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Characterization of Carbohydrate-Binding Cytochrome b_{562} from the White-Rot Fungus *Phanerochaete chrysosporium*

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cDNA encoding a hemoprotein similar to the cytochrome domain of extracellular flavocytochrome cellobiose dehydrogenase (CDH) was cloned from the white-rot fungus *Phanerochaete chrysosporium*. The deduced amino acid sequence implies that there is a two-domain structure consisting of an N-terminal cytochrome domain and a C-terminal family 1 carbohydrate-binding module (CBM1) but that the flavin-containing domain of CDH is not present. The gene transcripts were observed in cultures in cellulose medium but not in cultures in glucose medium, suggesting that there is regulation by carbon catabolite repression. The gene was successfully overexpressed in *Pichia pastoris*, and the recombinant protein was designated carbohydrate-binding cytochrome b_{562} (CBCyt. b_{562}). The resonance Raman spectrum suggested that the heme of CBCyt. b_{562} is 6-coordinated in both the ferric and ferrous states. Moreover, the redox potential measured by cyclic voltammetry was similar to that of the cytochrome domain of CDH. These results suggest that the redox characteristics may be similar to those of the cytochrome domain of CDH, and so CBCyt. b_{562} may have an electron transfer function. In a binding study with various carbohydrates, CBCyt. b_{562} was adsorbed with high affinity on both cellulose and chitin. As far as we know, this is the first example of a CBM1 connected to a domain without apparent catalytic activity for carbohydrate; this CBM1 may play a role in localization of the redox protein on the surface of cellulose or on the fungal sheath in vivo.

Wood, the most abundant biomaterial on earth, consists of cellulose, hemicellulose, and lignin. Of these constituents, lignin is the most recalcitrant polymer, and its decomposition requires a strong oxidative reaction. Although this property of lignin results in the high resistance of wood against microbial attack, a group of basidiomycetes known as white-rot fungi is able to destroy lignin through the reactions of various extracellular peroxidases, lignin peroxidases, manganese peroxidases, and versatile peroxidases (16, 33, 38, 54). Since hydrogen peroxide is required for activation of these enzymes, extracellular oxidases might participate in the redox networks leading to the fungal degradation of lignin (29, 30). However, mixtures of these enzymes have failed to degrade lignin in vitro.

In contrast to degradation of lignin, degradation of cellulose and hemicellulose has been thought to proceed only through the action of hydrolytic enzymes. However, Eriksson and coworkers have pointed out the importance of oxidative reactions in cellulose degradation (13, 14), and they isolated a cellobiose-oxidizing enzyme from a cellulolytic culture of the white-rot fungus *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) (4). This enzyme contains one *b*-type heme and one flavin adenine dinucleotide in two separate domains (20, 40), and it can oxidize the reducing-end groups of cellobiose and higher cellooligosaccharides to the corresponding lactones (4). Although this enzyme was originally named cellobiose oxidase (EC 1.1.3.25), its name has been changed to cellobiose dehydrogenase (CDH) (EC 1.1.99.18) (5), since Fe(III)-containing compounds are much more effective electron acceptors than molecular oxygen (31, 47). The flavin domain derived by proteolysis of CDH (20, 58) has cellobiose-oxidizing ability in the presence of quinone but does not utilize Fe(III)-containing compounds as electron acceptors (17). Thus, the reduction of ferricytochrome *c* requires the cytochrome domain of CDH, suggesting the significance of the heme as an Fe(III) reductase (47).

Eriksson and coworkers first proposed a physiological function of CDH in lignin degradation by white-rot fungi, because this enzyme can prevent reoxidation of the degradation products with quinone, phenoxy, and aromatic radicals in the presence of cellobiose (56, 57). More recently, it has been hypothesized CDH has a role as a source of hydroxyl radical in wood degradation (22, 32). However, we previously reported the localization of CDH on cellulose surfaces in vivo (23); we showed that there is a synergistic interaction between CDH and cellobiohydrolase I (24), and we identified a kinetic advantage of CDH over extracellular β-glucosidase for cellobiose metabolism (26). Moreover, transcription of the *cdh* gene responded positively to cellobiose, whereas transcription of the *bgl* gene was repressed under the same conditions (62). Although these observations clearly indicate the importance of

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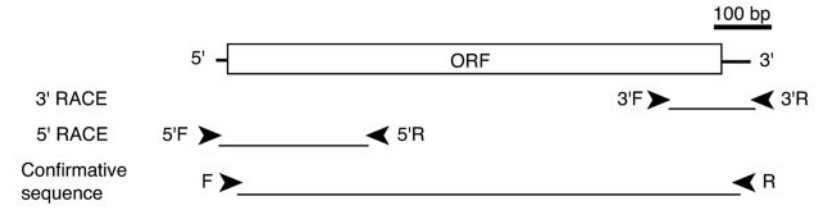

