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Structural and functional characterization of the catalytic domain of a cell-wall anchored bacterial lytic polysaccharide monooxygenase from *Streptomyces coelicolor*

Amanda K. Votvik\textsuperscript{1}, Åsmund K. Røhr\textsuperscript{1}, Bastien Bissaro\textsuperscript{1,2}, Anton A. Stepnov\textsuperscript{1}, Morten Sørlie\textsuperscript{3}, Vincent G. H. Eijsink\textsuperscript{1} & Zarah Forsberg\textsuperscript{1,10}*

Bacterial lytic polysaccharide monooxygenases (LPMOs) are known to oxidize the most abundant and recalcitrant polymers in Nature, namely cellulose and chitin. The genome of the model actinomycete *Streptomyces coelicolor* A3(2) encodes seven putative LPMOs, of which, upon phylogenetic analysis, four group with typical chitin-oxidizing LPMOs, two with typical cellulose-active LPMOs, and one which stands out by being part of a subclade of non-characterized enzymes. The latter enzyme, called *ScLPMO10D*, and most of the enzymes found in this subclade are unique, not only because of variation in the catalytic domain, but also as their C-terminus contains a cell wall sorting signal (CWSS), which flags the LPMO for covalent anchoring to the cell wall. Here, we have produced a truncated version of *ScLPMO10D* without the CWSS and determined its crystal structure, EPR spectrum, and various functional properties. While showing several structural and functional features typical for bacterial cellulose active LPMOs, *ScLPMO10D* is only active on chitin. Comparison with two known chitin-oxidizing LPMOs of different taxa revealed interesting functional differences related to copper reactivity. This study contributes to our understanding of the biological roles of LPMOs and provides a foundation for structural and functional comparison of phylogenetically distant LPMOs with similar substrate specificities.

**Abbreviations**

2,6-DMP  2,6-Dimethoxyphenol  
AscA  Ascorbic acid  
AA  Auxiliary activity  
CAZymes  Carbohydrate-Active enZymes  
CBM  Carbohydrate-binding module  
CbpC  Carbohydrate-binding protein C  
CD  Catalytic domain  
CHB  GH20 chitobiase  
Chi18  GH18 chitinase  
*Cj*  *Cellvibrio japonicus*  
CWSS  Cell wall sorting signal  
EPR  Electron paramagnetic resonance  
GlcNAcGlcNAc1A  Oxidized chitobiase  
GHS  Glycoside hydrolases  
HRP  Horseradish peroxidase

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**Results**

ScLPMO10D belongs to an uncharacterized AA10 clade populated by cell wall-anchored LPMOs. In 2008 Walter and Schrempf described a surface-located carbohydrate-binding protein, belonging to the carbohydrate-binding module (CBM) family 33, from *S. coelicolor* A3(2) called CbpC. Today the proteins and domains formerly referred to as CBM33 are known as AA10-type LPMOs or LPMO10s. In the study by Walter and Schrempf, it was shown that expression of CbpC was induced when chitin, cellulose or cellobiose, but not glucose, were used as the sole carbon source. It was also shown that the protein bound strongly to Avicel, and, to a lesser extent to chitinous substrates. Unlike other AA10-type LPMOs, CbpC possesses a C-terminal cell wall sorting signal (CWSS) and by using polyclonal anti-CbpC antibodies Walter and Schrempf showed that the protein was bound to the bacterial cell wall whereas a truncated version of CbpC, lacking the CWSS was translocated to the supernatant.

CbpC, hereafter called ScLPMO10D (Uniprot ID: Q9S296; Fig. 1), consists of 356 amino acids (Fig. 1B), making up an N-terminal signal peptide (residues 1–33), a family AA10 LPMO domain (residues 34–214; ScLPMO10CD), a low complexity region including a potential linker rich in glycine, alanine, aspartic acid, and serine (approximately residues 215–312), and a C-terminal CWSS (residues 313–356). The CWSS includes a characteristic LAETG-motif, followed by a stretch of disordered hydrophobic amino acids and a C-terminal tail.
rich in positively charged residues. The LAETG-motif represents a Streptomyces specific sorting signal, substituting the LPXTG-motif found in the cell wall-anchored proteins of Staphylococcus and Streptococcus species. The LAETG-motif makes up a recognition site for class E sortases (SrtE1), a type of transpeptidase that cleave and crosslink target proteins to the peptidoglycan matrix of the Gram-positive cell wall.

Phylogenetic analysis of 150 AA10 LPMO sequences (catalytic domains only), including 45 enzymes that have been experimentally characterized (see Supplementary Table S1), showed that ScLPMO10D does not cluster with any of the established subclades of AA10 sequences (Fig. 2). In a previous phylogenetic study by Book et al., two clades were defined: Clade I comprises subclades C and D, which both harbor chitin-oxidizing LPMOs, while Clade II comprises subclades A and B, of which subclade B harbors enzymes with mixed C1- and C4-oxidizing cellulose-activity in addition to C1-oxidizing activity on chitin, and subclade A harbors C1-oxidizing cellulose-active enzymes and the cell wall-anchored AA10s that are in focus in this study. Subclade A groups into three distinct clusters (Fig. 2) and has therefore, in this study, been divided into subclades A1-A3, of which subclade

Figure 1. Overview of predicted S. coelicolor A3(2) LPMOs (A) and primary structure of ScLPMO10D (B). (A) Shows the LPMO nomenclature that is based on the gene number shown below each enzyme in brackets, followed by domain architecture, the relative sizes (in number of amino acids and kDa) of the mature proteins and the substrates of the seven S. coelicolor LPMOs. The indicated substrates and oxidative regioselectivities of ScLPMO10B, ScLPMO10C, ScLPMO10G and ScLPMO10D (this study) are derived from experimental data, whereas the activity of the other LPMOs, marked with asterisks, is predicted based on sequence similarity and phylogenetic clustering (Fig. 2). (B) Shows the primary structure of ScLPMO10D, including its signal peptide (yellow). The copper-coordinating histidines (His34 and His134) and the LAETG sorting signal are shown with bold letters. The region of low complexity (approximately residues 215–312), including a potential linker rich in Gly, Asp, Ala, and Ser, that connects the LPMO domain to the LAETG-motif transpeptidase (sortase) recognition site is shown on a grey background. The C-terminal hydrophobic region is shown on a red background and is followed by a C-terminal tail containing six positively charged residues (Arg and His) shown on a blue background. The mature ScLPMO10D protein starts with His34 and ends with Thr316 crosslinked to the peptidoglycan of the S. coelicolor cell wall envelope. In this study a truncated version containing the catalytic domain (CD) only was used (ScLPMO10D; residues 34–214).
A1 contains C1-oxidizing cellulose active enzymes. Subclade A2 comprises the ScLPMO10D-like enzymes with LAETG motifs which, as shown below, have C1-oxidizing chitin activity, whereas subclade A3 contain proteins with as yet unknown activities (see below). Interestingly, subclade A2 also contains the only AA10 found in plants, namely Tma12 from the fern Tectaria macrodonta that has been shown to have insecticide properties, but no oxidative activity has been demonstrated to date. With Tma12 as an exception, subclade A exclusively harbors AA10s of actinobacterial origin, thus Tma12 may suggest an event of horizontal gene transfer from actinobacteria to plants.

In this study we have produced the catalytic domain of ScLPMO10D (previous CbpC33) and made attempts to produce the catalytic domain of Micromonospora aurantiaca LPMO10A (MaLPMO10ACD, see Fig. 2 and Supplementary Fig. S1), which are both found in subclades of uncharacterized AA10s i.e., A2 and A3, respectively. However, despite several attempts MaLPMO10ACD could not be produced as a soluble protein and its functional properties could thus not be studied.

