Kinetic insights into the role of the reductant in H$_2$O$_2$-driven degradation of chitin by a bacterial lytic polysaccharide monoxygenase

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Lytic polysaccharide monoxygenases (LPMOs) are monooxygenase enzymes that catalyze oxidative cleavage of glycosidic bonds in polysaccharides in the presence of an external electron donor (reductant). In the classical O$_2$-driven monoxygenase reaction, the reductant is needed in stoichiometric amounts. In a recently discovered, more efficient H$_2$O$_2$-driven reaction, the reductant would be needed only for the initial reduction (priming) of the LPMO to its catalytically active Cu(I) form. However, the influence of the reductant on reducing the LPMO or on H$_2$O$_2$ production in the reaction remains undefined. Here, we conducted a detailed kinetic characterization to investigate how the reductant affects H$_2$O$_2$-driven degradation of $^{14}$C-labeled chitin by a bacterial LPMO, SmlPMO10A (formerly CBP21). Sensitive detection of $^{14}$C-labeled products and careful experimental set-ups enabled discrimination between the effects of the reductant on LPMO priming and other effects, in particular enzyme-independent production of H$_2$O$_2$ through reactions with O$_2$. When supplied with H$_2$O$_2$, SmlPMO10A catalyzed 18 oxidative cleavages per molecule of ascorbic acid, suggesting a “priming reduction” reaction. The dependence of initial rates of chitin degradation on reductant concentration followed hyperbolic saturation kinetics, and differences between the reductants were manifested in large variations in their half-saturating concentrations ($K_{\text{m,red}}$). Theoretical analyses revealed that $K_{\text{m,red}}$ decreases with a decreasing rate of polysaccharide-independent LPMO reoxidation (by either O$_2$ or H$_2$O$_2$). We conclude that the efficiency of LPMO priming depends on the relative contributions of reductant reactivity, on the LPMO’s polysaccharide monoxygenase/peroxygenase and reductant oxidase/peroxidase activities, and on reaction conditions, such as O$_2$, H$_2$O$_2$, and polysaccharide concentrations.

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The kinetics of H$_2$O$_2$-driven oxidation of chitin (14C-labeled crystalline α-chitin nanowhiskers (CNWs)) by SmLPMO10A with 0.1 mM AsCA as reductant has been characterized in detail before (25). The $k_{cat}$ value for oxidative cleavage of CNWs was 6.7 s$^{-1}$, and $K_m$ values were 2.8 μM and 0.58 mg ml$^{-1}$ for H$_2$O$_2$ and CNWs, respectively (25). The quantification of the radioactivity in the supernatants of SmLPMO10A reactions enables sensitive detection of soluble products, the concentration of which is expressed in GlcNAC equivalents (NAG$_{eq}$). Under the conditions used in previous studies, the turnover of one molecule of H$_2$O$_2$ results in one oxidative cleavage, which is manifested in the release of four soluble NAG$_{eq}$ (25). Because about 50% of the oxidized groups remain associated with the insoluble substrate, the average degree of polymerization of soluble products is 8 NAG$_{eq}$ (25). Importantly, under these conditions (typically 1 mg ml$^{-1}$ CNWs, 42 nM SmLPMO10A, and reaction time < 10 min), and as long as enzyme inactivation and H$_2$O$_2$ depletion are avoided, release of NAG$_{eq}$ is linear over time up to levels of 190 μM released soluble NAG$_{eq}$ (25). Therefore, in the present study, we used 1 mg ml$^{-1}$ CNWs as initial substrate concentration, and the maximum levels of released NAG$_{eq}$ remained well below the 190 μM threshold. If not stated otherwise, the concentration of SmLPMO10A was 42 nM. Time courses of the release of NAG$_{eq}$ were measured at two initial H$_2$O$_2$ concentrations, 20 and 100 μM, and the nature and the concentration of the reductant varied.

Kinetic studies of LPMOs with H$_2$O$_2$ as cosubstrate in an aerobic environment are complicated by the simultaneous presence of O$_2$ and a reducing agent. Because of the low $K_m$ value of SmLPMO10A for H$_2$O$_2$ (25), low H$_2$O$_2$ concentrations must be used in kinetic studies, and care must be taken to account for the formation/depletion of the H$_2$O$_2$ in enzyme-independent and enzyme-dependent background reactions. For example, the formation of H$_2$O$_2$ in the reaction between AsCA and molecular oxygen is well-known (35). Furthermore, the oxidation of AsCA by O$_2$ is catalyzed by metal ions, like Cu(II) (36, 37), so that their presence even in trace amounts can significantly affect the formation of H$_2$O$_2$ and interpretation of the results of LPMO kinetics. The problem is further amplified by the occurrence of metal ions in polymeric substrates of LPMOs, such as cellulose and chitin (38–40).