Primer	Sequence	Direction
3'F	5'-GGAGGCACCGGATTCACT-3'	F
3'R	5'-GGCCACGCGTCGACTAGTAC-3'	R
5'F	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT-3'	F
	5'-AAGCAGTGGTAACAACGCAGAGT-3'	
5'R	5'-GCCAGTGTAGCCATTGGATACAGGTGC-3'	R
F	5'-TGAGCTATGGTATGCTGAGGAAGT-3'	F
R	5'-TTTCACGAAGTCTACATGAACTGGTT-3'	R

FIG. 1. Schematic diagram of cDNA encoding CBCyt. b_{562}, showing locations and nucleotide sequences of primers used for PCR. F and R indicate the forward and reverse directions, respectively. ORF, open reading frame.

cellobiose oxidation by CDH, the overall picture of the redox network operating in cellulose degradation remains unclear.

Recently, the DoE Joint Genome Institute in the United States has disclosed the total genome sequence of *P. chrysosporium* (39; http://genome.jgi-psf.org/whiterot1/whiterot1.home.html). In order to find homologues of CDH and candidate electron acceptors, we searched the database of the *P. chrysosporium* genome and found one homologous region for the cytochrome domain of CDH. This gene encodes not only a protein similar to the cytochrome domain of CDH but also the family 1 carbohydrate-binding module (CBM1) signature in the C-terminal region instead of the flavin domain. In the present study, therefore, cDNA encoding the carbohydrate-binding cytochrome was cloned from *P. chrysosporium* grown on cellulose, and the recombinant protein was successfully produced in *Pichia pastoris* for characterization of its properties.

MATERIALS AND METHODS

Materials. *P. chrysosporium* strain K-3 (27) was used as a source of target genes. *Escherichia coli* strain JM109 (Takara, Japan) and *P. pastoris* strain KM71H (Invitrogen, Carlsbad, CA) were used as the subcloning host and for the heterologous production of the recombinant protein, respectively.

Cloning and transcript analysis. The cloning procedure used was the procedure described previously (61). Total RNA was extracted from *P. chrysosporium* grown in cellulose medium as described previously (18). The oligonucleotide primers were designed based on available genomic information, as illustrated in Fig. 1. The first-strand cDNA for 3′ rapid amplification of cDNA ends (RACE) was synthesized with a Ready-To-Go You-prime first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ) using 3′ RACE adapter primer (Invitrogen, Carlsbad, CA), and the PCR was primed with 3′F and 3′R. The cDNA synthesized with a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA) was used for 5′ RACE as a template, and primers 5′F and 5′R were used for PCR. The nucleotide sequence, including the coding region, was confirmed by PCR amplification using specific primers F and R. For detection of transcripts, mycelia were grown in medium containing 2% glucose or cellulose as a sole carbon source, and the first-strand cDNA from each sample was used for reverse transcription PCR (RT-PCR) analysis with forward primer 5′-CGCTG GACTTCGGGCT-3′ and reverse primer 5′-CGCACTGACCCCACTCA-3′. The transcript level of the *actin* gene (accession no. AB115328), a housekeeping gene, was measured using primers actin-F (5′-GCCGTGTTCCCGTCCAT-3′) and actin-R (5′-CACTTGTAGATGGAGTTGTACGTCGT-3′).

Sequence analysis. The *P. chrysosporium* genome database (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) and the National Center for Biotechnology Information (NCBI) protein database (http://www.ncbi.nlm.nih.gov/BLAST/) were searched for amino acid sequences of CDH and carbohydrate-binding cytochrome b_{562} (CBCyt. b_{562}) using the tblastn algorithm (1, 2). All searches were performed with standard settings and the BLOSUM 62 matrix. Multiple-alignment analysis was performed using ClustalW (53) at GenomeNet (http://clustalw.genome.jp/). The candidate genes were searched against the conserved domain database (37) at the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and were scanned for the presence of signal peptides using the SignalP version 3.0 server (6, 43) at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/SignalP/).

