Characterization of the two *Neurospora crassa* cellobiose dehydrogenases and their connection to oxidative cellulose degradation

Christoph Sygmund,† Daniel Kracher,† Stefan Scheiblbrandner, Kawah Zahma, Alfons K. G. Felice, Wolfgang Harreither, Roman Kittl and Roland Ludwig*

*Food Biotechnology Laboratory, Department of Food Sciences and –Technology, BOKU – University of Natural Resources and Life Sciences, Muthgasse 18, A-1090 Vienna, Austria*

†C.S and D.K. contributed equally

*Corresponding author. Mailing address: Department für Lebensmittelwissenschaften und -technologie, Universität für Bodenkultur, Muthgasse 18/2, A-1190 Wien, Austria. Phone: +43 1 47654 6149. Fax: +43 1 47654 6199 E-mail: roland.ludwig@boku.ac.at*

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Abstract

The genome of *Neurospora crassa* encodes two different cellobiose dehydrogenases (CDH) with a sequence identity of only 53%. So far only CDH IIA, which is induced during growth on cellulose and features a C-terminal carbohydrate binding module (CBM), was detected in the secretome of *N. crassa* and preliminary characterized. CDH IIB is not significantly up-regulated during growth on cellulosic material and lacks a CBM. Since CDH IIB could not be identified in the secretome, both CDHs were recombinantly produced by *Pichia pastoris*. With the cytochrome domain dependent one-electron acceptor cytochrome c CDH IIA has a narrower and more acidic pH optimum than CDH IIB. Interestingly, the catalytic efficiencies of both CDHs for carbohydrates are rather similar, but CDH IIA exhibits 4-5 times higher apparent catalytic constants (*k*_cat and *K*_M values) than CDH IIB for most tested carbohydrates. A third major difference is the 65 mV lower redox potential of the heme b cofactor in the cytochrome domain of CDH IIA compared to CDH IIB. To study the interaction with a member of the glycoside hydrolase 61 family, the copper-dependent polysaccharide monooxygenase GH61-3 (NCU02916) from *N. crassa* was expressed in *P. pastoris*. A pH dependent electron transfer from both CDHs via their cytochrome domain to GH61-3 was observed. The different properties of CDH IIA and CDH IIB and their effect on the interaction with GH61-3 are discussed in regard to the proposed *in vivo* function of the CDH/GH61 enzyme system in oxidative cellulose hydrolysis.
Introduction

The extracellular fungal flavocytochrome cellobiose dehydrogenase (CDH, EC 1.1.99.18) constitutes a considerable fraction of the lignocellulolytic enzymes secreted by many cultures of wood degrading basidiomycetes, e.g., 0.5% in *Phanerochaete chrysosporium* (11), up to 1.2% in *Trametes spp.* (20), 2.4% in *Ceriporiopsis subvermispora* (9), 2.2% in *Sclerotium rolfsii* (19) and ascomycetes, e.g. 12% in *Corynascus thermophilus* (8), 2.3% in *Myriococcum thermophilum* (7) and 2.4% in *Neurospora crassa* (23). The widespread appearance of CDH implies an important function of this enzyme in wood degradation. Since its discovery in 1972, the exact catalytic role of CDH and its interaction with other fungal lignocellulolytic enzymes remained unclear. Several *in vivo* functions have been proposed (2, 11, 42). The most widely supported mechanism in the last two decades is related to CDH’s ability to produce hydrogen peroxide and concomitantly reduce Fe$^{3+}$, which potentially generates hydroxyl radicals by a Fenton type reaction. However, the catalytic efficiency of oxygen reduction is very low and about 100 times slower than the reduction of other electron acceptors such as quinones. Similarly, the reduction of weakly complexed Fe$^{3+}$ species (e.g. by carboxylic acids) occurring in plant material is much slower than ferricyanide turnover and additionally limited to pH values below 4.5. While white rot fungi generate such acidic pH values during growth the majority of ascomycete CDH producers degrade lignocellulose at higher pH values (34).

Evidence is growing that the main purpose of CDH *in vivo* is not to transfer electrons to small molecular weight electron acceptors like oxygen and Fe$^{3+}$, but to members of the glycoside hydrolase 61 (GH61) protein family. Recently, a polysaccharide monooxygenase activity for three members of the GH61 protein family in *N. crassa* has been described (17, 22) and copper was specified as active site constituent of these enzymes. Therefore, the name polysaccharide monooxygenase (PMO) has been suggested by Marletta and coworkers for the investigated GH61 proteins (22). The GH61 family is widely distributed in fungi. BLAST
searches of 7 CDH producing basidiomycetous and ascomycetous fungi revealed that cdh and gh61 genes occur together in the genomes of the CDH producing fungi Trametes versicolor, Phanerochaete chrysosporium, Chaetomium globosum, Thielavia terrestris, Podospora anserina, Glomerella graminicola and Aspergillus fumigatus. On the other hand, numerous fungi with gh61 genes in their genome do not have cdh genes (e.g. Hypocrea jecorina, ana. Trichoderma reesei). It has been also shown that the addition of certain T. terrestris or Thermoascus aurantiacus GH61 proteins has a high stimulatory effect on lignocellulose hydrolysis performance of T. reesei cellulases without involving CDH (10). Additionally, many of the tested members of the GH61 family have been shown not to cleave cellulose under the tested conditions. Therefore, it remains to be elucidated if the polysaccharide monooxygenase activity is a feature of all GH61 proteins.

The CDH enzyme family is a heterogeneous group of proteins with sequence identities as low as 35%. Phylogenetic analysis of these sequences (8, 41, 42) shows several well supported branches, which correlate partially with the species’ classification: Basidiomycete CDH sequences (from Atheliales, Corticiales and Polyporales) form the well-supported branch of class I CDHs. Class II consists only of sequences of ascomycete origin (Sordariales, Xylariales and Hypocreales). This class of CDHs partitions in two subclasses: class IIA CDHs contain a carbohydrate binding module (CBM) whereas class IIB CDHs do not. N. crassa is a member of the Sordariales and two cdh genes are found in its genome – one with a C-terminal CBM (CDH IIA) and one without (CDH IIB) (8). In Eurotiales, Helotiales and Pleosporales CDH encoding sequences of a separate phylogenetic branch were found by genome sequencing projects. The secretion of these class III CDHs has not been confirmed yet. Class II CDHs prefer cellobiose and cello-oligosaccharides as substrates, but can also oxidize other mono- and disaccharides although with lower catalytic efficiencies. This difference to class I CDHs might be an adaption to different habitats and substrates.
In the genome of *N. crassa* 14 gh61 genes are found (27), which are induced to various extents during growth on cellulose and xylan (28). Marletta and coworkers showed by HPLC and LC-MS measurements that class IIB CDH from *Myceliophthora thermophila* interacts with *N. crassa* GH61s (NCU01050, NCU07898 and NCU08760) (22) and investigated the reaction pathway for oxidative cleavage of cellulose by these GH61s (1). CDH is supposed to act as a reductase (via its heme b) for these PMOs, which catalyze the insertion of oxygen into C-H bonds adjacent to the glycosidic linkage. The oxygen atoms destabilize the glycosidic bond, which leads to its elimination and the formation of a sugar lactone or ketoaldose (22).