When setting up our experimental conditions, we first assessed the activity of SmLPMO10A in experiments without added H$_2$O$_2$, hereinafter referred to as “background activity.” Considerable variation in background activity was observed between different CNW batches in reactions containing AsCA (0.1 mM) as reductant (Fig. 1A). This indicates that the quality of the water and/or buffer components used in the preparation of CNW batches (see “Experimental procedures”) affects background activity. The addition of peroxidase completely removed the background activity, indicating that the formation of H$_2$O$_2$ drives catalysis under these conditions (Fig. 1B). The addition of EDTA (5 mM) also completely removed the background activity, suggesting that divalent metal ions are involved (41). The addition of catalase caused more than 10-fold reduction of the background activity (Fig. 1B). Collectively, these results suggest that, under the conditions used here, the background activity is related to the formation of H$_2$O$_2$ by divalent metal ion-catalyzed oxidation of AsCA by O$_2$.

It is worth noting that, whereas EDTA removed the background activity, the LPMO remained active when supplied with H$_2$O$_2$. The ability of LPMOs to withstand EDTA has been reported before (8) and coincides with very high reported copper affinities (8, 42). Apparently, the strong binding of copper and slow off rates (43) ensure that there is no significant transfer of copper from the LPMO to EDTA, provided that the contact time is kept short, as is the case here. Accordingly, in experiments with 0.1 mM AsCA as reductant and 20 μM load of H$_2$O$_2$, EDTA had no effect on the kinetics of SmLPMO10A. However, at lower AsCA concentration (10 μM), EDTA became inhibitory for the LPMO reaction (Fig. 1A). One may speculate that EDTA can sequester only the Cu(II) from LPMO and becomes inhibitory when the reduction of LPMO becomes less efficient. Con-
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Considered these complications, the use of EDTA was judged not applicable in further studies of reductant efficiency. To minimize complexity, in further work, we only used “low-background” CNW in the experiments with AscA as reductant. Unlike reactions with AscA, within the time scales used, there was no significant background signal when GA and methyl hydroquinone (MHQ) were used as reductants (Fig. S1), regardless of the CNW batch used. This is corroborated by a reported much higher stability of the latter reductants against oxidation (i.e. H$_2$O$_2$ production) compared with AscA (10). We also attempted to use 1,4-dihydroxy-2,6-dimethoxybenzene, an efficient reductant in supporting AA9’s activity (10), but the high background activities (data not shown) did not permit accurate measurement of SmLPMO10A kinetics, and this reductant was omitted from further studies. As found for AscA before (25), the order of the addition of reductant and H$_2$O$_2$ had no effect on the outcome of the experiment (Fig. S1). The experimental set-up where the reductant was added to pre-mixed CNW/SmLPMO10A 30 s before the reaction was started by the addition of H$_2$O$_2$ (zero time point) was used throughout this study.

Importantly, considering the above, and as a result of precisely tailoring the reaction conditions, in all of the experiments described below, the rates of SmLPMO10A without added H$_2$O$_2$ were always insignificant compared with reactions containing a 20 or 100 μM initial load of H$_2$O$_2$. Thus, in the experiments described below, the effects of various possible O$_2^-$, reductant-, and/or SmLPMO10A-dependent H$_2$O$_2$ producing pathways are negligible.

Time curves with different reductants

We studied the effect of reductants on chitin oxidation using either 20 or 100 μM H$_2$O$_2$ as initial load. We have previously shown (25), using 0.1 mM AscA as reductant, that at the lower H$_2$O$_2$ initial load (represented here by experiments with 20 μM H$_2$O$_2$), chitin oxidation by SmLPMO10A is fast enough compared with enzyme inactivation (i.e. most H$_2$O$_2$ is incorporated into oxidized products), whereas at the higher H$_2$O$_2$ initial load (represented here by experiments with 100 μM H$_2$O$_2$), the kinetics is governed by enzyme inactivation. A few experiments with the higher load of H$_2$O$_2$ were included in this study to illustrate the effect of reductant on H$_2$O$_2$-driven enzyme inactivation.