Production of the recombinant protein in *P. pastoris* and its purification. The expression vector was constructed as described previously (60). The following two oligonucleotide primers were designed, based on the nucleotide sequence of the mature protein, for ligation into the XhoI and XbaI sites of the *Pichia* expression vector pPICZα (Invitrogen, Carlsbad, CA): primer 5′ (5′-TTTCTC GAGAAAAGACAGTCCAGCTCCCAGTTTTG-3′) and primer 3′ (5′-TTTTC TAGACTAAGCTTGGCACTGGTAGTAATAAGG-3′). Approximately 10 μg of the DNA construct in pPICZα was linearized with Bpu1102I (Takara, Japan) prior to transformation of *P. pastoris*. Electroporation, selection of the transformant, and production of recombinant protein were carried out according to the instructions in the manual of an EasySelect *Pichia* expression kit (version G; Invitrogen, Carlsbad, CA). The culture was centrifuged (30 min, 5,000 × *g*), and ammonium sulfate (70% saturation) was added to the cell-free culture medium to precipitate the protein. The precipitate was dissolved in 20 mM sodium acetate, pH 6.0, and the solution was then mixed with 5% (wt/vol) bentonite (Sigma Chemicals, St. Louis, MO), followed by incubation for 30 min at 26.5°C. The bentonite was removed by centrifugation (30 min, 10,000 × *g*), and the supernatant was dialyzed against 20 mM sodium acetate, pH 6.0, using a PM-10 ultrafiltration membrane (Millipore, Bedford, MA). The crude protein was fractionated on a QAE-Toyopearl 550C column (44 mm by 170 mm) equilibrated with 20 mM sodium acetate, pH 6.0. The protein was eluted from the column with a linear 0 to 0.5 M NaCl gradient in 1,000 ml. The fractions containing the recombinant protein were collected and equilibrated against 20 mM potassium phosphate buffer containing 1 M ammonium sulfate, pH 7.0. The solution was applied to a Phenyl-Toyopearl 650S column (16 mm by 120 mm) equilibrated with the same buffer. The protein was eluted with a 500-ml reverse gradient to 20 mM potassium phosphate buffer, pH 7.0. The fractions containing the recombinant protein were collected and equilibrated against 20 mM sodium acetate buffer, pH 5.5. Deglycosylation was performed using endo-β-*N*-acetylglucosaminidase H (endo-H) (New England Biolabs, Beverly, MA) as described previously (60). After overnight incubation, the solution was equilibrated against 20 mM sodium acetate buffer, pH 4.5, and the recombinant protein was finally purified on a DEAE-Toyopearl 650S column (16 mm x 170 mm) equilibrated with the same buffer. A linear 0 to 0.15 M NaCl gradient was used to elute the protein. The purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

Spectroscopic analysis. All spectral analyses were carried out in 50 mM phosphate buffer, pH 7.0, at room temperature. The reduced form was prepared by addition of 1 mM ascorbic acid or sodium dithionite to obtain the working concentration. Optical absorption spectra were measured using a Shimadzu

