JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Oxidative Cleavage of Cellulose by Fungal Copper-Dependent Polysaccharide Monooxygenases

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Supporting Information

ABSTRACT: Fungal-derived, copper-dependent polysaccharide monooxygenases (PMOs), formerly known as GH61 proteins, have recently been shown to catalyze the O_2-dependent oxidative cleavage of recalcitrant polysaccharides. Different PMOs isolated from *Neurospora crassa* were found to generate oxidized cellodextrins modified at the reducing or nonreducing ends upon incubation with cellulose and cellobiose dehydrogenase. Here we show that the nonreducing end product formed by an *N. crassa* PMO is a 4-ketoaldose. Together with isotope labeling experiments, further support is provided for a mechanism involving oxygen insertion and subsequent elimination to break glycosidic bonds in crystalline cellulose.

Lignocellulosic biomass is a large, renewable, and untapped resource for production of fuels and chemicals.[1] Enzymatic conversion of lignocellulose to monosaccharides is a slow and expensive process, largely because cellulose is an insoluble crystalline substance.[2] For cellulase-catalyzed hydrolysis to occur, a cellulose chain must be separated from the crystalline surface and then drawn into the active site of the enzyme, where Asp or Glu residues function in general acid/base catalysis. The separation of the glucan chain from crystalline cellulose has been proposed to be the slow step in enzymatic hydrolysis of cellulose.[3]

Oxidative enzymes have recently been shown to be important for enzymatic degradation of cellulose and have been identified in multiple transcriptomic and proteomic analyses.[4−9] Polysaccharide monooxygenases (PMOs), previously called GH61 proteins, are a recently discovered class of copper-dependent metalloenzymes that oxidatively cleave glycosidic bonds on the surface of cellulose without requiring separation of a glucan chain.[10−14] These enzymes require molecular oxygen and reducing equivalents from cellobiose dehydrogenase.[10,13] A chemical reductant can also be used to drive the reaction.[11,13] PMOs have been shown to augment the hydrolysis of cellulose by cellulases,[10,12,13] and a deeper mechanistic understanding of these enzymes could be used to reduce further costs of cellulosic biofuels.

The metal center of PMOs contains a conserved type-2 copper binding site.[11] The bound copper is coordinated by a bidentate N-terminal histidine residue through the amino terminus and Nδ. The N-terminal histidine is also methylated at Nε, but the contribution of this post-translational modification to catalysis is unknown. The other equatorial coordination sites are occupied by the Nε of another histidine residue and a water molecule. A buried proximal tyrosine acts as the fifth ligand. The bidentate histidine binding motif is also present in CBP21, a structurally related chitin-degrading enzyme with less than 10% sequence identity to fungal PMOs.[15] CBP21 was reported to use Zn or Mg to catalyze oxygen-dependent cleavage of chitin. The products of CBP21 are aldonic acids, and it was reported that one oxygen atom from molecular oxygen was incorporated into the products.[16] In view of the similarity to PMOs, it is likely that Cu is the relevant metal in CBP21.

The oxidative chemistry catalyzed by fungal PMOs likely leads to incorporation of molecular oxygen into the products. We proposed a mechanism by which PMOs insert molecular oxygen into C−H bonds adjacent to the glycosidic linkage, leading to elimination of the adjacent carbohydrate moiety.[13] Two classes of PMOs were described. Type-1 PMOs generate products oxidized at C1, probably initially in the lactone form, which is then hydrolyzed spontaneously or enzymatically[17] to yield an aldonic acid. Following phosphorylation, gluconic acid could be metabolized by the pentose phosphate pathway. Type-2 PMOs generate products oxidized at the nonreducing end. Formation of a 4-ketoaldose could result from oxidative cleavage proceeding by the same general mechanism proposed for oxidation at C1. Another report with *Thermoascus aurantiacus* GH61A also observed oxidation at C1 and on the nonreducing end, proposed to be at C6.[11] No data has been reported that identifies the position on the carbohydrate of the nonreducing-end oxidation catalyzed by type-2 PMOs. This contribution presents evidence that further elucidates the mechanism of PMO action through the use of isotope-labeling experiments and definitive identification of the nonreducing-end product of a type-2 PMO.