First, we followed the formation of soluble NAGeq in time using a 20 μM initial H$_2$O$_2$ load. AscA, GA, and MHQ at different concentrations were used as reductants. Based on the stoichiometry of 4.0 ± 0.3 NAGeq released per H$_2$O$_2$ consumed (25), one can calculate that a maximum of 80 ± 6 μM NAGeq can be released in the experiments with 20 μM H$_2$O$_2$. Data presented in Fig. 2 show that the rate of NAGeq formation depends on the nature and concentration of the reductant. Although there is some uncertainty associated with the precise measurement of the plateau values for reactions with largely different rates, all time curves tend to reach a plateau value of NAGeq ([NAGeq]$_{max}$) close to 80 μM (Fig. 2). Of note, the consistency between the measured and expected [NAGeq]$_{max}$ values shows that side reactions leading to production or consumption of H$_2$O$_2$ are not significant under our experimental conditions.

As a clear exception, the experiments with AscA at low concentrations (0.2 and 0.5 μM) yielded lower values of [NAGeq]$_{max}$ (Fig. 2A). The addition of a fresh portion of AscA (up to 100 μM) at the point in the time curve where the formation of NAGeq had decayed caused a new burst in activity and subsequent leveling off at a [NAGeq]$_{max}$ around 80 μM (Fig. 2A). This indicates that at low AscA concentrations, depletion of the reductant limits the reaction. [NAGeq] values from experiments with low [AscA] were fitted to Equation 1 (25) using nonlinear regression analysis (Fig. 2A).

\[
[NAGeq] = [NAGeq]_{max} (1 - e^{-kt})
\]

(Eq. 1)

Plotting the [NAGeq]$_{max}$ values obtained for the three lowest AscA concentrations against the concentration of AscA results in a linear correlation, showing that 73 ± 20 NAGeq are released per molecule of AscA (Fig. 3). Considering that 4.0 ± 0.3 NAGeq are released per H$_2$O$_2$ consumed and that one H$_2$O$_2$ supports one oxidative cleavage (23, 25), we can thus estimate that an average of 18 ± 5 oxidative cleavages are performed by SmLPMO10A per reduction (priming) event under our experimental conditions.
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Figure 2. Time curves of the formation of soluble products (in NAGeq) in the presence of different reductants. All experiments were carried out in NaAc buffer (50 mM, pH 6.1) at 25 °C and contained CNWs (1.0 mg ml⁻¹), H₂O₂ (20 μM), and SmLPMO10A (42 nM). The reductant was AscA (A), GA (B), or MHQ (C). The concentration of the reductant is shown in the plot. The horizontal line indicates (shadowing shows S.D.) the maximum amount of soluble NAGeq that can be produced in the experiments with 20 μM H₂O₂ (80 ± 6 μM NAGeq). The arrows in A indicate the addition of fresh AscA to a final concentration of 100 μM. The solid lines show nonlinear regression analysis (up to the 1,200-s time point) according to Equation 1. Error bars, S.D. derived from at least two independent experiments.

Figure 3. Correlation between the maximum concentration of soluble products ([NAGeq]max) and the concentration of ascorbic acid. The [NAGeq]max values were found by nonlinear regression analysis of the data in Fig. 2A for the series with [AscA] of 0.2, 0.5, and 1.0 μM and for the time points up to 1,200 s according to Equation 1. The solid line shows linear regression of the data. Error bars, S.D. derived from at least independent experiments.

Figure 4. Time curves for the formation of soluble products (in NAGeq) at high H₂O₂ load. All experiments were carried out in NaAc buffer (50 mM, pH 6.1) at 25 °C and contained CNWs (1.0 mg ml⁻¹), H₂O₂ (100, 200, 500), and SmLPMO10A (42 nM). Gallic acid was used as reductant, and its concentration is shown in the plot. The solid lines show nonlinear regression analysis according to Equation 1. Error bars, S.D. derived from at least two independent experiments.