Crystal structure of the catalytic domain of ScLPMO10D. The structure of the catalytic domain of ScLPMO10D (residues 34–214, lacking the linker and the CWSS), hereafter called ScLPMO10DCD, was determined to 1.37 Å resolution with a single molecule in the asymmetric unit using the crystal structure of...
LPMO10A from *Tectaria macrodonta* (Tma12; PDB ID: 6IF7) as the starting model (Table 1). The structure shows the typical LPMO fold with a central β-sandwich built up by two distorted β-sheets that are connected by several loops and helices (Fig. 3). The first β-sheet is formed by three antiparallel β-strands (S1, S4, and S7) and the second β-sheet is built up by four antiparallel strands (S5, S6, S8, and S9). Two small β-strands (S2 and S3) are found before and after the long “L2 loop” that holds most of the α-helices (H1, H2, H3, and H5), in which one is a 310-helix (H5). Of note, most of the structural diversity observed among AA10s confines to the L2 loop, which accounts for approximately half of the substrate-binding surface.

The active site of ScLPMO10D is formed by His34 and His134, which coordinate the bound copper cofactor in a T-shaped geometry (Fig. 3B). No water molecules were found adjacent to the copper, which indicates that the copper had been photo-reduced during data collection. The residues in the secondary coordination sphere resemble those found in C1-oxidizing cellulose-active AA10 LPMOs, such as ScLPMO10C and in the atypical chitin-active CjLPMO10A. The second sphere includes a glutamate (Glu203) in what has been called the ‘gate-keeping’ position that has a close interaction with an arginine (Arg198). This glutamate is thought to affect polysaccharide binding, allow diffusion of small molecules (such as H2O, O2 or H2O2) through an active site access tunnel formed at the interface between the LPMO and the polysaccharide and, in the case of H2O2, activation of the co-substrate. In ScLPMO10D, an additional arginine (Arg79) is interacting with Glu203 that together with Arg198 forms a remarkable arrangement that has, to the best of our knowledge, not been observed in any other LPMO (Fig. 3B).

The closest structurally characterized homologue of ScLPMO10D (Fig. 3C). The sequence identity between ScLPMO10D and TmlLPMO10A is 57.4%, whereas the sequence identity with well-studied chitin-active CjLPMO10A and cellulose-active ScLPMO10C, is 42.7% and 41.5%, respectively. The sequence identity between ScLPMO10D and chitin-active SmLPMO10A is only 30.6%. MaLPMO10A, a putatively membrane bound AA10 LPMO lacking the LAETG/LPXTG-motif (see above and Supplementary Fig. S1), which could not be produced, shows a sequence identity of 44% to ScLPMO10D.

Figure 4 shows sequence details and structures of the catalytic centers of these LPMOs. Generally, it is worth noting the considerable variation in second sphere residues, the functional implications of which are largely unknown. It is also worth noting that the arrangement of a catalytically crucial glutamate (Glu203) interacting closely with two positively charged residues (Arg79 and Arg198) really stands out from the active site arrangement found in other LPMOs (see also Fig. 3). Like CjLPMO10A, ScLPMO10D shows a “hybrid”-type catalytic center with features known from both cellulose-active and chitin-active LPMO10s. While Arg198 and Glu203 in ScLPMO10D are common to cellulose-active LPMO10s, Asn76 and Thr131 are common to chitin-active LPMOs (Fig. 4). Interestingly, a recent study in which a mutant library of cellulose-oxidizing ScLPMO10C was screened for chitinolytic activity showed that both Phe82 (corresponding to Asn76 in ScLPMO10A) and Trp141 (corresponding to Thr131) had to be mutated to obtain such activity.

A structural model (AlphaFold) of MaLPMO10A shows an unusual active site that has not been seen in other LPMOs (Fig. 4E and Supplementary Fig. S2) where the residues corresponding to Arg198 and Glu203 in ScLPMO10D are replaced by Asn189 and Asp194, respectively. A recent study in which a mutant library of cellulose-oxidizing ScLPMO10C was screened for chitinolytic activity showed that both Phe82 (corresponding to Asn76 in ScLPMO10A) and Trp141 (corresponding to Thr131) had to be mutated to obtain such activity.

**Table 1.** Crystal data, diffraction data and refinement statistics for the ScLPMO10DC structure (PDB code 7ZJB). Values for outer shell in parenthesis. 
\[
\begin{align*}
R_{cryst} &= \frac{\sum |I - \langle I\rangle|}{\sum I} = \sum (|F_{obs}| - |F_{calc}|) / \sum |F_{obs}| \\
R_{free} &= \frac{\sum |F_{obs}| - |F_{calc}|}{\sum |F_{obs}|}
\end{align*}
\]

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respectively. Two residues that help shaping the copper site and that are commonly alanine and phenylalanine in AA10s (see Fig. 4), are replaced by an isoleucine and a tyrosine (Ile123 and Tyr196 in \( \text{MaLMO10A} \), in which the latter residue is fully conserved in sequences belonging to subclades A3 and B. Of note, two of the sequences that cluster with \( \text{MaLMO10A} \) (labelled with yellow stars in Fig. 2) lack one or both copper-binding histidines (Supplementary Fig. S3).

Substrate specificity of \( \text{ScLPMO10Dcd} \). \( \text{ScLPMO10DCD} \) was tested on several known LPMO substrates including \( \alpha- \) and \( \beta- \) chitin, chito-oligomers (DP5-6), phosphoric acid swollen cellulose (PASC), Avicel PH-101, bacterial microcrystalline cellulose (BMCC) and peptidoglycan isolated from \( \text{Streptomyces} \). Reaction products were analyzed by MALDI-ToF MS, HPAEC-PAD and UHPLC—with only oxidized products being produced for reactions with \( \alpha- \) and \( \beta- \) chitin (Supplementary Fig. S4). The degree of polymerization of the soluble products ranged from 4–7 and 4–9 for \( \alpha- \) and \( \beta- \) chitin, respectively. Quantitative comparison of product formation by \( \text{ScLPMO10DCD} \) and two other chitin-active active LPMOs, \( \text{SmLPMO10A} \) and \( \text{CjLPMO10ACD} \) (see below for more details), in reactions with the two chitin types showed that \( \text{ScLPMO10DCD} \) stands out in showing particularly low activity on \( \alpha- \) chitin relative to the activity on \( \beta- \) chitin (Supplementary Fig. S4).

Comparison of three phylogenetically distinct chitin-oxidizing LPMO10s. Figures 2 and 4 show that there is considerable sequence divergence among chitinolytic LPMOs. We set out to compare \( \text{ScLPMO10Dcd} \) (subclade A2) with two previously characterized chitin-active LPMO10s that are phylogenetically distant from \( \text{ScLPMO10Dcd} \), namely \( \text{SmLPMO10A} \) (subclade D) and \( \text{CjLPMO10ACD} \) (no subclade; Fig. 2). While \( \text{SmLPMO10A} \) is a single domain LPMO, \( \text{CjLPMO10ACD} \) is naturally appended to two CBMs (AA10-CBM5-CBM73)\(^{44} \), but in this study only the catalytic domain was used for comparative purposes.

Figure 5A shows time courses for \( \beta- \) chitin degradation using the three enzymes. While \( \text{SmLPMO10A} \) was active for the whole period (70 h) of the measurement, product formation by the two truncated LPMOs, \( \text{ScLPMO10DCD} \) and \( \text{CjLPMO10ACD} \), ceased after 30 h and 20 h of incubation, respectively. The fraction of soluble oxidized products, relative to the total amount of oxidized products, at the end of the reaction varied from 55%, for the least stable LPMO, \( \text{CjLPMO10ACD} \), to 75% and 78% for the other two (Fig. 5B).

