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Cellobiose Dehydrogenase and a Copper-Dependent Polysaccharide Monooxygenase Potentiate Cellulose Degradation by *Neurospora crassa*

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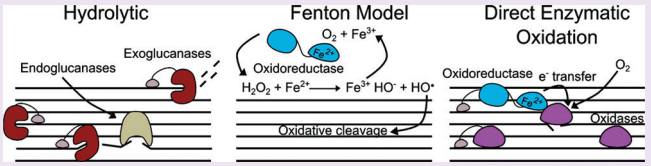
Supporting Information

ABSTRACT: The high cost of enzymes for saccharification of lignocellulosic biomass is a major barrier to the production of second generation biofuels. Using a combination of genetic and biochemical techniques, we report that filamentous fungi use oxidative enzymes to cleave glycosidic bonds in cellulose. Deletion of *cdh-1*, the gene encoding the major cellobiose dehydrogenase of *Neurospora crassa,* reduced cellulase activity substantially, and addition of purified cellobiose dehydrogenases from *M. thermophila* to the Δ*cdh-1* strain resulted in a 1.6- to 2.0-fold stimulation in cellulase activity. Addition of cellobiose dehydrogenase to a mixture of purified cellulases showed no stimulatory effect. We show that cellobiose dehydrogenase enhances cellulose degradation by coupling the oxidation of cellobiose to the reductive activation of copper-dependent polysaccharide monooxygenases (PMOs) that catalyze the insertion of oxygen into C−H bonds adjacent to the glycosidic linkage. Three of these PMOs were characterized and shown to have different regiospecifities resulting in oxidized products modified at either the reducing or nonreducing end of a glucan chain. In contrast to previous models where oxidative enzymes were thought to produce reactive oxygen species that randomly attacked the substrate, the data here support a direct, enzyme-catalyzed oxidation of cellulose. Cellobiose dehydrogenases and proteins related to the polysaccharide monooxygenases described here are found throughout both ascomycete and basidiomycete fungi, suggesting that this model for oxidative cellulose degradation may be widespread throughout the fungal kingdom. When added to mixtures of cellulases, these proteins enhance cellulose saccharification, suggesting that they could be used to reduce the cost of biofuel production.



Production of renewable transportation fuels that are both economically and environmentally sustainable is crucial for meeting global energy demand and reducing greenhouse gas emissions. Lignocellulosic biomass is an abundant renewable feedstock[1] that in principle could be broken down enzymatically, but the cost of cellulose-degrading enzymes is a major barrier to the economical production of these second generation biofuels.[2] Fungi play a central role in the degradation of cellulose in terrestrial environments, and glycoside hydrolases secreted by these fungi have been studied in great detail.[3] Considerable effort has been focused on the discovery and optimization of these cellulases, but improvement of catalytic activity has proved to be slow and challenging.

Recent transcriptomic and proteomic analyses of cellulolytic fungi have identified oxidative enzymes involved in degradation of plant biomass.[4−6] Despite the widespread occurrence of these enzymes in fungi, the specific function of these oxidative enzymes in cellulose degradation is unknown (Figure 1). The filamentous ascomycete *Neurospora crassa* is a well-known and genetically tractable organism that proficiently degrades plant cell walls. In addition to hydrolytic enzymes, *N. crassa* also produces cellobiose dehydrogenase (CDH).[7−9] The *N. crassa* genome contains two genes encoding predicted CDHs, but only one is expressed. CDH-1 is the major oxidoreductase secreted during growth on cellulose and catalyzes the oxidation of cellobiose or longer cellodextrins to the corresponding 1-5-*δ*-lactones.[10] These lactones hydrolyze spontaneously in solution, or enzymatically by lactonases, to generate aldonic acids.[11] All known CDH enzymes contain an N-terminal heme domain and a C-terminal flavin domain. The flavin domain is part of the larger glucose-methanol-choline oxidoreductase superfamily, which is widespread throughout all domains of life,[12] whereas homologues of the heme domain are only found in fungi.[13] Oxidation of cellobiose takes place in the flavin domain with subsequent electron transfer to the heme domain. The reduced heme is able to reduce a wide variety of substrates including quinones, metal ions, and organic dyes. Reduced CDH can also react with molecular oxygen, but at a 10- to 20-fold slower rate than organic dyes and metal ions.[14] Although most cellulolytic fungi produce CDHs, the biological function of these proteins is largely unknown. In this report we have used a gene deletion of CDH-1 in *N. crassa* to show the importance of oxidative

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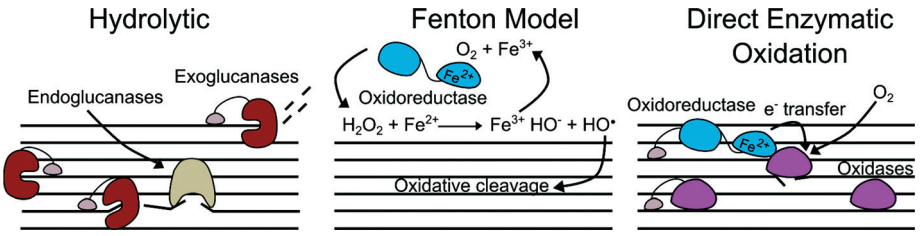

Figure 1. Schematic representing the hydrolytic and oxidative mechanisms proposed for cellulose breakdown. (Left) Hydrolytic chemistry: cellulases must gain access to a glucan chain and bring it into a cleft or tunnel active site where acid base catalysis is used to cleave glycosidic bonds. (Center) Fenton chemistry: hydroxyl radicals produced *via* the Fenton reaction from reduction of extracellular metal ions randomly oxidize cellulose. (Right) Enzymatic oxidation: direct enzyme-catalyzed oxidation by a combination of oxidases and oxidoreductases. Unlike hydrolases, oxidases could cleave glycosidic bonds without the energetically costly step of abstracting a glucan chain from crystalline cellulose.