Previous studies using 0.1 mM AscA as reductant showed that, under otherwise identical conditions, the initial addition of 100 μM H₂O₂ yields reaction kinetics that are dominated by enzyme inactivation. Under these conditions, a maximum of only 25 μM NAGeq was reached (i.e. 6.25% of the theoretical maximum) (25). Dose–response experiments with GA as reductant (Fig. 4) show that increasing the GA concentration leads to faster product formation and faster enzyme inactivation. For instance, fitting of the data to Equation 1 shows half-lives of 220 and 90 s for the reactions with 50 and 500 μM GA, respectively. At GA concentrations above 100 μM, the maximum levels of NAGeq reach 25 μM (Fig. 4), which is similar to what was observed for AscA with a 100 μM H₂O₂ load (25). The [NAGeq]max value is a result of the competition between (i) H₂O₂-driven catalysis of chitin oxidation by SmLPMO10A and (ii) irreversible inactivation of reduced SmLPMO10A by H₂O₂.

The fact that different [GA] yield different enzyme half-lives but similar [NAGeq]max values suggests that less efficient priming of SmLPMO10A slows down both catalysis and inactivation.

**Dependence of initial rates on the nature and concentration of the reductant**

The linear regions of the time curves of NAGeq formation were used to calculate the initial rates of reactions carried out with AscA, GA, and MHQ and an initial H₂O₂ load of 20 μM. Fig. 5A shows the results obtained for GA, and Fig. S2 shows the results obtained for the other reductants. Fig. 5B shows the dependences of the initial rates of NAGeq formation on the concentration of reductants. The initial rates versus [reductant] curves were in general accordance with hyperbolic saturation kinetics (Equation 2).

\[
V_i = \frac{V_{app,max} [R]}{K_{app} + [R]}
\]

(Eq. 2)

In Equation 2, the \( V_i \) is the initial rate of the formation of NAGeq, \([R]\) is the concentration of the reductant, and \( K_{app} \) and \( V_{app,max} \) represent the apparent half-saturating concentration of the reductant and apparent \( V_{max} \) of the formation of NAGeq. The \( V_{app,max} \) is related to the apparent catalytic constant for oxidative cleavage of CNWs (\( k_{app}^{cat} E_{tot} \)) according to \( V_{app,max} = n k_{app}^{cat} E_{tot} \) where \( n \) is an average number of NAGeq released per oxidative cleavage and \( E_{tot} \) is the total concentration of enzyme. The parameters of Equation 2 are referred to as apparent parameters because the reaction was studied at one set of substrate concentrations only (20 μM H₂O₂ and 1 mg ml⁻¹ CNWs). Table 1 lists the values of \( K_{app} \) and \( V_{app,max} \) and shows that differences between the reductants are primarily manifested by largely different \( K_{app} \) values amounting to 2, 86, and 700 μM, for AscA, GA, and MHQ, respectively. The differences between \( V_{app,max} \) values obtained with different reductants were less prominent (Table 1), which is as expected because close to full reduction of the LPMO should be reached in all cases, regardless of the reductant.

With GA as reductant, the \( K_{app} \) and \( V_{app,max} \) were determined for two different initial H₂O₂ loads, 20 and 100 μM. At the higher H₂O₂ load, the \( V_{app,max} \) decreased (indicating inhibition by H₂O₂), whereas the \( K_{app} \) increased (Table 1). The reasons for
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Figure 5. Initial rates of the formation of soluble products (in NAG eq) and their dependence on the nature and concentration of the reductant. All experiments were carried out in NaAc buffer (50 mM, pH 6.1) at 25 °C and contained CNWs (1.0 mg ml⁻¹) and SmLPMO10A (42 nM). A, linear regions of the progress curves (full progress curves are shown in Fig. 2B) obtained with 20 μM H₂O₂ and GA as reductant. Solid lines, linear regression of the data. The slopes of the lines correspond to the initial rates (v). For the linear regions of the time curves obtained with other reductants and with GA and 100 μM H₂O₂ see Fig. S2. B, dependence of initial rates on the nature and concentration of the reductant. The type of reductant and the concentration of H₂O₂ are indicated in the plot. Solid lines, nonlinear regression of the data according to Equation 2. Error bars, S.D. derived from at least two independent experiments.