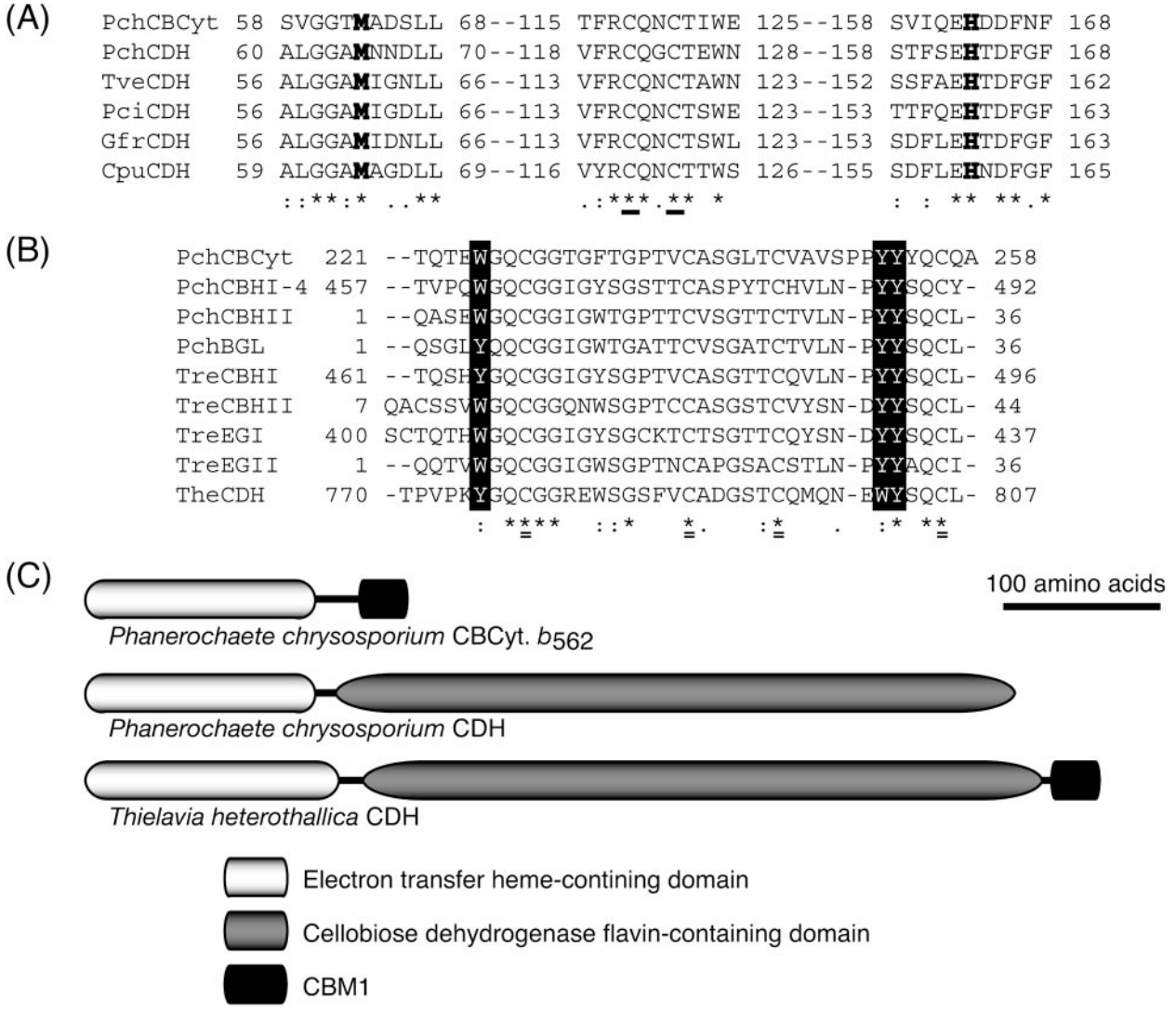

FIG. 2. (A) Multiple alignments of the cytochrome domains of CBCyt. b_{562} and basidiomycetes CDHs. Boldface type indicates methionine and histidine residues that are possible heme ligands, and conserved cysteines used for disulfide bond formation are underlined. PchCBCyt, CBCyt. b_{562} from *P. chrysosporium*; PchCDH, CDH from *P. chrysosporium* (accession no. X88897); TveCDH, CDH from *Trametes versicolor* (AF029668); PciCDH, CDH from *Pycnoporus cinnabarinus* (AF081574); GfrCDH, CDH from *Grifola frondosa* (AB083245); CpuCDH, CDH from *Coniophora puteana* (AB161046). (B) Multiple alignments of CBCyt. b_{562} CBM1 with other known CBM1s. Aromatic residues that are candidates for carbohydrate binding are indicated by solid boxes, and conserved cysteines used for disulfide bond formation are double underlined. PchCBHI-4, cellobiohydrolase I-4 from *P. chrysosporium* (accession no. L22656); PchCBHII, cellobiohydrolase II from *P. chrysosporium* (S76141); PchBGL, β-glucosidase from *P. chrysosporium* (AB081121); TreCBHI, cellobiohydrolase I from *Trichoderma reesei* (P62694); TreCBHII, cellobiohydrolase II from *T. reesei* (M16190); TreEGI, endoglucanase I from *T. reesei* (P07981); TreEGII, endoglucanase II from *T. reesei* (P07982); TheCDH, CDH from *Thielavia heterothallica* (AF074951). (C) Domain organization of CBCyt. b_{562} and CDH from *P. chrysosporium* and CBM1-carrying CDH from *T. heterothallica*.

UV-1600PC spectrophotometer. Raman spectra were obtained with a JASCO NRS-1000 spectrometer using a Kaiser Optical holographic notch-plus filter and a liquid N_2-cooled charge-coupled device detector with 4-cm^{-1} spectral resolution. Data were accumulated for 10 min. The excitation source was a Coherent Innova 90C Kr laser, and Raman spectra were collected using a backscattering geometry. Peak frequencies were calibrated relative to an indene standard and were accurate to ± 1 cm^{-1}.

Measurement of midpoint potential. The redox reaction was analyzed by cyclic voltammetry as described previously (25) with an ALS 624A electrochemical analyzer. All measurements were obtained using a three-electrode cell containing a 2-mercaptoethanol-modified gold working electrode, an Ag–AgCl–3 M NaCl reference electrode (205 mV versus the normal hydrogen electrode at 25°C), and a Pt wire counter electrode at room temperature under an N_2 atmosphere. The gold electrode modified with 2-mercaptoethanol was prepared by dipping the bare gold electrode in a 20.0 mM aqueous solution of 2-mercaptoethanol for 1 h. After dipping, the electrode was rinsed with water. The cyclic voltammogram was obtained in 50 mM sodium acetate buffer, pH 4.0, at 25°C.