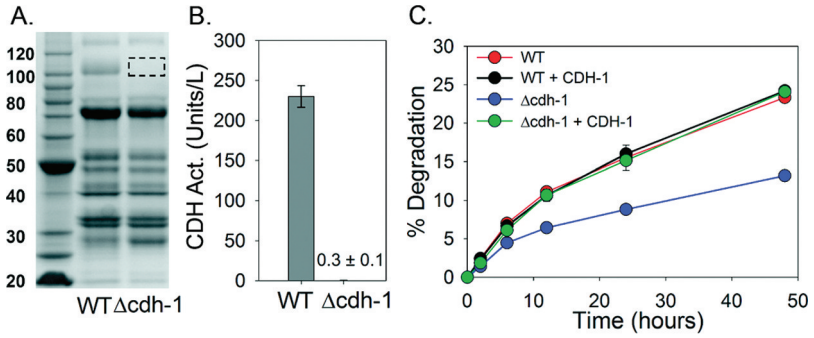

Figure 2. Characterization of Δ*cdh-1* strain. (A) SDS-PAGE of *N. crassa* WT (left) and Δ*cdh-1* strains. MW markers in kDa are to the left, and a box indicates the expected position of CDH-1. (B) CDH volumetric activity in the culture filtrate of WT and Δ*cdh-1* cultures grown on Avicel. (C) Cellulase assays with the WT and Δ*cdh-1* strains. Assays contained 10 mg mL^{-1} Avicel and 0.05 mg mL^{-1} secretome protein. CDH-1 from *Myceliophthora thermophila* was added at 0.004 mg mL^{-1}.

enzymes in fungi. Addition of CDH from *M. thermophila* was used to complement the Δ*cdh-1* strain, and a fractionation strategy was used to identify a novel family of copper-dependent enzymes that interact with CDH. These copper-dependent enzymes were then characterized and a mechanism proposed for their action on cellulose.

RESULTS AND DISCUSSION

Deletion of *N. crassa cdh-1*. A targeted gene replacement strategy was used to generate a clean deletion of *cdh-1* in *N. crassa*.[15,16] Proteins present in the secretome of the Δ*cdh-1* strain were similar to WT as judged by SDS-PAGE, except for the absence of CDH-1 (Figure 2A). CDH activity in the secretome of the Δ*cdh-1* strain was 800 ± 300-fold lower than in the WT secretome (Figure 2B). Cellulase activity of the Δ*cdh-1* strain secretome was 37−49% lower than that of WT and was restored to WT activity upon the addition of a purified *M. thermophila* CDH-1 (Figure 2C) or a partially purified *N. crassa* CDH-1 (Supplementary Figure 1). *M. thermophila* and *N. crassa* CDH-1 share 70% sequence identity and the same domain architecture. Due to difficulties in isolating pure *N. crassa* CDH-1, *M. thermophila* CDHs were used for the remainder of these studies. Addition of *M. thermophila* CDH-1 to WT secretome had no effect on cellulose hydrolysis (Figure 2C). Addition of *M. thermophila* CDH-2, which lacks a cellulose-binding module (CBM1), also stimulated cellulose hydrolysis; however, a 10-fold higher concentration was required compared to CDH-1 (Figure 3). The flavin catalytic domain of CDH-2, even at high concentrations, was not able to stimulate cellulose hydrolysis (Supplementary Figure 2). However, the flavin domain alone oxidizes cellobiose at rates identical to that of the full-length enzyme, suggesting that the N-terminal heme domain is required for the stimulatory effect. Methionine and histidine are the protein ligands to the CDH heme, and these residues are conserved in all CDHs. Given the ligation, electron transfer is likely to be outer sphere.[17] However, the physiological electron acceptor for the reduced CDH is unknown.

The most prominent hypothesis for the function of CDH involves generation of hydroxyl radicals formed *via* reduction of an extracellular ferric complex.[13,18] This ferrous iron can then take part in Fenton chemistry with hydrogen peroxide produced by CDH or another oxidase (Figure 1). Although the scope of these reactions is possible, control of hydroxyl radical reactivity would be particularly challenging. Several experiments were performed to determine if Fenton chemistry was responsible for the stimulation of cellulose degradation in *N. crassa*. Extensive buffer exchange of the Δ*cdh-1* strain secretome did not reduce the stimulatory effect of CDH on cellulose degradation. However, addition of the metal chelator EDTA completely blocked the stimulation (Supplementary Figure 3A). In control experiments where CDH-1 was incubated with EDTA, there was no change in the oxidation rate of cellobiose with DCPIP as an electron acceptor. Oxygen was also found to be required for stimulation of cellulase activity by CDH (Supplementary Figure 3B). Together these results suggested that metals and O_2 play a role in the enhancement, although low molecular weight metal complexes are not required. These results do not support a role for Fenton chemistry but do point toward the participation of a metal or metalloprotein.