It is well known that, under certain conditions, LPMOs suffer from autocatalytic oxidative damage leading to inactivation\(^{41,46,49,50} \). To assess whether the plateau in product formation in the reactions with \( \text{ScLPMO10Dcd} \) and \( \text{CjLPMO10ACD} \) was caused by enzyme inactivation, or reductant or substrate depletion, an experiment was set up in which fresh reactants were added to the reactions at a point where the product formation had stopped (Fig. 6A). The data showed that only addition of fresh LPMO, alone or in combination with fresh reductant, led...
to recovered product formation for both enzymes (Fig. 6B), indicating that the stagnation visible in the progress curves of Fig. 5A is primarily due to inactivation of the LPMO.

The detrimental effect of removing the CBMs on the stability of CjLPMO10A under turnover conditions has been correlated to the low affinity of the catalytic domain for chitin, which is weak in comparison to CBMs and natural single domain LPMOs such as SmLPMO10A. ScLPMO10D does not have a CBM and binding to both chitin and cellulose has been shown for a C-terminally polyHis-tagged variant of this protein. Comparative
binding studies of the three LPMOs with β-chitin (Supplementary Fig. S5) showed that ScLPMO10DCD binds with weak affinity to β-chitin, similar to CjLPMO10ACD, which may explain the lower stability visible in Fig. 5. Only SmLPMO10A displayed a clear binding curve over time and this enzyme showed superior performance in the experiments depicted in Fig. 5.

To assess whether stability differences (Fig. 5A) could also relate to differences in folding stability, the apparent melting temperature ($T_m$) of ScLPMO10DCD was determined by monitoring the effect of temperature on binding of a fluorescent dye (SYPRO orange). The obtained melting curve (see Supplementary Fig. S6) shows that copper-loaded ScLPMO10DCD has an apparent $T_m$ of 63 °C which is lower compared to the previously determined apparent melting temperatures of 71.2 °C for SmLPMO10A$^{47}$ and 70.2 °C for CjLPMO10ACD$^{51}$, but...
well above the temperature used in the experiments (40 °C). Also, we confirmed the stabilizing effect of copper as shown by a ca. 10 °C drop in Tm for the apo enzyme (Supplementary Fig. S6).

**Apparent H$_2$O$_2$-production, -consumption and redox potential.** To further assess differences between the three enzymes we looked at the initial oxidase rate, i.e., the rate of H$_2$O$_2$ generation in the absence of substrate, by using the Amplex red and horseradish peroxidase (HRP) assay as previously described[52,53]. The results showed that all three enzyme preparations were free of excess (i.e., non-LPMO bound) copper as ultrafiltrates of the enzyme preparations showed H$_2$O$_2$ production rates similar to the enzyme free reaction (Fig. 7A). Interestingly, CjLPMO10A$^{CD}$ which seemed the most unstable enzyme in the previous experiments (Fig. 5A), showed the highest apparent H$_2$O$_2$-production rate (26.5 ± 6.3 nM/s), ScLPMO10D$^{CD}$ and SmLPMO10A showed significantly slower rates, amounting to 5.8 ± 1.1 nM/s and 3.2 ± 0.5 nM/s, respectively.

As a control, the redox potential of copper was acquired through the same method. The error bars show ± S.D. (n = 3).

The peroxidase rate of the three LPMOs was measured using the assay described by Breslmayr et al.[54]. In this assay, the LPMO uses H$_2$O$_2$ to oxidize 2,6-dimethoxyphenol (2,6-DMP) to eventually form coerulignone, which can be detected spectrophotometrically at 469 nm. In this experiment, SmLPMO10A showed the fastest consumption rate followed by ScLPMO10D$^{CD}$ and CjLPMO10A$^{CD}$ (Fig. 7B). It is worth noting that, among the three LPMOs, there is an inverse correlation between the H$_2$O$_2$ production rates (Fig. 7A) and the initial peroxidase rates (Fig. 7B). It is remarkable that the variation in LPMO reactivity is so different for O$_2$ versus H$_2$O$_2$. Such a difference could be explained by the fact that catalytic rate in the peroxidase assay is limited by reduction of the LPMO by 2,6-DMP, which acts as both a reductant and a substrate[54]. In this latter case, differences in enzyme reactivities may provide a unifying explanation: a lower redox potential is likely to lead to faster oxidation by O$_2$ and slower reduction by 2,6-DMP. Indeed, one expects a correlation between the redox potential of the LPMOs and the observed oxidase activities as the first step in H$_2$O$_2$ production, i.e., formation of O$_2$, is endergonic.

In accordance with the high apparent oxidase activity and low peroxidase activity of CjLPMO10A$^{CD}$, the redox potential of the CjLPMO10A$^{CD}$-Cu(I)/CjLPMO10A$^{CD}$-Cu(I) redox couple was found to be 204 ± 7 mV, which is the lowest of the LPMOs investigated in this study. In comparison, the redox potential for the SmLPMO10A-Cu(I)/SmLPMO10A-Cu(I) and ScLPMO10D$^{CD}$-Cu(I)/ScLPMO10D$^{CD}$-Cu(I) redox couples were determined to be 302 ± 16 mV and 271 ± 7 mV, respectively (Fig. 7C). Thus, the oxidase rates, peroxidase rates and redox potentials are correlated, and the peroxidase reaction measured in the set-up proposed by Breslmayr et al. is likely limited by LPMO reduction.

**Electron paramagnetic resonance (EPR) spectroscopy.** The immediate environment of the active site copper ion in ScLPMO10D$^{CD}$ was analyzed by EPR spectroscopy as previously described[55] and the EPR spectrum was simulated (Supplementary Fig. S7). The estimated spin Hamiltonian parameters are summarized in Table 2 and compared to previously published data for SmLPMO10A[56], ScLPMO10C[55] and CjLPMO10A[44]. The g and A$^{Cu}$ tensors reflect the copper coordination in the LPMO active site, and of these the g and A$^{Cu}$ could be calculated with high accuracy. The g and A$^{Cu}$ tensors for ScLPMO10D$^{CD}$ (g = 2.275; A$^{Cu}$ = 139 × 10$^{-4}$ cm$^{-1}$) are different compared to chitin oxidizing LPMO10s in subclade D, such as SmLPMO10A, which yield values that fall between typical type 1 and type 2 copper enzymes[43,55]. With a higher A$^{Cu}$ value, the EPR spectrum of ScLPMO10D$^{CD}$ falls in between SmLPMO10A (A$^{Cu}$ = 116 × 10$^{-4}$ cm$^{-1}$) and CjLPMO10A$^{CD}$ (A$^{Cu}$ = 154 × 10$^{-4}$ cm$^{-1}$).