Structural studies of copper containing GH61 proteins revealed that their putative active sites show structural homologies to the active site of chitin binding protein-21 (CBP21), which is capable of cleaving chitin. The proposed reaction mechanism for CBP21 suggests a chitin oxygenase activity which consumes both oxygen and reducing agent as cosubstrates (35-37).

The first GH61 structure was obtained from Cel61A, a protein with endoglucanase activity found in *Hypocrea jecorina*. The structure does not show a copper atom in the active site but nickel, which was added to obtain better crystals and as source for anomalous scattering to obtain near atomic resolution (16). However, the authors state that nickel-containing enzymes are quite unusual and suggest that other transition metals could bind to Cel61A *in vivo*. The first structure showing a copper in the active site was from Quinlan et al. 2011 (24, 39), followed by the biochemical support for the function of the copper (22).

It was demonstrated that addition of *M. thermophila* CDH IIA (CDH-1 in Phillips et al. (22)) restores the cellulosytic activity of a Δcdh-1 *N. crassa* strain, whereas a tenfold higher concentration of CDH IIB (CDH-2) is necessary to replace CDH IIA (22). Earlier studies by Langston et al. showed that purified GH61 proteins have no demonstrable direct hydrolase activity, but *Thermoascus aurantiacus* GH61A in combination with *Humicola insolens* CDH cleaves cellulose into soluble, oxidized oligosaccharides (17).
We selected the model organism *N. crassa* for the first characterization of both CDH subclasses from one organism and to investigate their different physical and catalytic properties. To study the interaction of CDH IIA and CDH IIB with their proposed natural electron acceptor, GH61-3 from the same organism was expressed and purified. *N. crassa* GH61-3 is found in the same phylogenetic branch of GH61 proteins that showed PMO activity and its encoding gene NCU02916 is strongly upregulated during *N. crassa* cultivation on Miscanthus (32). *Pichia pastoris* was chosen to express all three enzymes without possibly interfering purification tags. After elucidating the physical and catalytic properties the interaction CDH/GH61-3 was studied. Published data of *N. crassa* transcriptome and secretome analyses are used to explore the induction and regulation of CDH IIA and CDH IIB as well as of members of the GH61 protein family.
2. MATERIALS AND METHODS

2.1 Organism, vectors and culture conditions

The genes coding for CDH IIA and IIB from *N. crassa* have been previously isolated (8) and were cloned in the CloneJET vector (Fermentas). The *N. crassa* gene NCU02916 encoding the GH61-3 protein was codon optimized for expression in *Pichia pastoris* (Figure S1), and commercially synthesized by Invitrogen. *P. pastoris* strain X-33 and the vectors pPICZA A and pPICZB are components of the Pichia Easy Select Expression System from Invitrogen. *P. pastoris* transformants were grown on YPD plates (10 g L\(^{-1}\) yeast extract, 20 g L\(^{-1}\) peptone, 10 g L\(^{-1}\) glucose and 15 g L\(^{-1}\) agar) containing 100 mg L\(^{-1}\) zeocin.

2.2 Construction of CDH IIA, CDH IIB and NCU02916 expression vectors

The published plasmids pNCIIA and pNCIIB (8) were used as templates for the amplification of *cdhIIA* and *cdhIIB* with the primers 5NCa-BstBI 5'-tatttcgaaacgatgaggaccacctcggcc-3', 3NCa-XbaI 5'-tatcacgtgctacacacactgccaatacc -3' and 5NCb-EcoRI 5'-tatgaattcatgaaggtcttcacccgc -3', 3NCb-NotI 5'-tatgcggccgctcatcttctccattttccc -3', respectively. PCR was performed with Phusion High Fidelity DNA Polymerase from New England Biolabs, a dNTP mix from Fermentas, oligonucleotide-primers from VBC Biotech (Vienna, Austria) and a C-1000 thermocycler from Bio-Rad Laboratories. The resulting *cdhIIA* cDNA and the *ncu02916* gene in the cloning vector were digested with *Bst*BI and *Xba*I and cloned into the equally treated vector pPICZA A. cDNA from *cdhIIB* and vector pPICZ B were digested with *Eco*RI and *Xba*I and ligated using the Rapid DNA Ligation Kit from Fermentas. The procedures resulted in genes encoding proteins with their native signal sequences cloned under the control of the methanol inducible AOX1 promoter. C-terminal tags for purification or antibody detection were omitted. Correct insertion of the genes and the absence of...
mutations were confirmed by DNA sequencing. Linearized, verified plasmids were used for transformation into electro-competent *P. pastoris* cells.

2.3 Microscale-screening for high producing CDH transformants

Cultivation and expression of both CDHs was done in 96-deep well plates according to Weis *et al.* (38) with small modifications. Cells were grown in 250 µL BMD1 (13.4 g L⁻¹ yeast nitrogen base, 0.4 mg L⁻¹ biotin, 10 g L⁻¹ glucose, 200 mM potassium phosphate, pH 6.0) at 25°C, 385 rpm and 60% humidity for approximately 60 h to reach the stationary growth phase. Induction was started by adding 250 µL of BMM2 medium (13.4 g L⁻¹ yeast nitrogen base, 0.4 mg L⁻¹ biotin, 1% methanol, 200 mM potassium phosphate, pH 6.0) to reach a final concentration of 0.5% methanol. After 70, 82 and 108 h of incubation 50 µL BMM10 (BMM2 with 5% methanol) was added to maintain inducing conditions. The cultivation was stopped after 130 h by centrifugation of the deep-well plates at 2600 × g and 4°C for 10 minutes. The supernatant of each well was analyzed for enzymatic activity with the 2,6-dichloroindophenol (DCIP) assay.

2.4 Enzyme production and purification

CDH IIA and CDH IIB were produced in a 7-L bioreactor (MBR) filled with 4 L of Basal Salts Medium. After sterilization, the pH of the medium was adjusted to pH 5.0 with 28% ammonium hydroxide and maintained at this level throughout the whole fermentation process. The cultivation was started by adding 0.4 L (9% (v/v)) preculture grown on YPD medium in 1 L baffled shaking flasks at 125 rpm and 30°C overnight. The cultivation was performed according to the Pichia Fermentation Process Guidelines of Invitrogen and the expression of recombinant protein was induced with methanol. The Invitrogen protocol was altered at the transition phase from glycerol to methanol according to Sygmund *et al.* (30).
temperature was 30°C, the airflow rate was 6 L min\(^{-1}\), and the stirrer speed 800 rpm. Samples were taken regularly.