Theoretical analysis of the reduction/oxidation of the LPMO in the absence of chitin

The reduced LPMO will be subject to nonproductive reoxidation primarily when it is not bound to substrate, because that exposes the reduced copper site to both O₂ and H₂O₂. To gain further insight into the dependence of kcat and vmax on the nature of the reductant and on the concentration of H₂O₂, we thus first considered oxidation/reduction of the LPMO in the absence of chitin, as outlined in Fig. 6A (the effects of including chitin into the reaction scheme are discussed below). In Fig. 6A, the chemically inert complexes ECu(I)–R, ECu(II)–O₂, and ECu(II)–H₂O₂ are omitted for clarity. The scheme of Fig. 6A assumes that both reduction and oxidation of the LPMO are irreversible. This assumption is plausible when analyzing initial rates, as product concentrations and, thus, rates of possible reverse reactions are negligible.

The catalytically active form of SmLPMO10A is the ECu(I) form, which has been proposed to bind to chitin followed by the binding of H₂O₂ and oxidative cleavage of the glycosidic bond (25, 44). The equation for steady-state [ECu(I)] is given in Fig. 6. The dependence between the initial rates of NAG eq formation (v) and [ECu(I)] is given by v = kcat(max)[ECu(I)], where v is the number of NAG eq released per oxidative cleavage and kcat(max) is the kcat for oxidative cleavages in the conditions where all enzyme is in ECu(I) form. Replacing [ECu(I)] with Ecat, one can arrive to Equation 2 and derive equations that show the dependence of kred and kcat on the concentration of O₂ and H₂O₂ (Fig. 6). Note that in doing so, we assume that concentration of CNWs has no effect on the steady-state [ECu(I)]. Using these equations, one can predict how the apparent catalytic properties of the LPMO depend on the efficiency of the reductant, kred/Kred, as shown in Fig. 6 (B–E).

Fig. 6B shows that in the presence of O₂ and H₂O₂, the concentration of the catalytically active ECu(I) form is always lower than Ecat. The equation for kapp shows that this parameter is always reduced compared with its maximum value, when all enzyme is expected to be in its Cu(I) form (kapp(max)), at least by a factor of 1 + [O₂]/Ko₂ + [H₂O₂]/KH₂O₂. This is evident by inspection of the expression of kcat in Fig. 6, assuming conditions of very efficient reduction (i.e., when the value of the rate constant for the reduction (kred) is much higher than the rate constants for oxidation of LPMO by O₂ (koxO₂) and H₂O₂ (koxH₂O₂). The similar vapp(max) values for different reductants (Table 1) indicate that their kred values are always higher than koxH₂O₂ and koxO₂. At very low concentration of both oxidants, O₂ and H₂O₂, the kcat approaches kapp(max) (regardless of kred), whereas the value of kapp decreases to zero (Fig. 6). Fig. 6C shows that, as expected, the kcat increases with an increasing kred value of the reductant (Fig. 6C), whereas the kapp decreases (Fig. 6D). The dependence of kapp/kapp(max) on the efficiency of the reductant (kred/Kred) was found to be linear (Fig. 6E).

Kinetics at low levels of O₂

Because the steady-state concentration of catalytically active Cu(I) form of LPMO is expected to depend in part on [O₂] (Fig. 6), we also measured the kinetics of the degradation of CNWs by SmLPMO10A under reduced [O₂] (i.e., under N₂ atmosphere). AscA, GA, and MHQ were used as reductants. The concentration of added H₂O₂ was 20 and 100 μM. In general, the kinetics (time curves and initial rates) measured in N₂ atmosphere were similar to those measured in normal air conditions (Fig. 7 and Fig. S3). This result is in line with earlier observations that, when provided with H₂O₂, the presence of O₂ has no effect on the activity of an LPMO (23). Of note, the binding of LPMO to cellulose has also been shown to be independent of the presence of O₂ (45). Collectively, these results suggest that in the experimental set-ups with initial H₂O₂ supply, the O₂-involving pathways in Fig. 6 are not significant.

Binding to CNWs in the absence of reductant

The binding of SmLPMO10A to CNWs in the presence of 0.1 mM AscA has been measured before (25) and resulted in the half-saturating concentration for CNWs of 0.68 ± 0.01 mg ml⁻¹. In the absence of reductant, the binding of LPMO to CNWs results in the formation of a nonproductive ECu(II)–CNW complex. In the case of nonefficient priming, this nonproductive complex can exist also in the presence of reductant but may be converted to a productive complex if reduction of LPMO within the complex with CNW is possible (e.g., through long-range electron transfer). To assess possible nonproductive binding, we measured the binding of SmLPMO10A to CNWs in the absence of reductant. SmLPMO10A was incubated with nonlabeled CNWs, and after separation of bound enzyme by centrifugation, the concentration of chitin-free enzyme was measured by measuring its activity in the supernatant using ¹⁴C-labeled CNWs (1.0 mg ml⁻¹) and H₂O₂ (20 μM) as substrates.
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Table 1
Kinetic parameters for different reductants in oxidation of chitin by SmLPMO10A