Adsorption of the protein on carbohydrates. Adsorption experiments were performed in 50 mM sodium acetate buffer, pH 4.0, using 0.1% cellulose, xylan, mannan, arabinan, or chitin. These insoluble carbohydrates were prepared as described elsewhere (3, 9, 44, 49, 52). The protein concentrations in the additive solutions were calculated from the absorbance at 420 nm (ε_{420} = 130 mM^{-1} cm^{-1}), which corresponded to the Soret band of *b*-type heme, and they ranged from 1.6 μM to 23 μM. The mixtures of the protein and carbohydrates were incubated with rotation (diameter, 20 cm; 20 rpm) for 1 h at 30°C and then were centrifuged for 10 min at 16,100 × *g*. The supernatants were centrifuged again to

remove the precipitates completely, and the protein concentrations in the supernatants were determined again. The equilibrium dissociation constant (K_d) and the adsorption maximum (A_{max}) were estimated as described previously (15).

Nucleotide sequence accession number. The nucleotide sequence of cDNA encoding CBCyt. b_{562} has been deposited in the DDBJ database under accession number AB193288.

RESULTS

Nucleotide and amino acid sequences. During the search of the *P. chrysosporium* genome database, a gene similar to the gene encoding the cytochrome domain of CDH was found at positions 110991 to 111711 in scaffold 87. The nucleotide sequence of the cDNA was determined by using RT-PCR and RACE with total RNA of *P. chrysosporium* K-3, and it was identical to that of the cellulose-binding iron reductase gene (*cir1*) recently described by Cullen (accession number AY682742). The N-terminal 19-amino-acid sequence was predicted to be a signal peptide by the SignalP program, suggesting that the mature protein consists of 258 amino acid residues with a molecular mass of 27.0 kDa and a pI of 3.95. The amino acid sequence contains two possible sites of N glycosylation and numerous possible sites of O glycosylation. In the sequence comparisons of the cDNA and *cir1* and of the deduced amino acid sequences of the mature protein, eight nucleotides and three amino acids (S4T, T195A, and A258V) were different.

In homology searches of several public databases (PIR, Swiss-Prot, DAD, PDB, and PRF) at NCBI, the N-terminal 188 amino acids of the mature protein exhibited 44 to 48% identity with the cytochrome domains of basidiomycete CDHs and 31 to 33% identity with cytochrome domains of ascomycete CDHs. The sequence was aligned with the cytochrome domain of basidiomycete CDHs by ClustalW, and two possible heme ligands (Met 63 and His 163) and two cysteine residues forming a disulfide bond (Cys 118 and Cys 121) were conserved among these proteins, as shown in Fig. 2A. The C-terminal CBM1 contains three aromatic residues (W225, Y252, and Y253), which might contribute to binding on the surface of carbohydrates (Fig. 2B). As shown in Fig. 2C, the domain structure of CBCyt. b_{562} includes a C-terminal CBM1 but no flavin-containing domain. It thus differs significantly from the domain structure of *Thielavia heterothallica* CDH, which includes both CBM1 and a flavin domain, and from the domain structure of *P. chrysosporium* CDH, which includes a flavin domain but no CBM1.

Effect of carbon sources on expression of the CBCyt. b_{562} gene. *P. chrysosporium* was grown on glucose or cellulose medium for 5 days, and RT-PCR analysis was carried out to detect expression of the CBCyt. b_{562} gene, using total RNA extracted from the mycelia. As shown in Fig. 3, transcripts of the CBCyt. b_{562} gene were detected in cellulose medium but not in glucose medium, as was the case for other carbohydrate-degrading enzymes, suggesting that transcription of the CBCyt. b_{562} gene is regulated by carbon catabolite repression.

Production and purification of the recombinant protein. Recombinant CBCyt. b_{562} (rCBCyt. b_{562}) was successfully produced in the expression system of *P. pastoris* and was purified from the culture solution by three-step column chromatography; the product gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, as shown in Fig. 4. The actual molecular mass of the protein is higher than that calculated from the amino acid sequence. The N-terminal amino acid sequence (Gln-Ser-Ser-Ser-Gln-Phe-X-Asp-Ser-Ser) was identical to the predicted N-terminal sequence, and treatment with endo-H resulted in a considerable decrease in the molecular mass, suggesting that the higher-than-expected molecular mass mainly reflects N glycosylation of the recombinant protein. However, since the molecular mass of the deglycosylated protein (49 kDa) is still higher than that calculated from the amino acid sequence, the rCBCyt. b_{562} might also have undergone O glycosylation.

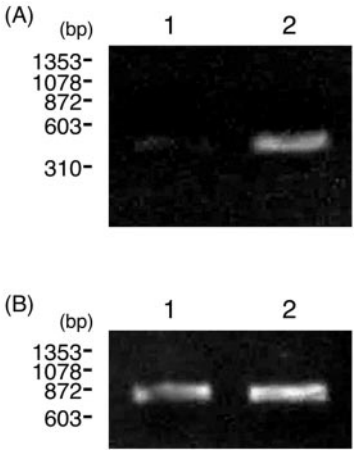

FIG. 3. RT-PCR analysis of CBCyt. b_{562} gene (A) and *actin* gene (B) expression in mycelium grown on different carbon sources. Lane 1, glucose medium; lane 2, cellulose medium. The PCR products were separated on a 2% agarose gel.