GH61-3 was produced in a Multifors fermenter (Infors HT, Bottmingen, CH) with a total volume of 500 mL. The fed-batch fermentation followed the Pichia Fermentation Process Guidelines of Invitrogen with slight modifications. The basal salts medium was supplemented with 0.1 mM CuSO\(_4\). The batch fermentation (300 mL starting volume) was inoculated with 25 mL preculture at 30°C. Air flow was kept constant at 2 L min\(^{-1}\) and the stirrer speed was set to 1000 rpm. The pH was maintained at 5.0 with ammonium hydroxide. After depletion of the glycerol in the batch medium the fed-batch phase was started with a constant feed of 2.4 mL h\(^{-1}\) 50% glycerol containing 12 mL L\(^{-1}\) PTM\(_1\) trace salts for 8 hours. For induction the feed was switched to 100% methanol containing 12 mL L\(^{-1}\) PTM\(_1\) trace salts at a low flow rate of 0.6 mL h\(^{-1}\) overnight for adaptation of the culture to methanol and the temperature was lowered to 25°C. Afterwards the feed rate was adjusted to keep the dissolved oxygen concentration around 4%. Antifoam was injected manually as required throughout the fermentation. Samples were taken for measurements of wet biomass and total soluble protein content.

Protein purification was started by centrifugation (6000 × g; 30 min at 4°C) of the fermentation broth. Saturated ammonium sulphate solution was slowly added to the clear culture supernatants containing CDH IIA (4.7 L), CDH IIB (4.5 L) and GH61-3 (0.35 L) to give a 20% (CDH IIA) or 30% (CDH IIB, GH61-3) saturated solution. Precipitate was removed by centrifugation (6000 × g; 20 min at 4°C) and the clear supernatant was loaded to a 600 mL PHE-Sepharose Fast Flow column (chromatographic equipment and materials from GE Healthcare Biosciences) equilibrated with 50 mM phosphate buffer pH 5.5 containing 20% (CDH IIA) or 30% (CDH IIB, GH61-3) ammonium sulphate. Proteins were eluted within a linear gradient from 20 to 0% ammonium sulphate within 5 column volumes or 30 to 0% within 7.5 column volumes and fractions collected in aliquots. Fractions containing the
enzymes of interest were pooled and diafiltrated with a hollow fiber cross-flow module (Microza UF module SLP-1053, 10 kDa cut-off, Pall Corporation). The diafiltrated CDH pools (~3 mS cm\(^{-1}\)) were applied to a 20-mL column packed with Q15-Source pre-equilibrated with 50 mM (CDH IIA) or 20 mM (CDH IIB) sodium acetate buffer, pH 5.5. Proteins were eluted within a linear salt gradient from 0 to 1 M NaCl within 10 column volumes. To obtain a homogenous preparation of CDH IIB, gel filtration with Superdex 200 was added as final polishing and buffer exchange step to 50 mM sodium acetate buffer, pH 5.5. HIC fractions containing GH61-3 (judged by A\(_{280}\) readings and SDS-PAGE) were concentrated and loaded onto a 470 mL gel filtration column (Sephacryl S-300) equilibrated with 100 mM sodium acetate buffer (pH 5.0). The purest CDH and GH61-3 fractions were pooled, concentrated, sterile filtered (0.2 µm), aliquoted and stored at -80°C.

2.5 Enzyme assays and protein determination

Activity of intact CDH was specifically determined by following the reduction of 20 µM cytochrome \(\text{c (cyt c, } \varepsilon_{550} = 19.6 \text{ mM}^{-1} \text{ cm}^{-1})\). This electron acceptor is exclusively reduced at the cytochrome domain. Activities of both the intact holoenzyme and its catalytically active proteolytic cleavage product, the dehydrogenase domain, were spectrophotometrically assayed using 0.3 mM DCIP (\(\varepsilon_{520} = 6.8 \text{ mM}^{-1} \text{ cm}^{-1}\)) as electron acceptor. The reaction was followed for 180 sec at 30°C in a Lambda 35 UV/Vis spectrophotometer featuring a temperature controlled 8-cell changer (Perkin Elmer). All assays were measured in McIlvaine buffer (21) at the indicated pH, containing 30 mM lactose as saturating substrate. One unit of CDH activity was defined as the amount of enzyme that oxidizes 1 µmol of the electron acceptor per min under the assay conditions. The interaction of GH61-3 with CDH IIA or IIB was measured by its interference with the cyt c assay. Molar ratios of cyt c:GH61-3 from 1:0.25 to 1:4 were measured and the highest interference found at a ratio of 1:4, which was used to obtain pH profiles of this interaction (buffer: sodium acetate pH 3–6, sodium
phosphate pH 6–8). The interaction of GH61-3 is given as the reduction of the cyt c activity (in % inhibition) and was measured in triplicates against a reference reaction without GH61-3. Protein concentrations were determined by the method of Bradford using a prefabricated assay from Bio-Rad Laboratories and bovine serum albumin as calibration standard. Spectra of homogeneously purified proteins were recorded at room temperature in both the oxidized and reduced state using a U-3000 Hitachi spectrophotometer. The proteins were diluted in McIlvaine buffer, pH 6.0 to an absorbance of ~1 at 280 nm and the spectrum was recorded before and immediately after the addition of reductant (cellobiose for CDH, ascorbate for GH61-3) to the cuvette.

2.6 Electrophoresis

SDS-PAGE was carried out using Mini-PROTEAN TGX precast gels (Bio-Rad Laboratories) with a gradient of 4–15%. Protein bands were visualized by staining with Bio-Safe Coomassie. Unstained Precision Plus Protein Standard was used for mass determination. All procedures were done according to the manufacturer’s recommendations (Bio-Rad Laboratories). To estimate the degree of glycosylation homogenous CDH samples were treated with PNGase F (New England Biolabs) under denaturing conditions according to the manufacturer’s instructions. GH61-3 was deglycosylated with 1000 U mg$^{-1}$ endoglucanase Hf (New England Biolabs) and 0.02 mg mg$^{-1}$ $\alpha$-mannosidase (Sigma) in 50 mM sodium acetate buffer, pH 5.0 containing 10 mM ZnCl$_2$. Chromatofocusing was used to determine the isoelectric points of both CDHs. Dialysed sample solutions containing the CDH and glucose oxidase (Sigma) with a known pI of 4.25 as internal standard were loaded onto a 10 mL Mono P column (GE Healthcare) equilibrated with 0.025 M imidazole-HCl, pH 7.4. The protein concentrations of applied samples were approx. 0.1 mg mL$^{-1}$. Proteins were eluted within 10 column volumes with a linear gradient from 0 to 100% Polybuffer 74, pH 3.6. Absorbances at 280 nm, 420 nm (heme $b$) and 450 nm (FAD) were measured online along with the pH value.
2.7 Voltammetric measurements