To show oxygen insertion from O_2, reaction mixtures containing 5.0 μM type-1 PMO from *Neurospora crassa* (NCU08760), 2.0 mM ascorbic acid, 50 mM ammonium bicarbonate (pH 7.8), and 5.0 mg/mL phosphoric acid swollen cellulose (PASC) were prepared anaerobically. The solutions were sealed in 1.0 mL vials, and then the headspace was replaced with $^{18}O_2$ on a Schlenk line or opened to atmospheric oxygen. Reactions were allowed to proceed at 40 °C for 1 h and then analyzed by LC−MS as previously described.[13] Cellulase

Received: November 17, 2011
Published: December 20, 2011

and PMO reactions are typically carried out at pH 5.0. A more basic pH was chosen here in order to limit the exchange of bulk water into the products. Figure 1 shows mass spectra

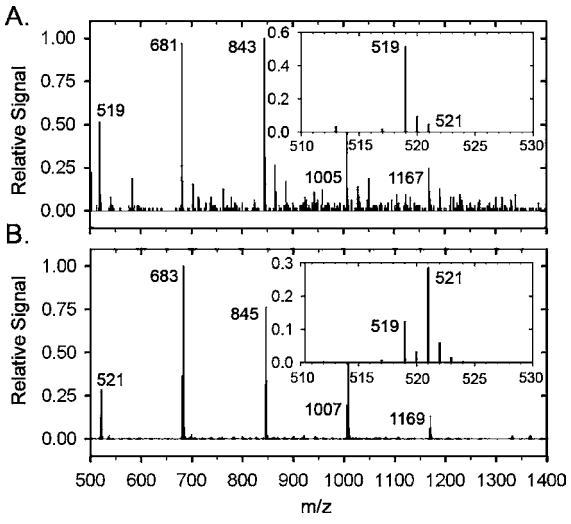


Figure 1. Mass spectra illustrating the incorporation of ^{18}O from $^{18}O_2$ into the aldonic acid products of a type-1 PMO (NCU08760). Assays were performed with (A) $^{16}O_2$ or (B) $^{18}O_2$, and the products were analyzed by LC−MS. Shown is a ladder of aldonic acid products (DP 3−7). DP = degree of polymerization. The inset is an expanded region around the mass of the cellotrionic acid ion.

confirming incorporation of 1 atom of ^{18}O into the aldonic acid products, providing evidence that the enzyme functions as a monooxygenase. Similar experiments in which the ascorbic acid was substituted with *Myceliophthora thermophila* cellobiose dehydrogenase 2 (*Mt*CDH-2) (Supplementary Figure 1) were also performed. ^{18}O incorporation into the products was also observed at both pH 5.0 and 7.8. A type-2 nonreducing-end PMO (NCU01050) did not produce products labeled with ^{18}O; however, the predicted 4-ketoaldose or dialdose products were present as hydrates in aqueous solutions and would rapidly exchange with bulk water.[18,19]

The identity of the nonreducing-end product generated by the type-2 PMO (NCU01050) was then investigated. Both C6 and C4 oxidation have been proposed to be the nonreducing-end modification catalyzed by some PMOs;[11,13] however, no evidence beyond mass measurements has been provided to support these hypotheses. To distinguish between these two possible products, a chemical modification strategy was used (Figure 2a). It is well-established that carbohydrates containing a C6 aldehyde can be oxidized to the corresponding uronic acids upon treatment with mild oxidants such as Br_2 or I_2.[20,21] If the nonreducing-end product contained a C6 aldehyde, oxidation with hypoiodite would convert it to a carboxylic acid. After chemical hydrolysis with trifluoroacetic acid (TFA), three monosaccharide products would be produced: glucuronic acid, derived from the nonreducing end; glucose, from internal carbohydrates; and gluconic acid, from oxidation at the reducing end. NCU01050 (5.0 μM) was incubated with 0.2 μM *Mt*CDH-2, 5.0 mg/mL PASC, and 50 mM sodium acetate buffer (pH 5.0) at 40 °C for 10 h to generate nonreducing-end products. PASC was removed by centrifugation, and the products were then oxidized with hypoiodite as previously described.[21] After oxidation, residual iodine was removed by addition of excess silver carbonate. Precipitated material was

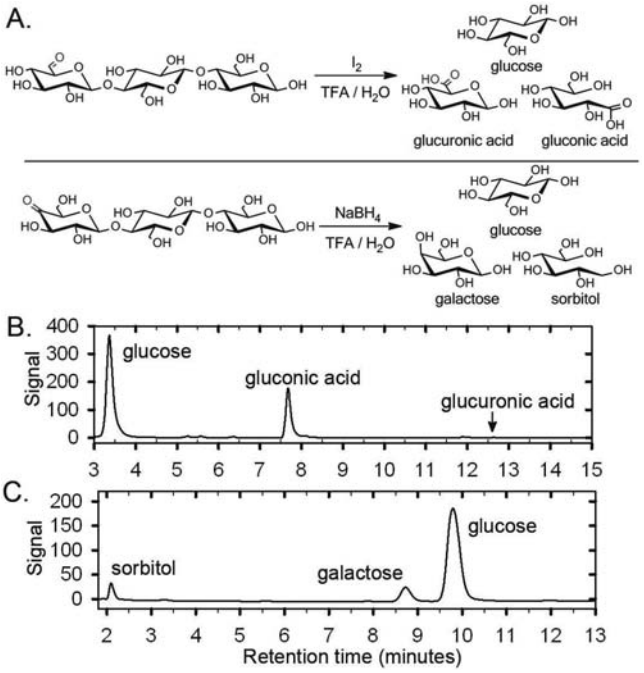


Figure 2. Product identification of a type-2 PMO. (A) Schematic showing the experimental design for identification of PMO products by oxidation or reduction followed by TFA hydrolysis. (B) HPLC chromatogram (Dionex PA-200) showing the products of a type-2 PMO (NCU01050) and CDH after hypoiodite oxidation and TFA hydrolysis. (C) HPLC chromatogram (Dionex PA-20) of NCU01050 products following reduction with sodium borohydride and TFA hydrolysis.