Spectral analysis of the recombinant protein. As shown in Fig. 5, the absorption spectrum of the oxidized form had broad peaks around 533 nm and 570 nm and a sharp peak at 420 nm. Upon reduction by addition of ascorbic acid or sodium dithionite, peaks appeared at 562, 533, and 430 nm, corresponding to the α-, β- and γ (Soret)-bands, respectively.

For further characterization of the heme in CBCyt. b_{562}, resonance Raman spectra were measured for both oxidized and reduced rCBCyt. b_{562} with excitation at 413 nm. The oxi-

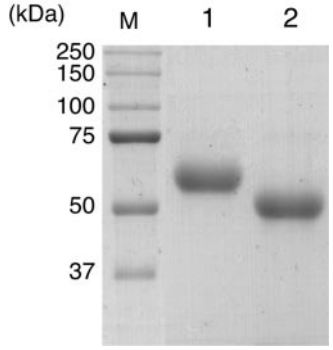

FIG. 4. Effect of deglycosylation on purified rCBCyt. b_{562}. Lane M, molecular mass standard; lane 1, rCBCyt. b_{562}; lane 2, rCBCyt. b_{562} after incubation with endo-H. Two micrograms of each sample was separated on a 12% polyacrylamide gel.

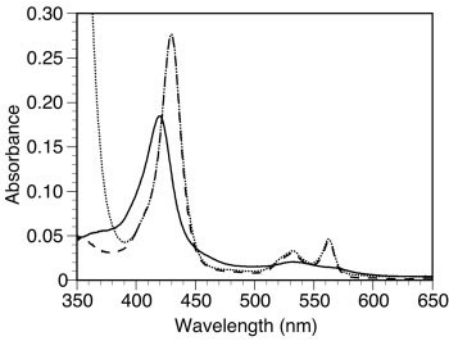

FIG. 5. Absorption spectra of rCBCyt. b_{562}. Solid line, oxidized form; dotted line, reduced form obtained by addition of sodium dithionite; dashed line, reduced form obtained by addition of ascorbic acid. All determinations were carried out in 50 mM phosphate buffer, pH 7.0, at room temperature.

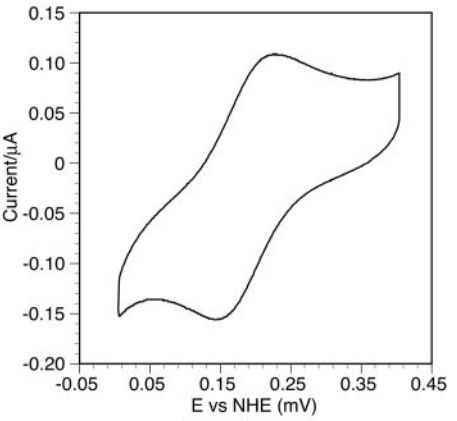

FIG. 6. Cyclic voltammogram of rCBCyt. b_{562}. The midpoint potentials were determined from the average of the cathodic and anodic peaks of the voltammogram.

dation state marker, υ_4, and core size markers, υ_2 and υ_3, of the oxidized form were present at 1,373 cm^{-1}, 1,578 cm^{-1}, and 1,504 cm^{-1}, indicating the presence of 6-coordinated, low-spin heme. These values shifted to 1,365 cm^{-1}, 1,582 cm^{-1}, and 1,494 cm^{-1}, respectively, in the reduced form prepared by addition of ascorbic acid. Therefore, the ferrous heme in CBCyt. b_{562} is also in a 6-coordinated, low-spin state (10). As shown in Table 1, the resonance Raman spectra of rCBCyt. b_{562} were almost identical to those of cytCDHs. These spectral analyses indicated that there is environmental similarity around the heme cofactor, including coordination and spin state, and, therefore, CBCyt. b_{562} should have properties similar to those of heme cofactors for the cytochrome domain of CDHs.

Electrochemical analysis of the recombinant protein. As shown in Fig. 6, apparent electron transfer was observed between CBCyt. b_{562} and the electrode without any mediator, and the midpoint potential of CBCyt. b_{562} was estimated to be 194 mV at pH 4.0. This value is slightly more positive than that for the cytochrome domain of CDH (180 mV) measured by the same technique (25).