The preparation of thiol-modified gold electrodes (BASi, West Lafayette, IN, USA, Ø = 1.6 mm, area of 0.02 cm²) for cyclic voltammetry and square wave voltammetry started with dipping the electrodes into a piranha solution (H₂SO₄:H₂O₂ = 3:1, ATTENTION: Piranha solution is highly corrosive and a strong oxidizer. Mixing the solutions is exothermic. Explosions might occur if the peroxide concentration exceeds 50%!) for 10 min, followed by electrochemical cleaning by cycling in 0.1 M NaOH with a scan rate of 100 mV s⁻¹ between 0 and -1000 mV vs. SHE (10 cycles). Afterwards the electrodes were cleaned mechanically by polishing on Microcloth (Buehler, Lake Bluff, IL, USA) in a Masterprep polishing suspension (0.05 µm, Buehler). The electrodes were rinsed with water, sonicated for 10 min in HQ water, followed by cycling in 0.5 M H₂SO₄ for 20 cycles with a scan rate of 200 mV s⁻¹ between 0 and +1950 mV vs. SHE, and finally rinsed with HQ water. Thiol self-assembled monolayer (SAM) formation at the electrode surfaces was done by immersing the electrodes in a 10 mM thioglycerol solution at room temperature overnight. Before exposure to CDH, the electrodes were carefully rinsed with HQ water. Then the electrodes were covered with a Teflon cap, which formed a cell volume of about 30 µL on the thiol-modified gold electrodes. Modification with CDH was made by filling the cavity with enzyme solution (20 mg mL⁻¹). A dialysis membrane (Carl Roth, molecular weight cut-off: 14,000 Da) was used to trap the enzyme in the cells (6). The dialysis membrane (pre-soaked in buffer) was pressed onto the electrode and fixed tightly with a rubber O-ring. All measurements were performed at room temperature. Cyclic voltammetry (scan rate = 10 mV s⁻¹) and square wave voltammetry were performed using a Gamry Reference 600 potentiostat (Gamry Instruments, Warminster, PA, USA) scanning between -50 and 350 mV vs. SHE. The square wave voltammograms were recorded at a frequency of 1 Hz, a step potential of 2 mV and amplitude of 20 mV. A standard three-electrode configuration was used with an Ag/AgCl reference electrode (saturated KCl,
Gamry Instruments) and a platinum wire as counter electrode. The buffers (McIlvaine buffer, optionally containing 10 mM lactose for catalytic experiments) used as electrolytes were carefully degassed under vacuum and purged with argon prior to all experiments. To maintain the inert atmosphere argon was blown over the solution during the measurements.

2.8 Kinetic measurements

Initial rates for the determination of pH profiles of various electron acceptors were determined at 30°C in McIlvaine buffer ranging from pH 2.5 to 9. Additional electron acceptors not mentioned in the enzyme assay section were: 1,4-benzoquinone ($\varepsilon_{290} = 2.24$ mM$^{-1}$ cm$^{-1}$) and ferrocenium hexafluorophosphate (FcPF$_6$, $\varepsilon_{300} = 4.3$ mM$^{-1}$ cm$^{-1}$). Due to the instability of FcPF$_6$ at alkaline pH values the buffer, lactose and enzyme solutions were pre-warmed and the reaction was started by addition of FcPF$_6$. The relative activities of both CDHs for carbohydrates (lactose, cellobiose, maltose, maltotriose, mannose, glucose, galactose and xylose) were measured with a 100 mM substrate concentration using DCIP (300 µM), cyt $c$ (20 µM), 1,4-benzoquinone (1000 µM) or FcPF$_6$ (100 µM) as electron acceptors. Catalytic constants for various carbohydrates were determined with 1,4-benzoquinone at pH 6.0. Catalytic constants were calculated using nonlinear least-squares regression by fitting the observed data to the Michaelis-Menten equation (Sigma Plot 11, Systat Software).
3. Results

3.1 Production and purification of recombinant CDH IIA, CDH IIB and GH61-3

The fermentations of CDH IIA (Figure 1A) and CDH IIB (Figure 1B) were performed as uniform as possible. By the end of the glycerol feed both cultures showed similar cell densities (CDH IIA: 170 g L\(^{-1}\); CDH IIB: 180 g L\(^{-1}\)). No CDH activity was detected at this time. After inducing the enzyme expression by feeding methanol, the specific growth rate \(\mu\) was reduced to 25–30% and the final cell densities were 270 g L\(^{-1}\) (after 84 h) and 310 g L\(^{-1}\) (after 96 h), respectively. The secretion of extracellular protein correlated with the biomass production. It was higher for CDH IIB (0.65 g L\(^{-1}\) after 98 h) than for CDH IIA (0.28 g L\(^{-1}\) after 96 h). CDH IIA was expressed with a higher volumetric activity (1700 U L\(^{-1}\), DCIP assay at pH 5.0; 360 U L\(^{-1}\), cyt \(c\) assay at pH 6.0) than CDH IIB (410 U L\(^{-1}\), DCIP assay at pH 5.0; 130 U L\(^{-1}\), cyt \(c\) assay at pH 6.0).

With no activity assay for GH61-3 at hand, NCU02916 transformants were checked for successful integration of the gene into the \(P.\) pastoris genome by PCR with the standard primers 5´AOX and 3´AOX. A positive clone was chosen for small scale fermentation. A steady increase of wet biomass could be observed throughout the fermentation reaching a wet biomass concentration of 355 g L\(^{-1}\) at time of harvest (Figure 1C). The specific growth rate was reduced by two-thirds after induction. The final extracellular protein concentration was 1.37 g L\(^{-1}\). Following the expression of GH61-3 by SDS-PAGE (Figure S2) it was found that it represents the major protein (band at ~50 kDa) in the culture supernatant. Its amount increases steadily during induction and the final concentration of GH61-3 in the culture supernatant was ~450 mg L\(^{-1}\).

CDH IIA was purified to a specific activity of 21.2 U mg\(^{-1}\) (DCIP assay, pH 5.0) or 8.3 U mg\(^{-1}\) (cyt \(c\) assay, pH 6.0) with a high yield (73%) by a two-step chromatographic purification procedure (Table S1). The purification of CDH IIB was more difficult because of its very low...
affinity to the HIC resin as well as the AIEX resin, which resulted in weak binding and losses. Whereas CDH IIA bound already at 20% ammonium sulphate saturation (125 mS cm⁻¹) to the PHE-Sepharose resin and was eluted at 11% (70 mS cm⁻¹), CDH IIB needed 30% saturation (180 mS cm⁻¹) and eluted at 20% (125 mS cm⁻¹). On the Q-Source resin CDH IIA eluted at a higher NaCl concentration ~150 mM (15 mS cm⁻¹) than CDH IIB ~110 mM (11 mS cm⁻¹). An additional gel filtration step had to be introduced to obtain homogenous CDH IIB, which resulted in a poor yield of only 11%. Purified CDH IIB has a specific activity of 5.1 U mg⁻¹ (DCIP assay, pH 5.0) or 3.0 U mg⁻¹ (cyt c assay, pH 6.0). GH61-3 bound to the HIC resin at 30% ammonium sulphate saturation and eluted at 8% (50 mS cm⁻¹). The fractions of the peak at 280 nm were pooled and concentrated giving 5.7 mL of a green solution having a protein content of 9 mg mL⁻¹. After a final gel filtration step the purest fractions were pooled and concentrated, giving 2 mL of a light blue solution with a protein content of 22.5 mg mL⁻¹.