Identification of a Copper Metalloenzyme. Next, an approach was developed to determine the mechanism by which CDH enhances cellulose degradation. Addition of CDH to a

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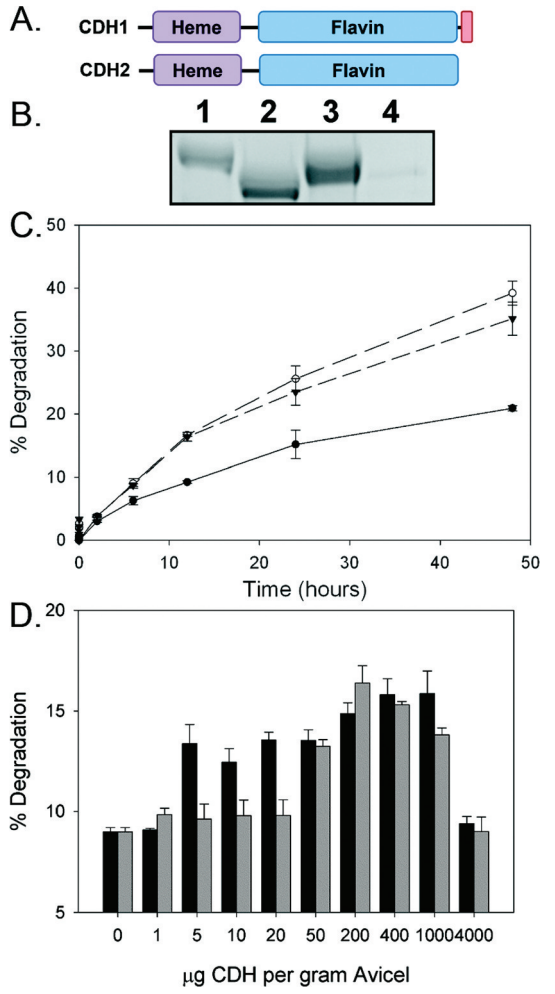

Figure 3. Stimulation of cellulose degradation by isoforms of CDH. (A) Domain architectures of *M. thermophila* CDH-1 and CDH-2. Red C-terminal domain on CDH-1 is a fungal cellulose-binding domain (CBM1). (B) Cellulose binding assay for *M. thermophila* CDH-1 and CDH-2. Lane 1, *M. thermophila* CDH-1; Lane 2, *M. thermophila* CDH-2; Lane 3, CDH-1 bound to Avicel; Lane 4, CDH-2 bound to Avicel. (C) Stimulation of cellulose degrading capacity of the Δ*cdh-1* culture filtrate (●) by addition of CDH-1 (○) or CDH-2 (▼). (D) Effect of the concentration of *M. thermophila* CDH-1 (black) and *M. thermophila* CDH-2 (gray) on cellulase activity of the Δ*cdh-1* culture filtrates. Values are the mean of three replicates. Error bars are the SD between these replicates.

mixture of purified cellulases showed no enhancement of activity (Supplementary Figure 4), suggesting that additional factor(s) were required. The strong inhibition by EDTA noted above was consistent with the hypothesis that these factors were metalloproteins. Analysis of the secretome showed that copper and zinc were the only metals present, with copper 10-fold more abundant than zinc (Supplementary Figure 5). However, no *N. crassa* proteins secreted during growth on cellulose are predicted to bind copper. Fractionation of the secretome (Supplementary Figure 6) resulted in two fractions that enhanced the cellulase activity of purified cellulases in a CDH-dependent fashion. Tryptic digests and subsequent LC–MS/MS analysis showed that each fraction contained two members of the GH61 protein family, erroneously classified as glycosyl hydrolases. GH61 proteins were previously reported to enhance cellulose degradation and were also inhibited by EDTA.[19] Further purification showed fractions containing GH61 proteins were also enriched in copper (Supplementary Figure 7). Phylogenetically diverse GH61 proteins encoded by NCU01050, NCU02240, NCU07898, and NCU08760 were purified (Figures 4 and 5A). Three of these were analyzed by

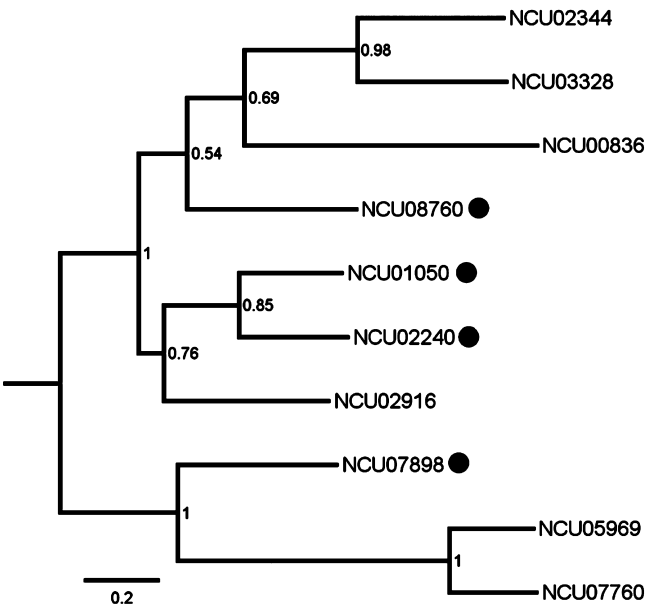

Figure 4. Phylogeny of GH61 proteins in *N. crassa.* Shown are the upregulated GH61 proteins. Of the 14 GH61 proteins in the *N. crassa* genome, 10 are upregulated >2-fold in response to growth on cellulose relative to sucrose.[7] Proteins identified *via* proteomics and purified here are marked (●).