Carbohydrate-binding properties of CBM1 in CBCyt. b_{562}. The amount of CBCyt. b_{562} adsorbed on insoluble carbohydrates was measured at various protein concentrations, and the calculated values for A_{max} and K_d are shown in Table 2. Although the A_{max} value of CBCyt. b_{562} for bacterial microcrystalline cellulose was the highest A_{max} value for the insoluble glycans tested, the binding efficiency (A_{max}/K_d) for chitin was highest and was approximately threefold higher than that for cellulose. Such binding behavior is often observed with CBM1 (e.g., in the case of *Trichoderma reesei* cellobiohydrolase II) (8, 35).

TABLE 1. Resonance Raman frequencies of CBCyt. b_{562} and cytochrome domain of CDH

Assignment	CBCyt.b_{562}(cm^{-1})		Cytochrome domain of CDH (cm^{-1})[a]	
	Ferric	Ferrous	Ferric	Ferrous
υ_{10}	1,619	1,618	1,622	1,618
υ_2	1,578	1,582	1,576	1,583
υ_{38}	1,565	1,556	1,561	1,555
υ_3	1,504	1,494	1,504	1,494
	1,431	1,433	1,431	1,450
υ_{20}		1,389		1,390
υ_4	1,373	1,365	1,370	1,362
	1,310	1,312	1,307	1,311
	1,231	1,226	1,228	1,224
		1,172		1,1173
$\upsilon_6 + \upsilon_8$	1,126	1,129	1,126	1,129
	996	1,002	991	994

[a] Data from reference 10.

DISCUSSION

Many carbohydrate-acting enzymes contain carbohydrate-binding modules (CBMs), which are defined as noncatalytic polysaccharide-recognizing modules (7). CBMs are classified into 42 families based on the similarity of amino acid sequences and the three-dimensional structures in the CAZY database (http://afmb.cnrs-mrs.fr/CAZY/), and they display diverse ligand specificities for various carbohydrates. Among them, family 1 CBMs (CBM1s), are often found in fungal carbohydrate-acting enzymes, and they are thought to contribute to binding to crystalline cellulose. Removal of the module causes a significant decrease in both binding and catalytic activity against cellulose (36, 50, 55), suggesting that the CBM1 is directly related to the catalytic function of these enzymes. Most CDHs do not have this type of module, although it is known that CDHs also bind to cellulose and that this ability is due to part of the flavin domain (20, 21, 46, 48). Since cellulose-bound

TABLE 2. Binding parameters of CBCyt. b_{562} for various carbohydrates[a]

Carbohydrate	Organism	K_d (μM)	A_{max} (μmol/g)	A_{max}/K_d
Cellulose	*Acetobacter xylinius*	5.16	7.48	1.45
Chitin	*Thalassiosira weissflogii*	0.54	2.34	4.33
Xylan	*Betula papyrifera*	4.29	0.38	0.09
Mannan	*Phytelephs macrocarpa*	6.06	1.63	0.27
Arabinan	*Beta vulgaris*	3.50	1.04	0.30

[a] The parameters were derived from adsorption data plotted as described in Materials and Methods.

TABLE 3. Genes encoding CBCyt. b_{562}, CDH, and unknown hemoproteins similar to the cytochrome domains of CBCyt. b_{562} and CDH

Protein	Organism	Accession no.	Feature	Reference
CBCyt. b_{562}	*Phanerochaete chrysosporium*	AB193288	Extracellular cytochrome, carries CBM1	This study
CDH	*Phanerochaete chrysosporium*	X88897	Extracellular flavocytochrome	45
	Trametes versicolor	AF029668	Extracellular flavocytochrome	12
	Pycnoporus cinnabarinus	AF081574	Extracellular flavocytochrome	41
	Grifola frondosa	AB083245	Extracellular flavocytochrome	61
	Athelia rolfsii	AY187232	Extracellular flavocytochrome	Database[a]
	Coniophora puteana	AB161046	Extracellular flavocytochrome	28
	Irpex lacteus	AB187223	Extracellular flavocytochrome	Database[a]
	Thielavia heterothallica	AF074951	Extracellular flavocytochrome, carries CBM1	51
	Humicola insolens	AF257654	Extracellular flavocytochrome	59
	Aspergillus nidulans	XM_411367	Extracellular flavocytochrome	Database[a]
	Gibberella zeae	XM_389261	Extracellular flavocytochrome, carries a CBM1	Database[a]
	Gibberella zeae	XM_383918	Extracellular flavocytochrome	Database[a]
	Gibberella zeae	XM_385048	Extracellular flavocytochrome	Database[a]
	Magnaporthe grisea	XM_360402	Extracellular flavocytochrome, carries CBM1	Database[a]
	Neurospora crassa	XM_325777	Extracellular flavocytochrome	Database[a]
	Neurospora crassa	XM_322291	Extracellular flavocytochrome, carries a CBM1	Database[a]
Unknown oxidoreductase	*Aspergillus nidulans*	XM_408099	Extracellular oxidoreductase, carries Rieske [2Fe-2S] domain	Database[a]
Unknown cytochrome	*Gibberella zeae*	XM_382527	Extracellular cytochrome, Shows short sequence at C-terminus	Database[a]
	Magnaporthe grisea	XM_368045	Intracellular cytochrome, shows short sequence at C terminus	Database[a]
	Magnaporthe grisea	XM_365000	Intracellular cytochrome	Database[a]
	Magnaporthe grisea	XM_369170	Extracellular cytochrome	Database[a]
	Neurospora crassa	XM_329062	Extracellular cytochrome	Database[a]
	Neurospora crassa	XM_325449	Intracellular cytochrome	Database[a]
	Magnaporthe grisea	XM_363329	Membrane-bound cytochrome, carries a transmembrane domain	Database[a]
	Neurospora crassa	XM_330633	Membrane-bound cytochrome, carries a transmembrane domain	Database[a]