3.2 Physical properties

Molecular masses were determined by SDS-PAGE. Both CDHs showed diffuse bands between 110 and 130 kDa, which can be caused by heterogeneous glycosylation and/or the commonly observed smearing of glycoproteins on SDS-PAGE (Figure S3). After deglycosylation under denaturing conditions with PNGase F single, sharp bands with a molecular mass of 85 kDa for CDH IIA and 88 kDa for CDH IIB are observed. The additional bands in the deglycosylated samples at 35 kDa originate from PNGase F. GH61-3 forms a diffuse band at ~55 kDa. After 48 h of deglycosylation with endoglucanase Hf and α-mannosidase the molecular mass is reduced to ~45 kDa. Chromatofocusing was used to determine the isoelectric points for CDH IIA and IIB, which are at pH 5.14 and pH 4.93, respectively. The UV/Vis spectra of the purified CDHs (Figures 2A and 2B) are characteristic. Upon reduction with lactose the typical Soret band shift is observed, while the α- and β-peaks appear at 563 nm and 533 nm, respectively. Concomitantly, the absorbance in
the region between 450 to 500 nm decreases due to reduction of the FAD. The molar absorption coefficients at 280 nm for all proteins were calculated using the mature amino acid sequence and the program ProtParam (http://web.expasy.org/protparam/). The molar absorption coefficients for CDH IIA ($\varepsilon_{280} = 174 \text{ mM}^{-1} \text{ cm}^{-1}$, $\varepsilon_{420}$ (ox) = 103 $\text{ mM}^{-1} \text{ cm}^{-1}$, $\varepsilon_{430}$ (red) = 142 $\text{ mM}^{-1} \text{ cm}^{-1}$, $\varepsilon_{532}$ (red) = 15.3 $\text{ mM}^{-1} \text{ cm}^{-1}$) and for CDH IIB ($\varepsilon_{280} = 157 \text{ mM}^{-1} \text{ cm}^{-1}$, $\varepsilon_{420}$ (ox) = 87 $\text{ mM}^{-1} \text{ cm}^{-1}$, $\varepsilon_{430}$ (red) = 119 $\text{ mM}^{-1} \text{ cm}^{-1}$, $\varepsilon_{532}$ (red) = 12.3 $\text{ mM}^{-1} \text{ cm}^{-1}$) were calculated from the spectra. The molar absorption coefficients for GH61-3 are: $\varepsilon_{280} = 47 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\varepsilon_{630}$ (ox) = 0.04 $\text{ mM}^{-1} \text{ cm}^{-1}$. The absorbance ratio ($A_{630}/A_{280}$) of GH61-3 is 0.00079 (Figure 2C).

Cyclic voltammetry and square wave voltammetry were employed to determine the redox properties of both CDHs cytochrome domains at pH 6.0 and 7.5 (Figure 3A and 3B). At pH 6.0, the specific catalytic currents are higher than at pH 7.5 and also the oxidative and reductive waves in the cyclic voltammograms are better defined than at pH 7.5. The determined midpoint potentials of the cytochrome domain’s heme $b$ cofactor are 99 mV vs. SHE at pH 6.0 and 158 mV vs. SHE at pH 7.5 for CDH IIB. The catalytic current at 350 mV vs. SHE in the presence of cellobiose is similar for both CDHs at pH 6.0 (12.5 $\mu\text{A cm}^{-2}$). At pH 7.5 the current is lower and slight differences are observed (CDH IIA: 5.3 $\mu\text{A cm}^{-2}$; CDH IIB: 4.5 $\mu\text{A cm}^{-2}$). The onset of the catalytic currents on the gold electrodes starts around 60 mV vs. SHE for CDH IIA and around 100 mV vs. SHE for CDH IIB. The onset was defined as the potential were 5% of the maximum current were measured. With GH61-3 no direct electron transfer could be observed on a thioglycerol modified gold electrode.

3.3 Catalytic properties
The pH dependency of the two-electron acceptor reduction by CDH IIA and CDH IIB (Figures 4A–4D) was similar for DCIP (optima at pH 5.0, >50% relative activity from pH 3.8 to 6.5) and 1,4-benzoquinone (optima at pH 7.0, >50% relative activity from pH 3.7 to >8.0). Due to quinhydrone formation in alkaline milieu, pH values above 8.0 were not measured with 1,4-benzoquinone.

The catalytic constants for the reduction of electron acceptors were determined at pH 5.0 for DCIP and pH 6.0 for the other electron acceptors (Table 1). In a preliminary screening we found that the relative activities of each CDH for various carbohydrates were independent of the used electron acceptor DCIP, cyt c or 1,4-benzoquinone (Table S2). In this experiment the most obvious difference between CDH IIA and CDH IIB was a 5 times higher glucose turnover relative to cellobiose by CDH IIB. By measuring the catalytic constants for cellobiose and lactose with DCIP as cosubstrate, we observed substrate inhibition for both CDHs (Table S3, Fig. S4 and S5). This behaviour was only found with DCIP as electron acceptor. As a consequence, the apparent catalytic constants of CDHs for carbohydrates were determined with the electron acceptor 1,4-benzoquinone (Table 2).

3.4 Interaction of CDH IIA and IIB with GH61-3

The interaction between CDH and GH61-3 was studied by the inhibition of the cyt c activity in the presence of varying concentrations of GH61-3. The rationale of experiments was to treat cyt c and GH61-3 as competing substrates for the intermolecular electron transfer from CDH’s cytochrome domain. In preliminary experiments with CDH IIA a constant concentration of 10 µM cyt c with varying GH61-3 concentrations was tested. Even with a low molar ratio cyt c:GH61-3 = 1:0.25 a significant reduction of the competing cyt c activity (10%) was found for both CDHs which increased up to 60% at a ratio of 1:4. Experiments using BSA and copper sulphate (in a four-fold excess over cyt c) as unspecific substitutes for GH61-3 showed little influence on the reduction of cyt c by CDH (Figure S6).
Initial rates were measured to guarantee steady-state conditions. A linear change in the absorbance indicates that indeed neither cyt c nor GH61-3 were depleted during the reactions. The measured pH dependency of the CDH IIA/GH61-3 (Figure 4E) and CDH IIB/GH61-3 (Figure 4F) interaction is expressed as % inhibition of cyt c activity. The highest inhibition of cyt c by the CDH/GH61-3 interaction is observed at acidic pH. The pH optimum of CDH IIB/GH61-3 interaction is more acidic for CDH IIA than for CDH IIB, but a much higher cyt c inhibition was found for CDH IIB/GH61-3 (81%) than for CDH IIA/GH61-3 (55%).
4. Discussion