inductively coupled plasma–atomic emission spectroscopy (ICP-AES) and found to bind copper with a 1:1 stoichiometry (Figure 5B). NCU02240 was not present in sufficient purity or yield for further characterization.

GH61 proteins have a highly conserved metal binding site that includes two histidine residues. One of the histidines is the N-terminal residue and functions as a bidentate ligand involving the amine and ring N2. Crystal structures of GH61 proteins with nickel,[20] magnesium, zinc,[19] or copper[21] have been reported; however, previous experimentation has not conclusively tied a specific metal to an activity. Redox chemistry involving O_2 generally requires a transition metal. Since copper is the metal natively bound to GH61 proteins from *N. crassa*, the correlation of activity with metal binding was investigated. In the presence of copper bound GH61, CDH activity increased nearly 10-fold, whereas apo or zinc bound GH61 only enhanced turnover 2-fold (Figure 5C). This suggests that GH61, and in particular GH61 bound to copper, can accept electrons from reduced CDH, thus increasing the oxidation of cellobiose. Hence, the copper in GH61 proteins could function as redox couple with CDH. Two other proteins that contain bidentate N-terminal histidine ligands were found in the protein databank. One of them, CopC, is part of the cop operon involved in copper resistance in bacteria and has been shown to bind Cu(II) with picomolar affinity (Figure 5D).[22] The other, particulate methane monooxygenase (pMMO), uses a trinuclear copper site to oxidize methane.[23] Combining our experimental results with the similar ligation in CopC and pMMO, we conclude that copper is the native metal in GH61 proteins.

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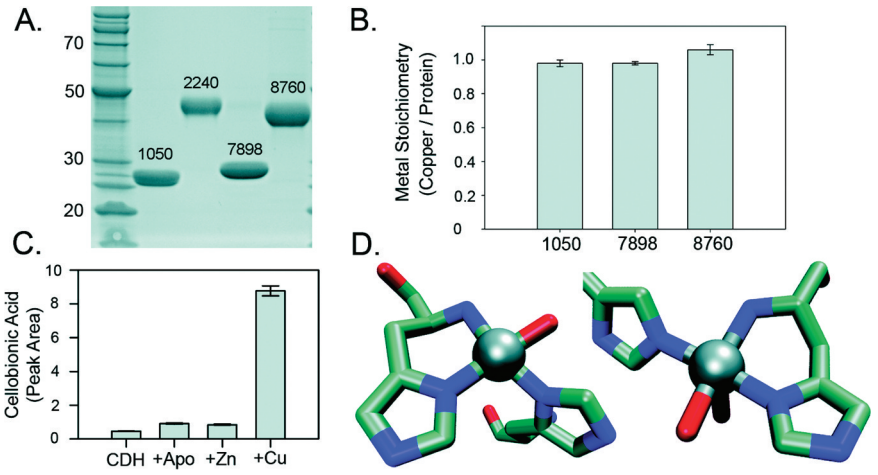

Figure 5. Purity and metal analysis of GH61 proteins. (A) SDS-PAGE of purified *N. crassa* GH61 proteins. (B) The number of bound copper molecules for each purified GH61 as determined by ICP-AES. (C) Effect of GH61 metal state on CDH activity. CDH-2 (200 nM) was mixed with 5.0 μM apo or metal reconstituted NCU01050 and 1.0 mM cellobiose. The reaction was incubated for 1 h at 40 °C, and products were analyzed by HPAEC. (D) Comparison of the metal binding sites in CopC (left) and GH61E (right) from *Thielavia terrestris*. Copper is bound in the CopC structure and zinc is bound in GH61E.

Product Analysis of Oxidative Cellulose Cleavage. The phylogenetic diversity of 10 *N. crassa* GH61s whose transcripts are upregulated during growth on cellulose[7] suggests that these enzymes may target a wide array of substrates in lignocellulose or generate different products (Figure 4). To investigate the reaction products of the purified GH61s, assays were performed on phosphoric acid swollen cellulose (PASC). When PASC was treated with GH61 and CDH, a series of aldonic acids two to nine glucose residues in length (A2−A9) were identified by high performance anion exchange chromatography (HPAEC). In addition to aldonic acids, the combination of CDH and GH61s NCU01050 or NCU07898 produced peaks at a later retention time (Figure 6A). Product analysis by liquid chromatography−mass spectrometry confirmed the presence of aldonic acids (Gx + 15 amu), as well as masses of Gx + 13 amu and Gx + 31 amu (Figure 6B). The Gx +13 mass is consistent with a doubly oxidized cellodextrin. Cellulose cleavage by these GH61s likely results in oxidation at the nonreducing end followed by oxidation at the reducing end by CDH. Given the necessity to cleave a 1,4-glycosidic bond, these products are likely oligosaccharides with a 4-keto sugar at the nonreducing end. The Gx + 31 mass is consistent with the hydrate of this product, a ketal. Ketoaldoses are unstable in aqueous solution and are known to decompose spontaneously into many different species.[24] The third purified GH61, NCU08760, did not form the late eluting peak on the HPAEC, or Gx + 13 and Gx + 31 species (Figure 6A and 6B), consistent with oxidation exclusively at the reducing end on C1 to form aldonic acids. Incubation of PASC with GH61 alone led to the formation of low amounts of hydrolytic products (Supplementary Figure 8). The formation of hydrolytic products could be due to low levels of cellulase contamination.