removed by centrifugation, and TFA was added to a concentration of 2.0 M. Oxidized oligosaccharides were heated at 121 °C for 1 h and then dried under a stream of nitrogen and washed twice with isopropanol. Products were then dissolved in 0.1 M sodium hydroxide and analyzed by Dionex high-performance anion-exchange chromatography (HPAEC) as previously described (Figure 2b).[13] The absence of glucuronic acid ruled out a PMO product containing a C6 aldehyde.

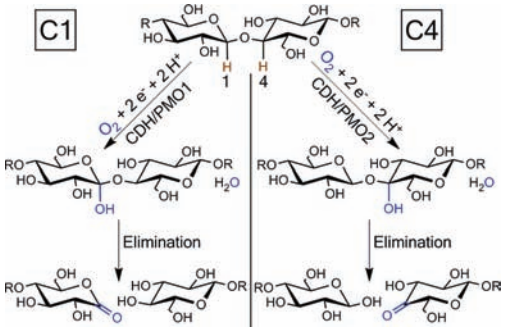
The formation of a 4-ketoaldose by NCU01050 was investigated next. Direct hydrolysis of the NCU01050 and *Mt*CDH-2 reaction products generated three peaks on a Dionex PA-200 column (Supplementary Figure 2). As expected, glucose and gluconic acid were formed from the internal carbohydrates and oxidation of the reducing end by CDH. The third peak, eluting at 6.4 min, was absent in control reactions with NCU08760 and *Mt*CDH-2 and probably is the PMO-oxidized nonreducing-end carbohydrate. If a ketone functional group were introduced in the ring, reduction with sodium borohydride would convert it back to a epimeric mixture of alcohols at that position (Figure 2a). Galactose is the C4 epimer of glucose and can be readily separated from glucose using a Dionex PA-20 column. Initial reaction products from the NCU01050 reaction were treated with 10 mg/mL sodium borohydride in 500 mM ammonium hydroxide for 2 h at room temperature. After reduction, residual borohydride was removed by addition of glacial acetic acid. The reduced sample was dried under a stream of nitrogen and hydrolyzed using TFA as described above. Analysis of the monosaccharide products using a Dionex HPAEC confirmed the formation of galactose (Figure 2C). In control experiments where

dx.doi.org/10.1021/ja210657t | J. Am. Chem.Soc. 2012, 134, 890−892

NCU08760 was substituted for NCU01050, 10-fold less galactose was formed (Supplementary Figure 3).

Together, these results provide support for the reaction pathway shown in Scheme 1. Type-1 PMOs insert oxygen at C1, while the NCU01050 type-2 PMO inserts oxygen at the 4 position. After oxygen insertion, the glycosidic bond is destabilized and likely broken by an elimination reaction, which may be catalyzed by the PMO or occur spontaneously. This elimination is irreversible because the carbon on the reducing or nonreducing end has been oxidized.

Scheme 1. Proposed Reaction Pathway for Oxidative Cleavage of Cellulose by PMOs



Insertion of oxygen likely occurs following PMO-mediated hydrogen abstraction from C1 or C4 to generate a substrate radical. This substrate radical could recombine with a copper–oxo species in the PMO active site, hydroxylating the glucan chain to form the intermediate shown. The C–H bond dissociation energy for C4 is higher than that for C1 and likely similar to those for the remaining positions on the pyranose ring, suggesting that a PMO could insert oxygen at any position.[22] The advantage of oxygen insertion at C1 or C4 is that a simple elimination reaction leads to bond cleavage. Oxygen insertion at other positions would require the involvement of additional amino acid residues on the surface of the PMO to potentiate bond cleavage, and there would be nothing to prevent reformation of the glycosidic bond on the cellulose surface. There is a vast amount of sequence variation in the PMO superfamily, and many highly cellulolytic fungi express more than 10 different PMOs during growth on cellulose.[4,8] The residues controlling regiospecificity in PMOs are unknown. Future work to identify the reaction products of divergent members of the PMO superfamily may reveal what factors control the position of oxygen insertion.

■ ASSOCIATED CONTENT

Supporting Information

Additional control experiments, experimental methods, and complete refs 5, 8, and 11. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

We thank S. Bauer and M. Pauly for helpful discussions on carbohydrate analysis. W.T.B. and C.M.P. were recipients of NSF predoctoral fellowships. This work was funded by a grant from the Energy Biosciences Institute to J.H.D.C. and M.A.M.

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