*N. crassa* is a model organism in the fields of genetics, biochemistry and fungal biology and has been studied for many decades. It is also recognized as a potent producer of lignocellulolytic and hemicellulolytic enzymes (5, 25, 28). When grown under cellulolytic conditions, a major part of the secretome consists of CDH IIA (2.4%) and members of the GH61 (14.6%) family – all together 17% (23) of the totally secreted protein mass, which indicates their importance in biomass degradation by *N. crassa*. In a transcriptome analysis of *N. crassa* done by Tian *et al.* (32), a tremendous increase of the cdhIIa gene transcription level during cultivation on Miscanthus was found (160-fold after 16 h), whereas the transcription of cdhIIb increased by only 2-3 times compared to growth on minimal medium. Some of the 14 gh61 genes found in *N. crassa* are also strongly upregulated during growth on Miscanthus whereas others are not (Figure 5). The transcription of 8 from the 14 putative gh61 genes found in the *N. crassa* genome is upregulated more than 5-fold, while 4 genes show a weak or no change (for 2 genes no transcriptional data are available). By comparison, a similar number of GH61s with/without a C-terminal CBM is upregulated, whereas only the CBM carrying CDH IIA is induced. The induction of CDH IIA during growth under cellulolytic conditions was shown to be even more pronounced in a strain carrying a deletion of cre-1. The authors of the study identified cdhIIA as a part of the CRE-1 regulon in *N. crassa* (27). cdhIIB seems to be unaffected by CRE-1 repression during growth on preferred carbon sources such as glucose. So far, expression of CDH IIB in *N. crassa* cultures could not be verified (8, 23). The comparison of CDH IIA and CDH IIB amino acid sequences (alignment see 8) show a low identity (53.2%). Interestingly, the flavodehydrogenase domains share more identical amino acids (59.2%) than the cytochrome domains (39%). In other ascomycete species CDH IIB is, however, an active enzyme and expressed solely (e.g. *Corynascus thermophilus*) or at much higher levels than CDH IIA (e.g. *Hypoxylon*...
haematostroma) (8). The reason for being missed in the purification of N. crassa culture supernatant by Harreither et al. (8) is most likely its low amount and the lower affinity to chromatography resins where the low quantity of CDH IIB was presumably not bound in the capture step and lost with the flow-through.

To investigate if N. crassa CDH IIB is an active enzyme and in which aspects it differs from CDH IIA, P. pastoris was chosen for heterologous overexpression of both CDHs. Since a previous attempt to express N. crassa CDH IIB in P. pastoris had failed (43), we chose a different approach for the construction of the expression cassette. Instead of using the S. cerevisiae α-factor pre-pro leader peptide the native leader sequence was used as secretion signal. This approach was already successfully applied for other FAD-dependent oxidoreductases from the GMC protein family (29, 30). To obtain both CDHs as similar as possible to the wild-type enzymes, any tags for purification or antibody detection were omitted. These changes in the expression strategy enabled the production of both CDHs in reasonable amounts. The higher volumetric activity measured in the culture supernatant of CDH IIA is due to the higher specific activity, the concentration of the CDHs was equal (80 mg L\(^{-1}\), including its proteolytic degradation product, the flavodehydrogenase domain). In comparison, the percentage of the total extracellular protein representing the recombinant enzyme was highest for GH61-3 (33%), slightly lower for CDH IIA (17% plus 11% flavodehydrogenase domain) and lowest for CDH IIB (7% plus 5% flavodehydrogenase domain).

The first difference in the physical properties of both CDHs was observed during purification. CDH IIA shows a strong interaction with the phenyl groups of the hydrophobic interaction resin, whereas CDH IIB is bound weaker and needs a higher ammonium sulphate concentration. A similar behaviour was found on the anion exchange resin, where CDH IIB is also bound weaker despite its lower pI. Since the number of putative glycosylation sites is identical in both CDHs (eight surface exposed sequons each) and also the amino acid
composition is similar, the different binding/desorption behavior is likely to originate from a
different composition of surface regions or the presence of the CBM. The low affinity of
CDH IIB to chromatography matrices reduced not only the yield by excessive tailing, but also
necessitated a further purification step by size exclusion chromatography. GH61-3, like CDH
IIB, needs 30% ammonium sulphate saturation to bind on the HIC matrix. It also bound
poorly on an anion exchange matrix (Q-Sepharose) and was therefore purified by size
exclusion chromatography. All enzymes were stable during purification and storage. The
absorbance ratios (A_{420nm}/A_{280nm}) for CDH IIA (0.58) and CDH IIB (0.55) are high and
confirm the purity of the obtained CDH preparations. Values for other ascomycetous CDHs
are e.g. 0.63 for *Humicola insolens* CDH IIB (13) or 0.60 for *C. thermophilus* CDH IIB (8).

In GH61-3 the very low absorption of the copper ion at 630 nm indicates a type-2 copper
complex.

The measured molecular masses of CDH IIA and CDH IIB are higher than that of CDH IIA
purified from the *N. crassa* proteome (90 kDa), which seems to be less glycosylated than the
recombinant CDHs. The mass difference between the glycosylated and deglycosylated
enzymes originates from N-glycans which can be cleaved off by PNGase F. The bands of the
deglycosylated CDHs are in perfect agreement with the masses calculated from the mature
amino acid sequences, which are 86.2 kDa for CDH IIA and 86.6 kDa for CDH IIB.

Recombinant GH61-3 is also glycosylated. However, since no reduction of the molecular
mass was observed after PNGase treatment we conclude that the contribution of N-glycans to
the glycoproteins molecular mass is small. The two N-glycosylation consensus sequences
(NXS/T) present in the GH61-3 sequence could increase the molecular mass by ~5 kDa, if
one assumes the typical high mannose-type N-glycan commonly reported for secreted
proteins from *P. pastoris*. Prolonged deglycosylation with a mixture of endoglucanase F and
α-mannosidase showed a decrease in mass by 10 kDa to ~45 kDa, which is still bigger than
the calculated mass of the mature protein sequence (34.3 kDa). We conclude that GH61-3 is
mostly O-glycosylated by mannose oligosaccharides (18, 33) possibly at the Ser/Thr rich 
linker before the C-terminal CBM. The isoelectric points of 5.14 for CDH IIA and 4.93 for 
CDH IIB as determined by chromatofocusing are notably higher than those of most other 
CDHs (~4.1), with the exception of Chaetomium sp. INBI (2-26-) CDH (5.0, (15)). In 
comparison to the theoretically calculated pI values of 6.7 for CDH IIA and 7.9 for CDH IIB 
this indicates a clear imbalance of surface exposed anionic and buried cationic amino acids. 
The previously reported pI of 6.8 for recombinantly expressed CDH IIA (43) is much higher 
and reflects most likely the addition of a His6-tag.