Since CDH is known to oxidize the C1 position of cellodextrins, a reaction with NCU08760 was carried out with ascorbic acid substituted for CDH. In the presence of ascorbic acid and copper, NCU08760 produced a ladder of aldonic acids (Figure 6C and Supplementary Figure 9). Under identical conditions, NCU01050 produced a ladder of products with a later retention time when analyzed by HPAEC and 100-fold less aldonic acid than NCU08760 (Figure 6D).

Proposed Mechanism of Polysaccharide Monooxygenases. The formation of oxidized sugars by the GH61s was oxygen dependent, suggesting that GH61s are oxidases (Supplementary Figure 10). Evidence that GH61s are copper enzymes requiring electron transfer from CDH to cleave cellulose in an oxygen-dependent manner provides the basis to propose a chemical mechanism for a new group of enzymes acting as polysaccharide monooxygenases (PMOs) (Figure 7). Precedent is drawn from the well-studied copper monooxygenases.[25,26] The work reported here supports one electron reduction of PMO-Cu(II) to PMO-Cu(I) by the CDH heme domain followed by oxygen binding and internal electron transfer to form a copper superoxo intermediate. Hydrogen atom abstraction by the copper superoxo at the 1-position (by NCU08760) or the 4-position (by NCU01050 or NCU07898) of an internal carbohydrate then takes place, generating a copper hydroperoxo intermediate and a substrate radical. The second electron from CDH then facilitates O−O bond cleavage releasing water and generating a copper oxo radical that couples with the substrate radical, thereby hydroxylating the polysaccharide at the 1- or 4-position. The additional oxygen atom destabilizes the glycosidic bond leading to elimination of the adjacent glucan and formation of a sugar lactone or ketoaldose. This elimination would be facilitated by a general acid, possibly a third absolutely conserved histidine that is located on the surface of all fungal PMO proteins near the metal binding site.[20] It is possible that a 2-electron reduction of oxygen to a Cu-OOH intermediate could abstract the hydrogen. However, peroxide is not able to shunt the reaction in the absence of CDH, and catalase showed no inhibitory effect (Supplementary Figure 11).

Conclusions and Outlook. In closing, we conclude that oxidative enzymes are key components of the enzyme cocktails secreted by fungi for plant cell wall degradation and have proposed a chemical mechanism for the action of a new family of metalloenzymes, the polysaccharide monooxygenases. While this manuscript was in preparation, similar results were published showing that an *H. insolens* CDH and a GH61 from *T. aurantiacus* act synergistically to depolymerize cellulose.[27] Shortly thereafter, the same group reported an X-ray crystal structure of *T. aurantiacus* GH61A bound to