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Apparent H$_2$O$_2$-production, -consumption, and redox potential. (A) Shows apparent H$_2$O$_2$ production by 2 μM ScLPMO10D$^{CD}$, CjLPMO10A$^{CD}$ or SmLPMO10A in 50 mM sodium phosphate buffer, pH 6.0, supplied with 1 μM ascorbic acid, 5 U/ml HRP, 100 μM Amplex Red, and 1% (v/v) DMSO. Excess copper control reactions (labelled “filtrate”) were set up using protein-free samples, obtained by ultrafiltration of the protein preparations. These samples contained the same amount of free copper as the LPMO preparation used in the experiment. There are two additional control reactions: “BG”, for background, representing a reaction without enzyme, and “Cu(II)SO$_4$”, a reaction in which enzyme is replaced by 2 μM CuSO$_4$. (B) Shows the apparent peroxidase activity of the three LPMOs measured by the method described by Breslmayr et al.[54]. The reactions contained 1 μM LPMO, 1 mM 2,6-DMP, and 100 μM H$_2$O$_2$ in 20 mM Pipes buffer, pH 6.0. The panel shows traces for two independent reactions per enzyme. (C) Shows the obtained redox potentials for the LPMO-Cu$^{3+/2+}$/LPMO-Cu$^{2+}$ couples, determined by monitoring a reaction between 150 μM reduced N,N,N',N'-tetramethyl-1,4-phenylenediamine (TMPred) and 35 μM (oxidized) LPMO-Cu$^{2+}$ under anaerobic conditions. As a control, the redox potential of copper was acquired through the same method. The error bars show ± S.D. (n = 3).
Studies of bacterial expansins have shown and type 2 copper enzymes. The crystal structure of ScLPMO10s contains both cellulose-active (subclade A1) and chitin-active (subclade A2) LPMOs. The crystal structure of ScLPMO10A contains twenty-four arginines, compared to, e.g., eight in ScLPMO10D and six in SmLPMO1A. Looking closer at the position of the arginines it seems that the majority are located on the surface (Supplementary Fig. S8) and the estimated net charge of MalLPMO10A (at pH 7.4) is predicted to be +5.03. As a comparison the predicted net charges of other LPMOs are −0.95 for ScLPMO1D, −3.8 for CjLPMO10A, −0.94 for SmLPMO1, −2.24 for PalLPMO10A (CbpD), −4.93 for TmLPMO10A, −2.94 for MalLPMO10B, and −11.98 for ScLPMO10C. Arginines are known to play important roles in binding negatively charged substances such as nucleic acids, cofactors, and effectors of protein active sites and for that reason some Arg-rich proteins have various interesting roles in biology, related to phenomena as diverse as gene expression, membrane-penetrating activity and pathogenesis-related defense. Studies of bacterial expansins have shown huge variations in protein pl and revealed that basic expansins bind to plant cell walls via electrostatic interactions with negatively charged polysaccharides (such as pectin and some hemicelluloses), while acidic expansins are repelled. It is conceivable that the high positive charge on the surface of MalLPMO10A contributes to substrate binding. Another possible biological function of the positively charged residues could be to provide binding sites for protein–protein complexation between MalLPMO10A and unknown partners.

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<td>11.7</td>
<td>42.3</td>
<td>12.3</td>
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<td>26.1</td>
<td>17.0</td>
<td>50.3</td>
<td>12.3</td>
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<td>A(No Cu)</td>
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<td>154</td>
<td>153</td>
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Table 2. Comparison of LPMO Spin hamiltonian parameters. Assuming collinear g- and A(Cu)—tensors in all simulations. aData from a previous study by Forsberg et al. bData from a previous study by Forsberg et al. c(10⁻⁴ cm⁻¹).

**Discussion**

In this study we have elucidated two subclades of previously uncharacterized enzymes in the AA10 phylogenetic tree that group with a subclade of strictly C1-oxidizing cellulose-active LPMOs of actinobacterial origin (subclade A1 in Fig. 2). The enzymes in subclades A2 and A3 do not only have another substrate specificity (only shown for A2) but are also different in that most of them are predicted to be cell wall anchored with (subclade A2) or without (subclade A3) crosslinking to the peptidoglycan matrix (Figs. 1, 2 and Supplementary Fig. S1). The enzymes in subclade A3 contain a high number of arginines. The catalytic domain of MalLPMO10A (residue 27–204) contains twenty-four arginines, compared to, e.g., eight in ScLPMO10D and six in SmLPMO1A. Looking closer at the position of the arginines it seems that the majority are located on the surface (Supplementary Fig. S8) and the estimated net charge of MalLPMO10A (at pH 7.4) is predicted to be +5.03. As a comparison the predicted net charges of other LPMOs are −0.95 for ScLPMO1D, −3.8 for CjLPMO10A, −0.94 for SmLPMO1, −2.24 for PalLPMO10A (CbpD), −4.93 for TmLPMO10A, −2.94 for MalLPMO10B, and −11.98 for ScLPMO10C. Arginines are known to play important roles in binding negatively charged substances such as nucleic acids, cofactors, and effectors of protein active sites and for that reason some Arg-rich proteins have various interesting roles in biology, related to phenomena as diverse as gene expression, membrane-penetrating activity and pathogenesis-related defense. Studies of bacterial expansins have shown huge variations in protein pl and revealed that basic expansins bind to plant cell walls via electrostatic interactions with negatively charged polysaccharides (such as pectin and some hemicelluloses), while acidic expansins are repelled. It is conceivable that the high positive charge on the surface of MalLPMO10A contributes to substrate binding. Another possible biological function of the positively charged residues could be to provide binding sites for protein–protein complexation between MalLPMO10A and unknown partners.

ScLPMO10D, our representative from subclade A2, oxidizes both α- and β-chitin with an unusually strong preference for the latter substrate (Supplementary Fig. S4). This experimental result shows that subclade A of the AA10 phylogenetic tree that group with a subclade of strictly C1-oxidizing cellulose-active LPMOs of actinobacterial origin (subclade A1 in Fig. 2). The enzymes in subclades A2 and A3 do not only have another substrate specificity (only shown for A2) but are also different in that most of them are predicted to be cell wall anchored with (subclade A2) or without (subclade A3) crosslinking to the peptidoglycan matrix (Figs. 1, 2 and Supplementary Fig. S1). The enzymes in subclade A3 contain a high number of arginines. The catalytic domain of MalLPMO10A (residue 27–204) contains twenty-four arginines, compared to, e.g., eight in ScLPMO10D and six in SmLPMO1A. Looking closer at the position of the arginines it seems that the majority are located on the surface (Supplementary Fig. S8) and the estimated net charge of MalLPMO10A (at pH 7.4) is predicted to be +5.03. As a comparison the predicted net charges of other LPMOs are −0.95 for ScLPMO1D, −3.8 for CjLPMO10A, −0.94 for SmLPMO1, −2.24 for PalLPMO10A (CbpD), −4.93 for TmLPMO10A, −2.94 for MalLPMO10B, and −11.98 for ScLPMO10C. Arginines are known to play important roles in binding negatively charged substances such as nucleic acids, cofactors, and effectors of protein active sites and for that reason some Arg-rich proteins have various interesting roles in biology, related to phenomena as diverse as gene expression, membrane-penetrating activity and pathogenesis-related defense. Studies of bacterial expansins have shown huge variations in protein pl and revealed that basic expansins bind to plant cell walls via electrostatic interactions with negatively charged polysaccharides (such as pectin and some hemicelluloses), while acidic expansins are repelled. It is conceivable that the high positive charge on the surface of MalLPMO10A contributes to substrate binding. Another possible biological function of the positively charged residues could be to provide binding sites for protein–protein complexation between MalLPMO10A and unknown partners.

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Notably, the A(Cu) of CjLPMO10A resembles that of cellulose oxidizing LPMOs, such as ScLPMO10C, which yield EPR signals typical for type 2 copper centers, based on the Peisach-Blumberg classification of type 1 and type 2 copper enzymes.