Another difference between both CDHs was found in the electrochemically measured redox 
potential of the heme b cofactor in the cytochrome domain. CDH IIA showed with 95 mV vs. 
SHE a 65 mV lower midpoint potential than the CDH IIB counterpart. The two values are in 
good agreement with other published midpoint potentials, but represent the upper and the 
lower values reported so far. The lower value for CDH IIA is in good agreement with C. 
thermophilus CDH IIB (31). For other class II CDHs higher values around 130 mV at neutral 
pH conditions were reported. The midpoint potential does only change by 5 mV between pH 
6.0 and 7.5, which was also found for CDH IIB from H. insolens and CDH IIA from M. 
thermophilum (3, 13).

Steady-state kinetic measurements for various electron acceptors revealed identical pH optima 
of both CDHs for the two-electron acceptors DCIP (pH 5.0) and 1,4-benzoquinone (pH 7.0), 
which are reduced at the flavodehydrogenase domain. However, the pH profiles for the one-
electron acceptors cyt c and FePF₆ differed, which can be seen from the normalized insets. 
Cyt c is strictly dependent on the presence of CDH’s cytochrome domain for the 
intermolecular electron transfer. CDH IIA has a bell shaped optimum for cyt c turnover (pH 
6.0) while CDH IIB shows a broad plateau from pH 4.0 to 9.0 with two optima at pH 5.0 and 
7.5. Thus CDH IIB has an extended activity in the acidic and the alkaline pH range. The pH 
optimum of CDH IIB for FePF₆ is much higher than for CDH IIA. The pH optima of various
*N. crassa* cellulolytic enzymes range from pH 5.0 to 7.0 with significant residual activities from pH 3.0 to 8.5 (4, 40). Judged from the pH profiles of the one-electron acceptors CDH IIB works over a broader pH range and at alkaline pH values.

For all tested electron acceptors the K$_M$ values were in the low micromolar range. The catalytic efficiency for the two-electron acceptors DCIP and 1,4-benzoquinone were 3.9 and 4.6 times higher for CDH IIA, the catalytic efficiency for FePF$_6$ is even 24 times higher for CDH IIA. Cyt c is the exception – it is 1.5 times more efficiently reduced by CDH IIB. For the determination of the catalytic constants for carbohydrates 1,4-benzoquinone was used to avoid the limitation of the oxidative FAD cycle by either the contribution of the intramolecular electron transfer (IET) on the measurements when using cyt c, nor to limit the reaction by the inhibition found with DCIP for *N. crassa* CDH turnover. The substrate inhibition in the presence of DCIP was observed for both CDHs, but is stronger for CDH IIB. The K$_i$ values for cellobiose and lactose remain relatively constant between pH 4.0 and 7.0.

The highest catalytic efficiencies for electron donors are found for the β-1,4-linked disaccharide cellobiose and cello-oligosaccharides. Although the catalytic efficiencies are very similar, the CDHs differ again in their apparent k$_{cat}$ and K$_M$ values. CDH IIA has approx. 4-5 times higher k$_{cat}$ values whereas CDH IIB has approx. 4-5 times lower K$_M$ values. Nevertheless, observed catalytic constants for a number of sugar substrates reveal a broad substrate spectrum. Beside the cellobiose mimicking substrate lactose, both CDHs can convert the β-1,4-linked hemicelluloses xylobiose and xylotriose, with K$_M$ values in the low millimolar range and only 50–100 times lower catalytic efficiencies than cellobiose. This demonstrates that CDH IIA and IIB can be efficiently reduced by xylobiose and xylotriose in vivo and might reflect the lifestyle of *N. crassa* as degrader of hemicellulose-rich plant cell walls. The catalytic efficiencies for the monomers glucose and xylose are 25,000–35,000 times lower than for the di- or polysaccharides built of them. The discrimination of glucose in respect to cellobiose is nearly as high as in basidiomycetous class-I CDHs (e.g. *P.*
chrysosporium CDH: 87,000, (12)). Starch derived maltose and malto-oligosaccharides featuring α-1,4-linkages are moderate substrates for CDH IIB but poor substrates for CDH IIA. 2α-mannobiose with an α-1,2-linkage was not converted, which stands in contrast to previously published data (43). To verify measurements 2α-mannobiose was ordered from two different suppliers (Sigma and Santa Cruz Biotechnology Inc.). Mannopentaose was however a modest substrate. The comparison of further catalytic constants for N. crassa CDH IIA reported in this manuscript with previously reported data by Zhang et al. is limited, because of the different used electron acceptors and pH values (DCIP at pH 4.5, (43). Other hemicellulose constituents such as mannose, arabinose and galactose are no in vivo substrates. Cyt c, which is exclusively oxidized at CDH’s cytochrome domain provides a measure of the IET rate between the flavodehydrogenase and the cytochrome domain when it is assumed that the cytochrome domain/cyt c interaction is very fast and not rate limiting. High bimolecular rate constants have been indeed reported for P. chrysosporium CDH (1.75 × 10^7 M⁻¹ s⁻¹ (26) and 6.6 × 10^6 M⁻¹ s⁻¹ (14)). From the catalytic efficiencies measured for cyt c with CDH IIA (9.5 × 10^5 M⁻¹ s⁻¹) and CDH IIB (1.4 × 10^6 M⁻¹ s⁻¹) a similarly fast electron transfer reaction can be assumed. Interestingly, the k_cat value was 6.9 times higher for CDH IIA despite the 65 mV lower midpoint potential of its cytochrome domain. Both CDHs show IET up to pH 9.0. Fast kinetic studies will be necessary to elucidate the IET and the cytochrome domain/cyt c interaction in more detail.

The second investigated reaction in which IET is involved to transfer reduction equivalents to a macroscopic, terminal electron acceptor was the interaction of CDH with SAM gold electrodes. It is found that at pH 6.0 the direct electron transfer (DET) to the macroscopic electron acceptor limits the whole reaction. This is assumed from the similar catalytic currents obtained from both CDHs at pH 6.0 and 7.5. If this last electron transfer step would not be rate limiting, the different IET rates indicated by the above discussed cyt c turnover should result in a higher current for CDH IIA. The lower midpoint potential of the CDH IIA
cytochrome domain is also reflected by the lower onset potential of CDH IIA’s catalytic current. This different midpoint potential might be of importance for the interaction with GH61 polysaccharide monooxygenases.