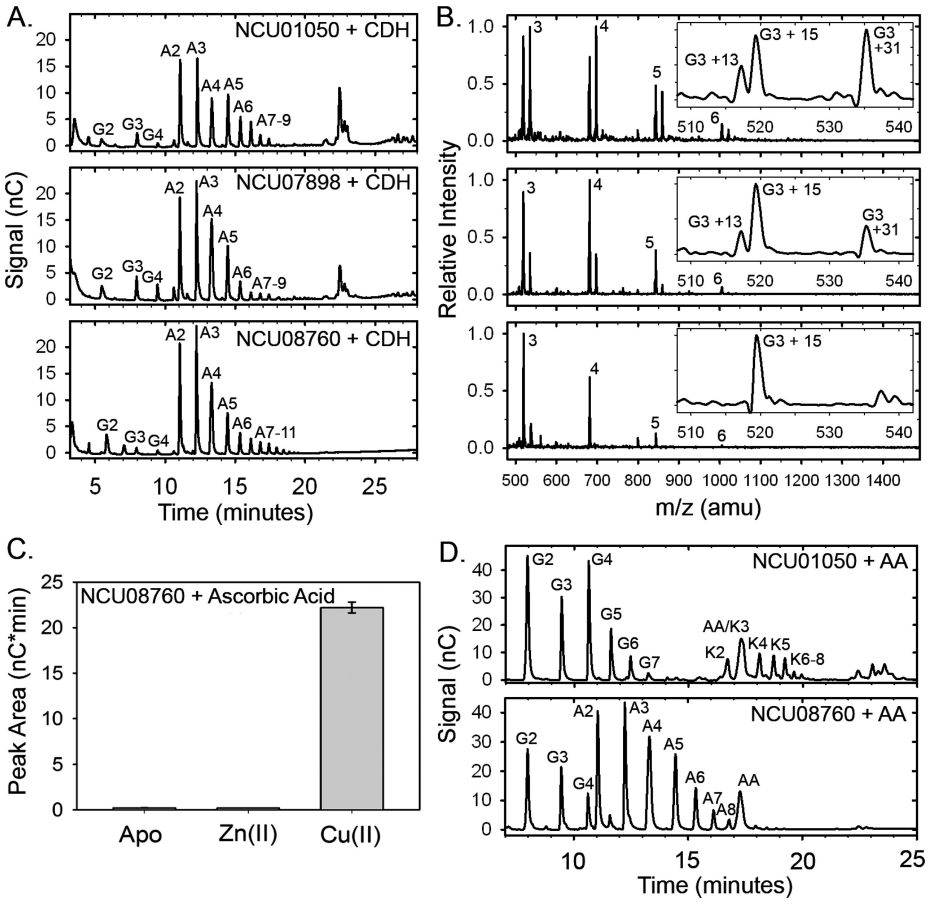

Figure 6. Reaction products of cellulose cleavage by CDH and GH61. GH61 (5.0 μM) and 0.5 μM CDH-2 were incubated with 5 mg mL^{-1} phosphoric acid swollen cellulose for 2 h in 10 mM ammonium acetate pH 5.0 at 40 °C. Products of CDH-2 in combination with each GH61 protein (NCU01050, top; NCU07898, middle; or NCU08760, bottom) were analyzed by HPAEC (A) and LC−MS (B). HPAEC standards were used to determine retention times of cellodextrins (G1−G6) and the respective aldonic acid derivatives (A2−A9). LC−MS of reaction mixtures in negative ion mode shows reaction mixtures comprised of masses consistent with a ladder of aldonic acids (Gx + 15 amu) separated by an anhydroglucose unit. Inset is a zoom around the G3 series. NCU01050 and NCU07898 show additional masses consistent with a keto-aldonic acid (Gx + 13 amu) or the hydrate of that product, a ketal-aldonic acid (Gx + 31 amu). (C) Incubation of 5.0 μM apo-, Zn-bound, or Cu-bound NCU08760 with 2 mM ascorbic acid confirms that Cu-bound GH61 is required for generation of oxidative products. Error bars represent standard deviation of assays performed in triplicate. (D) Ascorbic acid (2 mM) and 5.0 μM GH61 were assayed on PASC and analyzed by HPAEC. Products of NCU01050 include a ladder of cello-oligosaccharides (G2−G7) and a ladder of later eluting products that are likely oxidized at the nonreducing end to generate 4-keto sugars (K2−K8). Products of NCU08760 include a ladder of cello-oligosaccharides (G2−G5) and aldonic acids (A2−A8). Trace amounts of aldonic acids were produced by NCU01050, but these are 100-fold less abundant than those produced by NCU08760, suggesting different regiospecificities for the 2 proteins. AA designates the peak due to ascorbic acid.

copper(II) with a ligation similar to that previously described. Interestingly, the N-terminal histidine of this protein was methylated at ring N3. In the presence of nonprotein reductants, GH61A generated oxidized sugars modified at the reducing or nonreducing end, and nonreducing end modification was proposed to occur at C6; however, no evidence was provided to support this.[21] Hydroxylation at C6 could lead to breakage of the glycosidic bond *via* elimination to form a 4,5-unsaturated aldehyde. No evidence for a dehydrated product was observed, though it is possible that hydration could occur in solution as is the case with some hexenuronic acids. The unequivocal identification of aldonic acids as the product of NCU08760 would seem to argue against a pathway involving C6 oxidation. Oxidation at the 4-position could lead to cleavage of the glycosidic bond *via* the same general mechanism as that proposed for cellulose cleavage by oxidation at C1 (Figure 7).

In contrast to the widely discussed Fenton model, our data supports a direct enzymatic oxidation of cellulose leading to glycosidic bond cleavage. The genetic and biochemical experiments reported here with CDH show that, in *N. crassa*, CDH-1 functions as the sole extracellular enzyme that exhibits reductase activity toward PMOs and that the heme domain of CDH is required for enhancement of cellulose degradation. In many highly cellulolytic ascomycetes, CDH contains a C-terminal cellulose-binding module that targets the enzyme to the cellulose surface, probably to facilitate electron transfer to phylogenetically diverse PMOs. Basidiomycete CDHs do not contain CBMs; however, some CDHs have been shown to bind cellulose[28] and many basidiomycetes secrete high levels of a protein with a CDH heme domain fused to a CBM.[29] These "free" heme domains would require electrons from another, unidentified reductase, to potentiate the action of PMOs. Oxidoreductases functionally similar to CDH presumably exist in bacteria, but to our knowledge none have been identified.

