAGC1 transformant and the wild-type strain confirmed that the 90,000 molecular-weight band corresponding to CDH molecular weight is produced only in the pAGC1-transformed *P. chrysosporium* (Fig. 2). Time courses for the appearance of secreted CDH activity in stationary cultures of the pAGC1 transformant, a pOGI18 transformant, and wild-type *P. chrysosporium* strain OGC101 are shown in Fig. 3. Cytochrome *c* reduction indicative of active CDH was observed only in the pAGC1 transformant over the 10-day period. Only the extracellular medium from shaking or stationary culture of pAGC1 transformant, over a 10-day period, exhibited cytochrome *c* reduction in the presence of cellobiose, which was indicative of active CDH production. Cytochrome *c* reduction activity was not detectable either in the wild-type strain or the pOGI18 transformant, which did not have the *cdh-1* insert. All of the above evidence strongly suggests that the CDH activity observed in pAGC1 cultures must be due to recombinant CDH. Expression of rCDH is apparently affected by the culturing method. Stationary cultures produced 600 U/liter of CDH in 9 days, whereas shaking cultures in the same period produced only 240 U/liter (Fig. 3).

The lower yield in shaking cultures may be due to a combination of factors, including a lower level of rCDH production and secretion, proteolytic hydrolysis of rCDH, or the instability of rCDH under shaking conditions. Based on the cytochrome *c* activity and protein assay, rCDH constituted approximately 25% of the total extracellular protein. SDS-PAGE analysis of total extracellular proteins also suggested that rCDH is the predominant protein in the extracellular medium (Fig. 2). The level of rCDH production in glucose cultures was approximately 15-fold higher than that of rMnP or rLiP production (26, 27). This may be due to the inherent stability of CDH (2).

rCDH from stationary cultures of the pAGC1 transformant was purified in three steps, involving ammonium sulfate precipitation, size exclusion, and anion-exchange chromatography (Table 1). The R_z value (A_{420}/A_{280}) of the purified rCDH was ~0.55. The specific activity, measured with cytochrome *c* as the electron acceptor, was 8 U/mg. SDS-PAGE (Fig. 3A) showed a single band with a molecular mass of 90 kDa. Isoelectric focusing indicated a major and a minor protein band around a pI of 3.6. These characteristics are very similar to those of wtCDH, suggesting that rCDH is

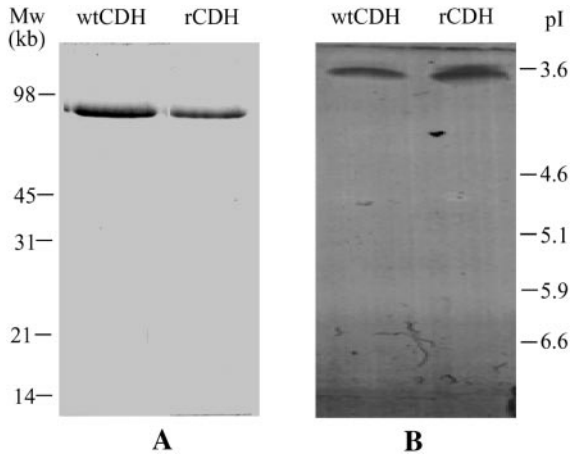

FIG. 4. (A) SDS-PAGE of wtCDH and rCDH. (B) Isoelectric focusing of wtCDH and rCDH.

homogeneous and that the post-translational processing of rCDH, including folding, insertion of prosthetic groups, signal peptide cleavage, and glycosylation, is similar to that of the wtCDH.

The UV-visible absorption spectrum of oxidized rCDH exhibits a Soret maximum at 420 nm, a shoulder at 450 nm, and two visible bands at 529 and 570 nm (Fig. 4). The shape and intensities of absorption bands are very similar to those of wtCDH. The ratio of protein to flavin to heme in the expressed CDH was approximately 1:1:1. The steady-state kinetic parameters (k_{cat}, K_m and K_{cat}/K_m) for cytochrome *c* and DCPIP reduction and cellobiose oxidation were similar to the values determined for the wtCDH (Table 2). The similarity of the spectral and kinetic properties of rCDH and wtCDH suggests that heme b and FAD were correctly incorporated into the protein (Fig. 5).

In summary, homologous expression of rCDH in *P. chrysosporium* has been successfully achieved. To our knowledge, this is the first report of the recombinant expression of CDH in any organism. CDH mutants that will be useful in protein engineering studies are now being prepared using this expression system.

TABLE 2

Steady-State Parameters for rCDH and wtCDH from *P. chrysosporium*[a]

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
CDH	cellobiose	11.1	22.0	$5.0 \cdot 10^5$
wtCDH	cellobiose	12.0	25.0	$4.8 \cdot 10^5$
CDH	cytochrome *c*	11.7	0.7	$1.7 \cdot 10^6$
wtCDH	cytochrome *c*	12.0	0.7	$1.7 \cdot 10^6$
CDH	DCPIP	24.0	2.3	$1.0 \cdot 10^6$
wtCDH	DCPIP	23.1	3.8	$6.1 \cdot 10^6$

[a] Reactions were performed in 20 mM sodium succinate (pH 4.5). K_m and k_{cat} for cellobiose were determined using 12.5 μM cytochrome *c*. K_m and k_{cat} for cytochrome *c* were determined using 400 μM cellobiose. K_m and k_{cat} for DCPIP were determined using 400 μM cellobiose.

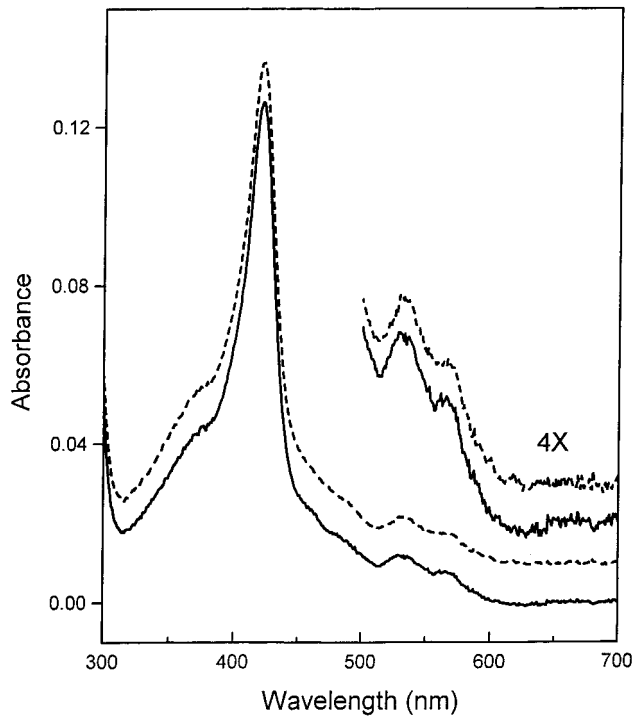

FIG. 5. Absorption spectra of rCDH (—) and wtCDH (---). Samples contained ~0.6 μM protein in 20 mM sodium succinate (pH 4.5).

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