Experimental data from a growing body of literature emphasize the concept that CDH enhances the depolymerisation of crystalline cellulose in a synergistic mechanism together with GH61 polysaccharide monooxygenases. Not much about the interaction of CDH and GH61 enzymes is known yet, but it was shown that the electron transfer proceeds via the cytochrome domain since the flavodehydrogenase domain alone showed no effect (22). However, these studies used CDHs and GH61 enzymes from different organisms. N. crassa GH61-3 (NCU02916) used in this study is a close relative of NCU0224 and NCU01050, which were both identified as polysaccharide monooxygenases (22). The interaction with both N. crassa CDHs was measured by using a competing substrate approach. Cyt c was chosen as competing substrate for GH61-3, since in both reactions IET is, as postulated, involved. From the strong inhibition of the reaction with cyt c, which is an electron acceptor with a high catalytic efficiency/bimolecular rate constant – it is obvious that the interaction of CDH with GH61-3 is of similarly high efficiency. This effect was found to be more pronounced for CDH IIB. The cyt c reaction was inhibited by 81% in the presence of GH61-3 at pH 4.0 and 5.0, whereas for CDH IIA only an inhibition of 55% at pH 4.0 was found. Similar to the pH profiles of the one-electron acceptors of cyt c and FcPF6, CDH IIB showed a more efficient interaction with GH61-3 also at neutral/alkaline pH. At pH 8.0 GH61-3 inhibited the cyt c reduction by of CDH IIB to 43%, but of CDH IIA only to 19%. Whether CDH IIB generally interacts more efficiently with N. crassa polysaccharide monooxygenases from the GH61 family or just in the case of GH61-3 remains to be elucidated. The higher pH optimum of the CDH IIB/GH61-3 interaction is probably a result of the higher redox potential of CDH IIB’s cytochrome domain.
This work characterized in detail the properties of *N. crassa* CDH IIA, CDH IIB and GH61-3 and their interaction for electron transfer. The two CDHs differ strongly by their transcription level, the presence of a CBM, the redox potential of the cytochrome domain and the pH optima with positively charged one-electron acceptors and GH61-3. We suggest that CDH IIA and CDH IIB fulfil different functions at different fungal growth phases, under different pH conditions or with different GH61 enzymes. Their ability to obtain electrons from cellobiose or cello-oligosaccharides and xylobiose or xylo-oligosaccharides suggests that, depending on the reduced GH61 polysaccharide monooxygenase, CDHs are involved in cellulose and hemicellulose degradation.

5. Acknowledgements

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FIGURE CAPTIONS

FIG. 1. Production of recombinant CDH IIA (A), CDH IIB (B) and GH61-3 (C) in *P. pastoris*. Black circles, wet biomass; grey triangles, volumetric activity (DCIP assay at pH 5.0); black diamonds, extracellular protein concentration (Bradford assay). The measurements were done in duplicates, the difference between the values was <5%.

FIG. 2. Spectral characterization of CDH IIA (A), CDH IIB (B) showing the oxidized (black) and reduced (gray) spectra. Lactose was used for reduction. The difference spectra (oxidized-reduced) are given as insets. Spectrum of oxidized GH61-3 (C) with an inset showing the spectrum of the type-2 copper atom in its oxidized (black) and reduced (grey) state. Ascorbate was used to reduce the enzyme.

FIG. 3. Electrochemical measurements of both CDHs on thiol-modified gold electrodes at pH 6.0 (A) and pH 7.5 (B). Midpoint potentials of both CDHs were calculated from cyclic voltammograms (left) and square wave voltammograms (middle) in the absence of substrate. Cyclic voltammetry (right) in the presence of substrate was used to measure the current of direct electron transfer to a gold electrode.

FIG. 4. pH dependency of CDH IIA and IIB activities for the artificial electron acceptors ferrocenium hexafluorophosphate (A), cytochrome c (B), 1,4-benzoquinone (C) and DCIP (D) using lactose as electron donor. CDH IIA, circles; CDH IIB, triangles. Activities from pH 2.5-9 were measured with McIlvaine buffer. pH dependent interaction of CDH IIA (E) and CDH IIB (F) with the competitive substrates cyt c and GH61-3. Turnover rates of cyt c in the absence of GH61-3, diamonds; turnover rates of cyt c in presence of GH61-3, squares; the
ratio of the turnover numbers, which is used as a measure for the inhibition of the cyt c reduction by GH61-3, filled circles.

FIG 5. Phylogenetic tree of the *N. crassa* GH61 protein family. Sequence alignment was performed with Clustal using the default parameters, phylogenetic analysis was done with MEGA 5 using the Maximum likelihood method and 1000 Bootstrap replicates. Relative expression levels of *N. crassa* gh61 genes during growth on Miscanthus (16 h) are shown. Data taken from Tian et al. (32).


Table 1. Apparent kinetic constants of CDH IIA and CDH IIB for electron donors were determined with lactose (30 mM) in McIlvaine buffer at the indicated pH values.

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Stoichiometry</th>
<th>pH value</th>
<th>CDH IIA</th>
<th>CDH IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_M$ [µM]</td>
<td>$k_{cat}$ [s$^{-1}$]</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>2</td>
<td>6</td>
<td>67.9 ± 5.8</td>
<td>64.8 ± 1.7</td>
</tr>
<tr>
<td>FePF$_6$</td>
<td>2</td>
<td>6</td>
<td>5.2 ± 0.2</td>
<td>75.4 ± 2.1</td>
</tr>
<tr>
<td>1,4-Benzoquinone</td>
<td>1</td>
<td>6</td>
<td>26.2 ± 3.0</td>
<td>49.2 ± 0.7</td>
</tr>
<tr>
<td>DCIP</td>
<td>1</td>
<td>5</td>
<td>33.8 ± 4.8</td>
<td>38.1 ± 0.8</td>
</tr>
</tbody>
</table>

The sample standard deviation was calculated from n=3 experiments.
Table 2. Apparent kinetic constants of CDH IIA and CDH IIB for electron donors were determined with 1,4-benzoquinone (1 mM) in McIlvaine buffer, pH 6.0.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>CDH IIA</th>
<th>CDH IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[mM]</td>
<td>[s⁻¹]</td>
</tr>
<tr>
<td>Cellophane</td>
<td>0.090</td>
<td>45.8</td>
</tr>
<tr>
<td>Cellotriose</td>
<td>0.230</td>
<td>49.7</td>
</tr>
<tr>
<td>Cellotetraose</td>
<td>0.204</td>
<td>46.0</td>
</tr>
<tr>
<td>Cellopentaose</td>
<td>0.196</td>
<td>46.6</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.290</td>
<td>49.3</td>
</tr>
<tr>
<td>Maltose</td>
<td>17.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>34.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>35.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>3700*</td>
<td>55*</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>1.5**</td>
</tr>
<tr>
<td>Mannopentaose</td>
<td>39.2</td>
<td>19.8</td>
</tr>
<tr>
<td>Mannobiose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mannose</td>
<td>10000*</td>
<td>4.7</td>
</tr>
<tr>
<td>Xylobiose</td>
<td>3.6</td>
<td>45.4</td>
</tr>
<tr>
<td>Xylootriose</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Xylose</td>
<td>2770*</td>
<td>1.5</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>0.1**</td>
</tr>
</tbody>
</table>

* extrapolated K_m and k_cat values
** turnover number measured with 1000 mM galactose

The relative standard error (n=3) for all reported K_m and k_cat values is <10%.