Bacterial proteins with structural homology to fungal PMOs have recently been biochemically characterized and shown to oxidize cellulose[30] and chitin.[31] These bacterial proteins have the same conserved metal ligands as fungal PMOs and are likely

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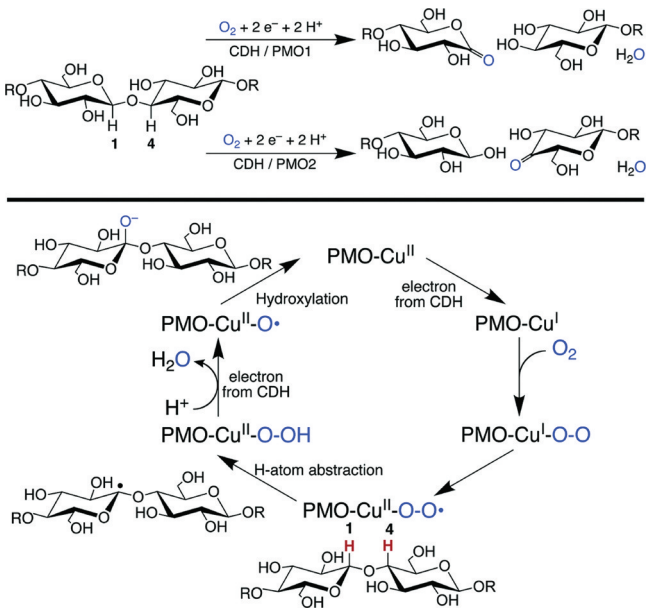

Figure 7. PMO reactions and proposed mechanism. (Top) Type 1 PMOs abstract a hydrogen atom from the 1 position leading to formation of sugar lactones. Type 2 PMOs catalyze hydrogen atom abstraction from the 4 position leading to formation of ketoaldoses. (Bottom) PMO mechanism: an electron from the heme domain of CDH reduces the PMO Cu(II) to Cu(I) and then O_2 binds. Internal electron transfer takes place to form a copper superoxo intermediate, which then abstracts a $H^{\bullet}$ from the 1 or 4 position on the carbohydrate. A second electron from CDH leads to homolytic cleavage of the Cu-bound hydroperoxide. The copper oxo species ($Cu-O^{\bullet}$) then couples with the substrate radical, hydroxylating the substrate. Addition of the oxygen atom destabilizes the glycosidic bond and leads to elimination of the adjacent glucan.

copper metalloenzymes employing a similar mechanism. Further work is needed to confirm that the activity of these proteins is dependent on bound copper. PMOs are conserved in every cellulolytic fungus studied to date and are generally expressed at very high levels during growth on cellulose. In the white rot fungus *P. chrysosporium* and the brown rot fungus *S. lacrymans*, expression profiling experiments revealed that a PMO is the most highly upregulated protein in response to growth on biomass.[4,6]

N. crassa has a potent genome defense mechanism, RIP, which prevents nearly all gene duplications.[32] Surprisingly, in the *N. crassa* genome, there are 14 genes encoding predicted PMOs. Previous expression profiling studies showed that expression of at least 10 of the PMOs was induced during growth on cellulose. The average pairwise sequence identity between PMOs in *N. crassa* is only 33%, suggesting that these proteins may have diverse functions. The biochemical characterization of three members of the PMO superfamily reported here showed that different PMOs catalyze reactions with different regiospecificity. At this point, phylogenetic inference in uncharacterized clades does not allow for regioselective prediction. Further work exploring the activity of the PMOs encoded by NCU00836, NCU03328, and NCU07760 may reveal new reactions or phylogenetic trends that allow for functional prediction.

In addition to their prevalence in nature, supplementation of cellulase cocktails with PMOs can significantly reduce the enzyme loading required for saccharification of lignocellulose. Because several different PMOs are produced by fungi and PMOs work through a mechanism completely orthogonal to that of cellulases, it is likely that addition of multiple PMOs to cellulase cocktails will further reduce enzyme loadings. Additional mechanistic insights into this large family of enzymes may facilitate their development for commercial applications.

METHODS

Preparation of Δ*cdh-1* Strains. The DNA cassette used to delete *cdh-1* was provided by the *Neurospora* functional genomics project. Details about how the cassette was generated are available online (http://www.dartmouth.edu/~neurosporagenome/protocols.html). FGSC 9717 was grown on Vogel's minimal media[33] slants for 21 days at RT. Conidia from the FGSC 9717 slant were transformed by electroporation with 1 μg of the Δ*cdh-1* (ΔNCU00206) cassette and plated onto media containing hygromycin. Positive transformants were then crossed with wild-type *N. crassa*. Ascospores were germinated on media containing hygromycin, and several hygromycin-resistant transformants were harvested and screened for production of CDH. The genotypes of three transformants that showed good growth on cellulose and lacked CDH activity were confirmed by PCR using primers specific to *cdh-1* and the hygromycin resistance cassette.

Growth of *N. crassa*. Wild-type or Δ*cdh-1 N. crassa* was inoculated onto slants of Vogel's minimal media and grown for 3 days at 30 °C in the dark followed by 7 days at RT with ambient lighting. A conidial suspension was then inoculated into 100 mL of Vogel's salts supplemented with 2% Avicel PH101 (Sigma) in a 250 mL Erlenmeyer flask. After 7 days of growth on Avicel, cultures were filtered over 0.2 μm polyethersulfone (PES) filters.

CDH Activity Assays. Spectrophotometric assays were performed at RT by the addition of an appropriate amount of CDH or culture filtrate to a mixture containing 1.0 mM cellobiose, 200 μM DCPIP, and 100 mM sodium acetate pH 5.0. Detection of CDH activity in the Δ*cdh-1* strain required concentrating the culture filtrate 100-fold before performing the assay. Reduction of DCPIP was monitored spectrophotometrically by the decrease in absorbance at 530 nm. One unit is equivalent to the conversion of 1 μmol min^{-1}.