One final interesting feature of the crystal structure of ScLPMO10DCD is the presence of a second arginine (Arg79), in addition to Arg198, that interacts with the catalytically crucial Glu203 (Fig. 3B). This arginine is also found in MalLPMO10A and compared to subclade A2 where only 26% of the sequences possess an arginine at this position, it is fully conserved in the Arg-rich subclade A3. One would expect that the presence of a seemingly strong salt bridge involving Glu203 would affect the catalytic properties of this residue, and it would therefore be interesting to assess this in future studies. So far, we have no basis for speculating about the impact of Arg79 on LPMO reactivity.
Table 3 summarizes key properties of the three LPMO catalytic domains that are compared in this study. Comparison of the chitin-degrading abilities of ScLPMO10D<sup>CD</sup> (subclade A2), SmLPMO10A (subclade D) and CjLPMO10A<sup>CD</sup> (no subclade, Fig. 2), showed mainly variation in enzyme stability. For comparative purposes we used catalytic domains only, meaning that both ScLPMO10D<sup>CD</sup> and CjLPMO10A<sup>CD</sup> lacked their linkers, CWSS or CBMs. The results show that only the naturally single module LPMO, i.e., SmLPMO10A, binds well to the chitin substrate in the conditions used (Supplementary Fig. S5). Reduced LPMOs that are not bound to their substrate are more likely to take part in ‘off-pathway’ events, meaning that if a reduced LPMO reacts with H₂O₂ in the absence of substrate, or if the binding is weak or unprecise, for instance as a result of a CBM truncation, the reaction may lead to oxidation of the active site and inactivation of the enzyme<sup>17,50</sup>. It is thus not surprising that the two weak-binding LPMOs are less stable under turnover conditions.

Another factor determining LPMO performance may be the ability of the LPMO-reductant system to generate the H₂O₂ co-substrate. Production of H₂O₂ was measured using the Amplex Red/HRP method in the absence of substrate and showed that CjLPMO10A<sup>CD</sup> produces some fourfold and eightfold more H₂O₂ compared to ScLPMO10D<sup>CD</sup> and SmLPMO10A, respectively (Fig. 7A). In accordance with a previous observation for an LPMO<sup>11</sup>, the three enzymes showed a correlation between low redox potential and high apparent oxidase activity, with CjLPMO10A<sup>CD</sup> having the lowest redox potential (204 ± 7 mV) and the highest apparent H₂O₂ production rate (26.5 ± 6.3 nM/s), followed by ScLPMO10D<sup>CD</sup> (redox potential: 271 ± 7 mV; H₂O₂ production rate 5.8 ± 1.1 nM/s) and SmLPMO10A (redox potential: 302 ± 16 mV; H₂O₂ production rate 3.2 ± 0.5 nM/s). Of note, substrate-binding inhibits LPMO oxidase activity<sup>49,61</sup>. Based on these observations it is not surprising that CjLPMO10A<sup>CD</sup> and ScLPMO10D<sup>CD</sup> inactivated more rapidly than SmLPMO10A, since the former two enzymes bind the substrate weakly and produce considerable amounts of H₂O₂, a combination that makes the enzymes prone to autocatalytic inactivation. SmLPMO10A, on the other hand, shows the strongest binding and lowest H₂O₂ production, leading to stable progress curves.

From EPR data, redox potentials, peroxidase data and oxidase data, it is clear that the reactivities of the three LPMOs differ (see Table 3). These differences in enzyme behavior are accompanied by variation in structural features of the copper coordination sphere. The extraarginine in ScLPMO10D provides one example, but cannot really be linked to functional variation, since both CjLPMO10A<sup>CD</sup>, with higher oxidase activity, and SmLP- MO10A, with lower oxidase activity, lack this arginine. Further studies are needed to specifically link these structural variations to the observed functional differences.

The closest characterized homologues of ScLPMO10D<sup>CD</sup> are Tma12 (57.4% sequence identity), an LPMO from a fern with reported insecticidal properties<sup>39</sup>, and CbpD (57.3% sequence identity) from *Pseudomonas aeruginosa*, a chitin-oxidizing virulence factor that promotes survival of the bacterium in human blood<sup>37</sup>. Intriguingly, chitin-active LPMOs are remarkably abundant, occurring in a multitude of bacteria, some of which do not seem capable of, or at least do not regularly engage in, chitin degradation. Based on the above considerations, it may very well be that observed chitin-activity for LPMOs such as ScLPMO10D and CbpD does not reflect the may very well be that observed chitin-activity for LPMOs such as ScLPMO10D and CbpD does not reflect the possible chitin-like, LPMO substrates. Thus, it is not surprising that ScLPMO10D and CbpD, which have evolved not only to protect the cell-wall anchored *Sc* LPMO10D relates to cell wall dynamics and turnover<sup>33,64</sup>. Building on the early work by Walter & Schrempf<sup>33</sup>, we here provide the first biochemical evidence of LPMO activity for ScLPMO10D (previously called CbpC). However, we were not able to detect activity towards isolated peptidoglycan from a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ScLPMO10D&lt;sup&gt;CD&lt;/sup&gt;</th>
<th>CjLPMO10A&lt;sup&gt;CD&lt;/sup&gt;</th>
<th>SmLPMO10A</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subclade</td>
<td>A2</td>
<td>non</td>
<td>D</td>
<td>2</td>
</tr>
<tr>
<td>Substrate binding&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>~ 20%</td>
<td>~ 60%</td>
<td>S5</td>
</tr>
<tr>
<td>T&lt;sub&gt;onset&lt;/sub&gt;</td>
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<td>70.2 °C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.2 °C</td>
<td>S6</td>
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<tr>
<td>Oxidase activity</td>
<td>5.8 nM/s</td>
<td>26.5 nM/s</td>
<td>3.2 nM/s</td>
<td>7A</td>
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<tr>
<td>H₂O₂ consumption&lt;sup&gt;d&lt;/sup&gt;</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>7B</td>
</tr>
<tr>
<td>Redox potential</td>
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<td>204 mV</td>
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</tr>
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<td>Operational stability&lt;sup&gt;e&lt;/sup&gt;</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>5A</td>
</tr>
</tbody>
</table>

Table 3. Overview of the properties of three phylogenetically distinct chitin-oxidizing LPMOs. <sup>a</sup>Measured as protein adsorbed to the substrate after 2 h incubation (see Figure S5). <sup>b</sup>Data from a previous study by Madland et al.<sup>31</sup>; <sup>c</sup>pH = 7.0. <sup>d</sup>Data from a previous study by Jensen et al.<sup>37</sup>; <sup>e</sup>pH = 6.0. <sup>f</sup>Initial activity in the 2.6-DMP assay; shown as highest (+++) to lowest (+). <sup>g</sup>Stability under turnover conditions; shown as highest (+++) to lowest (+).
**Streptomyces** sp., which could be attributed to the substrate not being in its natural crystalline and insoluble context. Indeed, it is well known from research on lignocellulose-active LPMOs that certain hemicellulolytic activities are only detectable when the hemicellulose in question is adsorbed to cellulose.

Streptomyces have a complex multicellular lifestyle alternating between mycelial growth and the formation of reproductive spores, a process that involves cell wall remodeling at apical sites of the hyphae during cell elongation as well as during degradation of vegetative mycelium. Intriguingly, available transcriptomic data from Yagüe et al. show that ScLPMO10A (SCO0481; subclade C) and ScLPMO10D (SCO1734; subclade A2) are upregulated whereas ScLPMO10E (SCO2833; subclade C) is downregulated when the bacteria differentiate from vegetative growth to the reproductive stage, i.e., sporulation (Supplementary Fig. 59). Interestingly, ScLPMO10E has been linked to cell wall remodeling and degradation of peptidoglycan although the true substrate of this “chitin-active” LPMO remains unknown. Using knock-out mutations, Zhong et al. showed that the absence of this LPMO leads to morphological changes and make the bacterium more sensitive to lysozyme. Moreover, gene expression studies have shown that *S. coelicolor* only upregulates the expression of two LPMO genes when grown in chitin-rich conditions, namely ScLPMO10E and ScLPMO10G (SCO7225; subclade C), of which the latter displayed a five times higher increase in expression level than the former when grown in chitin-enriched soil. Hence, ScLPMO10G is more likely to be the main oxidizing force during chitin catabolism, while ScLPMO10E seems to play a role in cell wall remodeling. Supplementary Fig. 59 shows that the down-regulation of ScLPMO10E correlates with the upregulation of ScLPMO10D, which we here suggest may also be involved in cellular development.