[a] GenBank/EMBL/DDBJ/PDB.

CDH retains cellobiose-oxidizing activity (20) and the enzyme is localized on cellulose surfaces in vivo (23), the cellulose-binding ability of CDH helps its catalytic activity, as is the case for many CBM-containing enzymes. In the present study, we found that CBCyt. b_{562} has a unique structure; i.e., the protein lacks the cellobiose-oxidizing flavin domain of CDH but carries a C-terminal CBM1 instead. As far as we know, this is the first example of a CBM1 connected to a domain without apparent catalytic activity toward carbohydrate. The CBM1 in CBCyt. b_{562} nevertheless presumably contributes to the localization of the protein. In this study, we tested the binding ability of the protein not only for cellulose but also for other polysaccharides, and we observed a high affinity of CBCyt. b_{562} for chitin, as well as cellulose. Although the surface condition of these glycans strongly depends on the source organism and it is not easy to compare binding parameters, the results clearly suggest the possibility of localization of CBCyt. b_{562} on the fungal sheath, as well as on the surface of cellulose. Further cytochemical analysis is required to identify the localization of CBCyt. b_{562} in vivo.

The cytochrome domain of CBCyt. b_{562} shows significant homology with that of CDHs, and residues essential to ligate heme (19) are well conserved. Moreover, the Raman spectrum showed that the heme of CBCyt. b_{562} is 6-coordinated in both the ferric and ferrous states, suggesting that CBCyt. b_{562} has an electron transfer function, like that of the cytochrome domain of CDH. In RT-PCR analysis, the CBCyt. b_{562} gene transcripts were detected in cellulose medium but not in glucose medium, as is the case for *cdh* (34). This result led us to suspect that the function of CBCyt. b_{562} is as a physiological electron acceptor of CDH. Indeed, when CBCyt. b_{562} was mixed with CDH in vitro, it was partially reduced by addition of cellobiose (data not shown). However, the similar values for the midpoint potentials of the cytochrome domains of CBCyt. b_{562} and CDH would be thermodynamically disadvantageous for accepting electrons from CDH. Recently, Martinez and coworkers found various unknown genes encoding extracellular oxidoreductases in the *P. chrysosporium* genome (39). Therefore, some of these enzymes are candidates for the natural electron donor of CBCyt. b_{562}. On the other hand, functional and structural similarities between CBCyt. b_{562} and CDH indicate that the heme in CBCyt.b_{562} may have Fe(III)-reducing ability. There-

fore, Fe(III)-containing compounds might be candidates for the possible electron acceptor for CBCyt. b_{562}.

To date, the cytochrome domain of CDH is the only hemoprotein with an immunoglobulin-like β-sandwich fold in the Structural Classification of Proteins (SCOP) (11, 42). During BLAST searches of various genome databases, however, we found many genes that may encode similar hemoproteins which share the putative ligands of heme and the two cysteine residues for forming a disulfide bond (19). A comparison of the deduced amino acid sequences indicated that some of these proteins have an N-terminal signal peptide and some do not, as shown in Table 3. In addition to the flavin-binding domain or CBM1, moreover, there are hemoproteins carrying both the flavin domain and CBM1 (*Gibberella zeae*, *Magnaporthe grisea*, *Neurospora crassa*, and *Thielavia heterothallica*), a Rieske [2Fe-2S] center (*Aspergillus nidulans*), or a transmembrane domain (*M. grisea*, *Neurospora crassa*) in the C-terminal region. These results clearly suggest that these proteins might have evolved by fusion of ancestral functional domains in order to acquire various redox systems, and it seems likely that the hemoproteins function not only extracellularly but also in the intracellular region or membrane. The occurrence of such hemoproteins, as well as CBCyt. b_{562} as described here, may provide unique insight into the novel redox networks in filamentous fungi.

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