Copper Stoichiometry of Apo-PMOs. Apo-PMO stocks of NCU01050, NCU07898, and NCU08760 were diluted to a final concentration of 1.0 mg mL^{-1} in 10 mM Tris pH 8.5 buffer and incubated with 200 μM copper sulfate at RT for 16 h. After reconstitution, the protein was diluted 5-fold into 10 mM Tris pH 8.5 and desalted using a 26/10 desalting column to remove unbound copper. The desalted protein was concentrated to a final volume of 2.5 mL using 3,000 MWCO PES spin concentrators. The absorption at 280 nm was recorded for each sample and used to determine the concentration of the protein. The concentration of copper in the sample was measured using a Perkin-Elmer 7000 series ICP-AES. The wavelengths used for copper quantification were 327.393 and 324.752 nm.

Cellulase Assays on PASC. Phosphoric acid swollen cellulose (PASC) was prepared by addition of 10 g of Avicel to 500 mL of 85% phosphoric acid and blended for 30 min. Cellulose was precipitated by the addition ice-cold water and washed with water multiple times. The concentration of PASC was determined by the phenol-sulfuric acid assay. Assays contained 5.0 μM PMO, 0.5 μM CDH-2, and 5 mg mL^{-1} PASC in 10 mM ammonium acetate pH 5.0 and were performed at 40 °C unless otherwise noted. In some assays, 2 mM ascorbic acid was used in place of CDH-2.

Product Analysis by HPAEC. Cellulase assays were mixed with 9 parts of 0.1 M NaOH for an overall 10 fold dilution to remove the supernatant. Samples were analyzed on a Dionex ICS-3000 HPAEC-PAD. Products were separated on a PA-200 HPAEC column using 0.1 M NaOH in the mobile phase with the concentration of sodium acetate increasing from 0 to 140 mM (14 min), 140 to 300 mM (8 min), 300 to 400 mM (4 min), and then held constant at 500 mM (3 min) before re-equilibration in 0.1 M NaOH (4 min). The flow rate was set to 0.4 mL min^{-1}, the column was maintained at a temperature

of 30 °C, and samples were detected on an electrochemical detector. Authentic standards of glucose, cellodextrins, glucono-δ-lactone, and cellobiono-δ-lactone were used to determine retention times and for quantification. Cellobiono-δ-lactone was synthesized as previously described.[11]

Product Analysis by LC−MS. Samples were analyzed by an Agilent HPLC (1200 series) connected to an electrospray ionization emitter in a linear ion trap mass spectrometer (LTQ XL, Thermo Scientific). Carbohydrates were separated using a SeQuant ZIC-HILIC column (150 mm × 2.1 mm, 3.5 μM 100 Å) with a SeQuant ZIC-HILIC guard column (20 mm × 2.1 mm, 5 μm). Solvent A was 5 mM ammonium acetate pH 7.2, and solvent B was 90% acetonitrile and 10 mM ammonium acetate pH 6.5. Samples were prepared by centrifugation of the assay mixture followed by the addition of 1 volume of 100% acetonitrile and 1% formic acid to the supernatant. Sample injection was set to 5 μL. The elution program consisted of a linear gradient from 80% B to 20% B over 14 min followed by 5 min at 20% B and then re-equilibration for 2 min at 80% B. The column temperature was maintained at 25 °C, and the flow rate was 0.2 mL min^{-1}. Mass spectra were acquired in negative ion mode over the range m/z = 310−2000. Data processing was performed using Xcalibur software (version 2.2, Thermo Scientific).

Metal Dependence of PMO Activity. Apo-PMO was prepared by treatment of as purified PMO with 10 mM EDTA for 24 h. Protein was then concentrated in a 3 kDa spin concentrator and loaded onto a Sephacryl S100 column with 10 mM Tris pH 8.0 and 100 μM EDTA in the mobile phase. Following elution, Sigma TraceSELECT grade buffers, metals, and water were used for all assays, and only extensively washed and rinsed plastics were used due to problems with copper contamination. The protein was buffer exchanged >100-fold into 10 mM sodium acetate (Sigma cat. nos. 59929 and 07692) in water (Sigma cat. no. 14211) to a final concentration of >40 μM PMO. Cu- or Zn-bound PMO was then produced by reconstitution with a 2-fold molar excess of $CuSO_4$ (Sigma cat. no. 203165) or $ZnSO_4$ (Sigma cat. no. 204986).

Assays to quantify CDH activity in the presence or absence of PMO protein were performed in the presence of 1.0 mM cellobiose, 50 mM sodium acetate pH 5.0, and 200 nM CDH-2. NCU01050 (5 μM) or NCU01050 reconstituted with Zn or Cu was added to the reaction, and after 30 min assays were quenched by addition of 0.1 M NaOH and analyzed for cellobionic acid production by Dionex HPAEC. PASC assays to determine the metal dependence of NCU08760 activity were performed as described above except TraceSelect grade buffer and water were used with equimolar amounts of apo-, Zn-, or Cu-bound PMO. Before the assay, PASC was mixed in a large volumetric excess of 100 μM EDTA for 48 h. The PASC was then washed multiple times with TraceSelect water until the concentration of residual EDTA was <1 nM.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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