An analysis of the loci of the different LPMO genes of *S. coelicolor* showed that only ScLPMO10D (SCO1734) and ScLPMO10E (SCO2833) lie within the central region of the linear chromosome (SCO1440–5869). The central region encodes conserved core functions, which include cellular development, while the segments flanking the central region, referred to as the left and the right arm, are suggested to encode auxiliary functions upregulated at specific conditions. The other LPMO genes are either found on the left (SCO0481, ScLPMO10A; SCO0643, ScLPMO10B; SCO1188, ScLPMO10C) or right (SCO6345, ScLPMO10F; SCO7225, ScLPMO10G) arm. Thus, in line with a possible function in cellular development, one may conclude that ScLPMO10D and ScLPMO10E serve more critical functions, compared to the other LPMOs. It is conceivable that the relatively high number of LPMO-encoding genes in the genomes of actinobacteria (*S. coelicolor* and *M. aurantiaca*), compared to other prokaryotes, is related to the complex cellular processes of these organisms.

### Methods

#### Bioinformatics analysis.

The amino acid sequences of ScLPMO10D, MaLPMO10A and the six other *S. coelicolor* LPMO10s were analyzed using CAZy (http://www.cazy.org) and InterPro (http://www.ebi.ac.uk/interpro) for information on domains and functional sites as well as for identifying potential motifs for sortase-mediated cell wall-anchoring. Prediction of transmembrane helices was carried out using the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM).

To construct a phylogenetic tree, 150 AA10 sequences (catalytic domains only) were aligned using the MUSCLE online tool provided by EMNL-EBI. To construct the pool of sequences containing representatives from all clades of AA10 LPMOs, as previously defined by Book et al., the sequences of 45 previously characterized LPMOs from each clade as queries for protein–protein BLAST (blastp) searches against the non-redundant NCBI database. We aimed to add approximately 20 protein sequences to fill the subclades while not exceeding a total number of 150 sequences. The added blastp sequences were selected based on their sequence identity to their queries and other subclade members, mostly rejecting sequences that did not add to the diversity of the subclades (e.g., >90% of 150 sequences. The added blastp sequences were selected based on their sequence identity to their queries and other subclade members, mostly rejecting sequences that did not add to the diversity of the subclades (e.g., >90% of 150 sequences). The resulting multiple sequence alignment was employed as input to build the phylogenetic tree, using PhyML available via the online platform NGPphylogeny.fr. The final phylogenetic tree (Fig. 2) was visualized using the iTOL platform. C-terminal sequences were included to the completed phylogenetic tree to illustrate the diversity of domain architecture and other unique characteristics, such as the lack of catalytic histidines or to show sequences of non-bacterial origins.

**Cloning, expression, and purification of recombinant LPMO10s.** Codon-optimized genes encoding the N-terminal domains of *S. coelicolor* A3(2) ScLPMO10D, including its native signal peptide (residues 1–214; UniProt ID: Q95929; ScLPMO10DCD (CD for catalytic domain)), and *M. aurantiaca* ATCC 27,029 MaLPMO10A, also with its native signal peptide (residues 1–205; UniProt ID: D9TC53; MaLPMO10A), were purchased from GenScript (Piscataway, NJ, USA). The genes were amplified using gene specific primers containing overhangs for cloning in the pRSET B vector (bold) and cleavage sites for histidines or to show sequences of non-bacterial origins.

To clone the genes, the N-terminal regions of *S. coelicolor* (ScLPMO10D) and *M. aurantiaca* (MaLPMO10A) were amplified from the respective genomes using specific primers containing restriction sites for *Hind III* (underlined in the reverse primers) and *Hind III* (underlined in the reverse primers), respectively, as follows; forward primer ScLPMO10D 5’-CGGAAACGCGGAATGGCAGCAGGTGATCCATGGGGCA-3’; reverse primer ScLPMO10DCD 5’-CAGCCGATCAGCTTTTAACCAAGGTTAACAT-3’; forward primer MaLPMO10ACD 5’-CCGCGAATGTCACCGTGTCGCCGACAG-3’; reverse primer MaLPMO10ACD 5’-CAGCCGATCAGCTTTTAACCAAGGTTAACAT-3’. The amplified genes were inserted into pre-linearized pRSET B vector (by the two restriction endonucleases *Bsm I* and *Hind III*) containing the signal peptide of *S. lividans* (residue 1–27) using the In-Fusion® HD cloning kit (Clontech) as previously described. Consequently, the native signal peptides of ScLPMO10D and MaLPMO10A are substituted for the signal peptide of *S. lividans* LPMO10A, which is known to result in efficient translocation of LPMOs to the periplasmic space during *Escherichia coli* expression.

To prepare expression strains, plasmids, whose sequence had been verified, were transformed into One Shot® BL21 Star (DE3) chemically competent *E. coli* cells (Invitrogen), according to the supplier’s protocol. Transformed cells were grown in Terrific Broth medium supplemented with 100 µg/mL ampicillin at 30 °C, using a
LEX-24 Bioreactor (Harbinger Biotechnology, Canada) with compressed air for aeration and mixing. After 20 h and no induction, cells were harvested by centrifugation and periplasmic proteins were extracted using cold osmotic shock with magnesium, as previously described48. The resulting periplasmic fractions were sterilized by filtration through a 0.22-µm syringe filter. No soluble protein was obtained for MaLPMO10A(CD) despite several expression attempts using different types of media and different temperatures. The pH of successfully produced ScLPMO10DCD solution was adjusted to 9.0 by adding Tris/HCl buffer to 50 mM final concentration (buffer A), prior to protein purification. The pH-adjusted extract was loaded onto a pre-equilibrated (buffer A) 5-ml Q-Sepharose FF column (GE Healthcare) connected to an ÄKTA purifier FPLC system (GE Healthcare). Under these conditions, most native E. coli proteins bound to the column, whereas ScLPMO10DCD appeared in the flow-through. The pooled flow-through fractions were concentrated using Amicon Ultra-15 centrifugal filters with a 10 kDa molecular weight cut-off (Merck Millipore), with concomitant buffer exchange to 20 mM Tris–HCl, pH 7.5, and then loaded onto a HiLoad 16/60 Superdex 75 size-exclusion column. Fractions containing protein of high purity, analyzed by SDS-PAGE, were pooled and concentrated as described above. The protein concentration was determined by measuring absorption at 280 nm, using the theoretical extinction coefficient (34,170 M⁻¹ cm⁻¹)49 and the Beer-Lambert law.

Additional enzymes, namely the LPMOs GjLPMO10A(CD) and SmLPMO10A, the chitinases SmChi18A and SmChi18C, and a GH20 chitobiase SmCHB were expressed and purified as described previously50,51. Before use, all LPMOs were incubated with a three-fold molar surplus of CuSO₄ as described previously52, followed by desalting using PD MidiTrap G-25 columns (GE Healthcare) equilibrated with 20 mM sodium phosphate, pH 6.0. The negligible level of residual free copper in preparations of copper-saturated LPMOs was assessed by measuring the apparent H₂O₂ production in reactions with LPMO-free filtrates, as described below in the section “Measuring apparent H₂O₂ production.”

Crystallization, diffraction data collection, structure determination, and model refinement. Crystals of the catalytic domain of ScLPMO10DCD (residues 34–214) were obtained with the hanging drop vapor diffusion method. Equal volumes (1 µl) of Cu(II)-saturated LPMO (in 20 mM Tris/HCl buffer, pH 8.5) and different reservoir solutions were mixed at room temperature. Crystals were obtained in 2.0 M ammonium sulfate and 0.1 M sodium cacodylate pH 6.5 at a protein concentration of 7.7 g/L. Protein crystals were soaked in a cryo-solution containing mother-liquor with 35% glucose (w/v) before they were flash frozen in liquid nitrogen. Diffraction data were collected at the ID23-1 beamline at ESRF, Grenoble, France. The CCP4ii package was used to solve the structure by molecular replacement ( Phaser)53 and the structure was refined using REFMAC54. Model manipulations were carried out using Coot55 and molecular graphics were generated using PyMOL2 (The PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC.).

The 3D model of MaLPMO10A (UniProt ID: D9TCT5, residues 27–298) without the signal peptide was built using AlphaFold 2.0.0.1 at the Saga HPC cluster (Sigma2, Norway)56.

Substrate specificity of ScLPMO10DCD. LPMO activity was primarily assessed for β-chitin (10 g/L), using 1 µM enzyme and 1 mM ascorbic acid in 50 mM sodium phosphate buffer, pH 6.0. Unless stated otherwise, all reactions were incubated in 2 mL Eppendorf tubes at 800 rpm in an Eppendorf thermomixer set to 40 °C (Eppendorf, Hamburg, Germany). Extracted and deproteinized β-chitin from squid pen (batch 20,140,101, France Chitin, Orange, France), was ball-milled and sieved to produce separate fractions of particles with 75–200 µm and 500–850 µm size. Activity was also tested towards 10 g/L shrimp shell (Pandalus borealis) α-chitin [purchased from Chitinor AS; Senjahopen, Norway; demineralized by hydrochloric acid treatment and subsequently deproteinized by alkaline (NaOH) treatment] and cellulotic substrates such as 5 g/L phosphoric acid swollen cellulose (PASC; prepared from Avicel PH-101 as described by Wood57); 10 g/L Avicel PH-101 (Sigma-Aldrich, St. Louis, MO, USA) and 1 g/L bacterial microcrystalline cellulose58 (kindly provided by Dr. Pritt Väljamäe, University of Tartu). Furthermore, activity was tested towards chitin oligomers (DP5-6, 2 mM) purchased from Megazyme (Bray, Ireland), and peptidoglycan isolated from Streptomyces sp. (3 g/L) purchased from Merck (Darmstadt, Germany).

Activity on β-chitin. Unless otherwise specified, all reactions were carried out in triplicates with 1 µM LPMO (ScLPMO10DCD), 10 g/L β-chitin (75–200 µm particle size), and 1 mM ascorbic acid in 50 mM sodium phosphate buffer, pH 6.0. Control reactions without ascorbic acid and/or the LPMO were included in all experiments.

To quantify soluble oxidized products, aliquots were withdrawn at selected time points and the soluble fractions were immediately separated from the insoluble substrate by filtration using a 96-well filter plate (Millipore) and a Millipore vacuum manifold. By separating soluble and insoluble fractions the reactions were quenched, as the LPMOs used in this study do not oxidize soluble chito-oligosaccharides to a significant extent49,50. The filtrate containing solubilized oxidized chito-oligosomers was subsequently mixed with an equal volume of a solution containing chitobiase from S. marcescens (SmCHB; 1 µM final concentration) followed by an overnight incubation at 37 °C. SmCHB, a GH20 β-hexosaminidase, cleaves off single GlcNAc units from chito-oligosaccharides until GlcNAc is the final product. However, if the chito-oligosaccharide is oxidized at the C1 position, as in the case for chitin-oxidizing AA10 enzymes, the final product of SmCHB treatment is an oxidized dimer (chitobionic acid; GlcNAcGlcNAc1A)57. As a result of this treatment, all soluble oxidized products are converted to chitobionic acid, which can easily be quantified.

To determine the total amount of oxidized sites, an aliquot of the crude reaction mixture (containing both soluble and insoluble products) was diluted five times (i.e., to 2 g/L chitin), incubated at 98 °C for 10 min to
terminate the LPMO reaction, and mixed with an equal volume of a chitinase cocktail (2 µM SmChi18A, 2.5 µM SmChi18C, and 1 µM SmCHB, final concentrations), followed by overnight incubation at 37 °C. This treatment turned out to be sufficient to completely degrade all chitin and convert all oxidized products to chitobionic acid.

**Probing the cause of enzyme inactivation.** Reactions with 10 g/L β-chitin (75–200 µm particle size) were set up under standard conditions, as described above, for all three enzymes and samples were taken and vacuum-filtered at 30 and 35 h, when product formation likely had ceased. Of note, SmLPMO10A showed no signs of inactivation at these time points and was therefore excluded for the next step of the experiment. After the 35-h time-point, the remaining ScLPMO10D and GlpLPMO10A reaction mixtures were divided into six identical fractions which were further supplemented with an equal volume of either (i) fresh buffer, (ii) fresh substrate (to a final concentration of 10 g/L), (iii) fresh enzyme (1 µM), (iv) fresh reductant (2 mM), (v) fresh reductant and substrate, or (vi) fresh reductant and enzyme, all in 50 mM sodium phosphate, pH 6.0. The incubation was continued overnight before terminating the reactions via filtration, followed by chitobiase treatment and quantification of chitobionic acid.

**Qualitative and quantitative analysis of oxidized chito-oligosaccharides.** Product mixtures in reaction supernatants were qualitatively assessed using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) UltrafleXtreme mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany), equipped with a Nitrogen 337-nm laser. Reaction mixtures (1 µL) were applied to an MTP 384 ground steel target plate TF (Bruker Daltonics) together with 2 µL of 9 mg/mL of 2,5-dihydroxybenzoic acid (DHB) dissolved in 30% acetonitrile, followed by drying under a stream of air. Data collection and analysis were performed using the Bruker FlexAnalysis software.

Quantification of oxidized chitobiase (GlcNAcGlcNAc1A) was achieved using a Dionex Ultimate 3000 UHPLC system (DionexCorpor., Sunnyvale, CA, USA) equipped with a Rezex RFQ-Fast Acid H+ (8%) 7.8 × 100 mm column (Phenomenex, Torrance, CA) operated at 85 °C. 8 µL samples were injected into the column and the reaction products were eluted isocratically, using 5 mM sulfuric acid as mobile phase, and detected via UV absorption at 194 nm. Data collection and analysis were performed with the Chromleon 7.0 software. Standards were generated in-house by complete oxidation of N-acetyl-chitobiase (Megazyme; 95% purity) with a chitoooligosaccharide oxidase from *Fusarium graminearum* (ChitO)85, as previously described77.

**Chitin binding.** Binding to β-chitin in the absence of an electron donor was analyzed spectroscopically using an A320, method as previously described44. 10 g/L β-chitin (500–850 µm particle size) and 3 µM LPMO in 50 mM sodium phosphate, pH 6.0, were incubated at 40 °C and 800 rpm in an Eppendorf thermomixer (Eppendorf, Hamburg, Germany). At t = 0, 2.5, 5, 15, 30 and 60 min, samples were rapidly vacuum-filtered using a 96-well filter plate (0.45-µm) to separate unbound protein from protein bound to substrate. Thus, substrate binding could be monitored by measuring the A280, which reflects unbound protein in the reaction supernatants, at various time points.

**Apparent melting temperature (Tm).** The apparent Tm of ScLPMO10D, with or without the bound copper cofactor, was determined using a Protein Thermal Shift Kit (Thermo Fisher Scientific, Waltham, MA, USA), in the presence of a fluorescent dye—SYPRO Orange. The fluorescence of the dye is significantly increased upon binding to hydrophobic regions of the protein that become accessible as the protein unfolds46. A StepOnePlus Real-Time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA) was used to monitor the fluorescence with Ex/Em wavelengths set to 490/530 nm. Apo enzyme was prepared in 50 mM sodium phosphate, pH 6.0, by preincubating ScLPMO10D in 10 mM ethylenediaminetetraacetic acid (EDTA), a metal-chelating agent, for 10 min at room temperature. Reactions were prepared in quadruplicates (n = 4) with 0.7 g/L copper saturated LPMO (with or without 10 mM EDTA) in 50 mM sodium phosphate, pH 6.0, and heated in the presence of the dye in a 96-well plate from 25 to 99 °C over a 50-min time period. The apparent Tm was derived from the first derivative of the fluorescence emission with respect to temperature (∆DF/DT).

**Electron paramagnetic resonance.** A 250 µL sample containing 400 µM Cu(II)-saturated ScLPMO10D in 50 mM MES, pH 6.0, was prepared and frozen in liquid nitrogen. EPR spectra were recorded using a BRUKER EleXsys 560 SuperX instrument equipped with an ER 4122 SHQE SuperX high-sensitivity cavity and a cold finger. The microwave power was set to 1 mW, the modulation amplitude was 10 G and the spectra were recorded at 77 K. The EasySpin toolbox developed for Matlab was used to simulate and fit EPR spectra87.

**Determination of the redox potential (E⁰).** The cell potentials for the redox couple Cu(II)-LPMO/Cu(I)-LPMO were determined for *Sc*, *N*, *N'*-tetramethyl-1,4 phenylenediamine (TMPox) and Cu(II)-LPMO were determined using a Protein Thermal Shift Kit (Thermo Fisher Scientific, Waltham, MA, USA), in the presence of a fluorescent dye—SYPRO Orange. The fluorescence of the dye is significantly increased upon binding to hydrophobic regions of the protein that become accessible as the protein unfolds46. A StepOnePlus Real-Time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA) was used to monitor the fluorescence with Ex/Em wavelengths set to 490/530 nm. Apo enzyme was prepared in 50 mM sodium phosphate, pH 6.0, by preincubating ScLPMO10D in 10 mM ethylenediaminetetraacetic acid (EDTA), a metal-chelating agent, for 10 min at room temperature. Reactions were prepared in quadruplicates (n = 4) with 0.7 g/L copper saturated LPMO (with or without 10 mM EDTA) in 50 mM sodium phosphate, pH 6.0, and heated in the presence of the dye in a 96-well plate from 25 to 99 °C over a 50-min time period. The apparent Tm was derived from the first derivative of the fluorescence emission with respect to temperature (∆DF/DT).

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**Determination of the redox potential (E⁰).** The cell potentials for the redox couple Cu(II)-LPMO/Cu(I)-LPMO were determined for ScLPMO10D42, GlpLPMO10A42, and SmLPMO10A, as previously described86. For each enzyme, equal volumes (50 µL) of anaerobic solutions of reduced N,N,N',N'-tetramethyl-1,4 phenylenediamine (TMPox) and Cu(II)-LPMO in 20 mM PIPES buffer (pH 6.0, T = 28 °C) were mixed to give a final concentration of 150 µM and 35 µM, respectively. The extent of the reaction was determined by detecting the absorbance of the formed TMP radical cation (TMPox) at λ = 610 nm. To find the concentration of TMPox, which equals the concentration of Cu(I)-LPMO, an extinction coefficient for TMPox of 14.0 mM−1cm−1 was applied89. From the determined concentrations of TMPox (i.e., the concentration of reduced LPMO), the equilibrium constant (K) was computed to yield the cell potential of the reaction between TMPred and Cu(II)-LPMO. Finally, the different cell potentials for the Cu(II)-LPMO/Cu(I)-LPMO redox couples were calculated by subtracting the
known cell potential for TMP\textsuperscript{red}/TMP\textsuperscript{ox}, 273 mV\textsuperscript{90}, to the calculated cell potentials of the different equilibrium reactions of TMP\textsuperscript{ox} and Cu(II)-LPMO.

**Measuring apparent H\textsubscript{2}O\textsubscript{2} production.** Production of H\textsubscript{2}O\textsubscript{2} by the LPMO and/or through abiotic oxidation of the reductant, possibly catalyzed by free copper was analyzed according to Stepnov et al.\textsuperscript{85}, based on the method described by Kitzl et al.\textsuperscript{82}. Reaction mixtures containing 2 μM LPMO-Cu(II), 5 U/ml horse radish peroxidase (HRP), 100 μM Amplex Red and 1% (v/v) DMSO, were prepared in 50 mM sodium phosphate buffer, pH 6.0 and pre-incubated in a 96-well microtiter plate for 5 min at 30 °C, before initiating the reaction by adding ascorbic acid to 1 mM final concentration. Each experiment included control reactions lacking one of the reaction components, as well as H\textsubscript{2}O\textsubscript{2} standard curves including reductant. In some of the control reactions the LPMO stock solution was substituted with an equal volume of water or with an equal volume of an LPMO-free filtrate that was prepared via ultrafiltration of the LPMO stock solution. The latter control experiments were done to establish whether the LPMO preparations contained significant amounts of free copper, as described previously\textsuperscript{83}. For the standard curve, H\textsubscript{2}O\textsubscript{2} dilution series were prepared in 50 mM sodium phosphate, pH 6.0 containing 5 U/ml HRP, 100 μM Amplex Red and 1 mM ascorbic acid. Hydrogen peroxide formation was monitored over time using a Varioscan LUX plate reader at 30 °C (Thermo Fisher Scientific, Waltham, MA, USA), by following resorufin absorption at 563 nm. For all reactions, the standard curve was used to convert the measured A\textsubscript{563} values into micro molar concentrations of H\textsubscript{2}O\textsubscript{2}. Apparent H\textsubscript{2}O\textsubscript{2}-production rates were derived from the initial linear parts of the resorufin accumulation curves, i.e., 0.5–4 min.

**Oxidation of 2,6-DMP.** ScLPMO10D\textsuperscript{CD}, CjLPMO10A\textsuperscript{CD}, and SmLPMO10A were assessed for their peroxidase activity using a method described by Breslmayr et al.\textsuperscript{84}. This assay features a spectrophotometric method with 2,6-dimethoxyphenol (2,6-DMP) as a chromogenic substrate and H\textsubscript{2}O\textsubscript{2} as co-substrate. The LPMO carries out a peroxidase-like reaction that converts 2,6-DMP into coerulignone, the formation of which can be measured spectrophotometrically at A\textsubscript{469}. A solution containing 2,6-DMP and H\textsubscript{2}O\textsubscript{2}, and another solution containing the LPMO, were preincubated separately for 5 min at 30 °C in 50 mM sodium phosphate, pH 6.0. Reactions were initiated by mixing equal volumes of the two solutions, giving a final volume of 100 μL and a final concentration of 1 μM LPMO, 1 mM 2,6-DMP and 100 μM H\textsubscript{2}O\textsubscript{2}. Immediately after mixing, product formation was recorded in a Varioscan LUX plate reader (Thermo Fisher Scientific, Waltham, MA, USA), which was used to monitor the A\textsubscript{469} over a period of 10 min.

**Data availability**

The dataset generated and analyzed during the current study is available in the Protein Data Bank (PDB ID code 7ZJB).

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**References**

**Author contributions**
A.K.V. designed, performed, and analyzed the biochemical experiments and wrote the paper. Å.K.R. determined the crystal structure of ScLPMO10D^CD, performed the EPR spectroscopy and contributed to writing the paper. B.B. performed cloning and crystallization and carried out bioinformatics analysis. A.A.S. and M.S. designed and analyzed the experiments in Fig. 7 and M.S carried out supervision. V.G.H.E. contributed to supervising the work, interpreting data, and writing the manuscript. Z.F. supervised the work, designed, and analyzed experiments and wrote the manuscript. All authors have reviewed the results and approved the final version of the manuscript.

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**Competing interests**
The authors declare no competing interests.

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