| Reductant           | V_{\text{max}}^{\text{app}} \mu M NAG eq s^{-1} | k_{\text{cat}}^{\text{app}} \text{oxidative cleavages s}^{-1} | K_{\text{m}}^{\text{app}} \mu M | k_{\text{cat}}^{\text{app}}/K_{\text{m}}^{\text{app}} \text{s}^{-1} |
|---------------------|-------------------------------|---------------------------------|-----------------|-----------------|
| Ascorbic acid       | 0.53 ± 0.05                   | 3.2 ± 0.3                       | 2.0 ± 0.9       | 1,600,000 ± 700,000 |
| Gallic acid         | 0.30 ± 0.01                   | 1.8 ± 0.03                      | 86 ± 7          | 21,000 ± 1,500   |
| Methylhydroquinone  | 0.38 ± 0.02                   | 2.3 ± 0.1                       | 700 ± 100       | 3,300 ± 400      |
| Gallic acid\*       | 0.20 ± 0.01                   | 1.2 ± 0.1                       | 180 ± 30        | 6,700 ± 1,000    |

* Results obtained using 100 \mu M H_{2}O_{2}; in all other cases, the initial H_{2}O_{2} load was 20 \mu M.

Figure 6. Theoretical analysis of the reduction/oxidation of LPMO in the absence of polysaccharide substrate. A, in the scheme, E represents LPMO, and Cu(I) and Cu(II) denote the oxidation state of the active-site copper. The reductant (\(R\)) and oxidants (O_{2} and H_{2}O_{2}) are allowed to bind only to the Cu(I) and Cu(II) forms of LPMO, respectively. The routes shown in gray are not included in the analysis. These include (i) formation of H_{2}O_{2} that is subsequent to reduction of Cu(I) by the LPMO and (ii) oxidation of LPMO by H_{2}O_{2} that leads to the irreversible inactivation (i.e., \(pK_{\text{Cu}}^{\text{H}_{2}O_{2}}\)), where \(p\) is the probability that LPMO is inactivated in the reaction with H_{2}O_{2}. The equations show the dependence of the Cu(II) form of the LPMO and the parameters of Equation 2 (i.e., the apparent catalytic constant for chitin oxidation (\(k_{\text{cat}}^{\text{app}}\)) and the apparent half-saturating concentration of the reductant (\(K_{\text{red}}^{\text{app}}\)) on the concentration of O_{2} and H_{2}O_{2}. The steady-state equations were derived using the assumption that only the Cu(I) form of LPMO is catalytically active. The binding of Cu(I) to LPMO forms a Cu(I):LPMO complex (\[Cu(I):LPMO\]), (i.e., \(\mathcal{E}\)), whereas the reductant \(R\), oxidants \(O_{2}\), and \(H_{2}O_{2}\) (in Equation 3) are equal (0.02 s^{-1}). The values of second-order rate constants (\(k_{\text{red}}\) and \(k_{\text{red}}^{\text{app}}\)) were calculated using the equations shown in the figure. In all calculations, the values of first-order dissociation rate constants were arbitrarily set to 100 s^{-1} for \(k_{\text{red}}\) and \(k_{\text{red}}^{\text{app}}\) and to 10^3 s^{-1} for \(k_{\text{red}}\). These results in the values of 1, 0.1, and 0.1 mM for \(K_{\text{red}}^{\text{app}}\), \(K_{\text{red}}\), and \(K_{\text{red}}^{\text{app}}\), respectively. The concentrations of O_{2} and H_{2}O_{2} were set to 0.2 and 0.02 mM, respectively. In B, the value of the catalytic rate constant for reduction (\(k_{\text{red}}^{\text{app}}\)) was varied as shown in the plot, and the catalytic rate constants for oxidation by O_{2} (\(K_{\text{red}}^{O_{2}}\)) and H_{2}O_{2} (\(K_{\text{red}}^{H_{2}O_{2}}\)) were set to 0.01 and 0.1 s^{-1}, respectively. The latter implies that the rate of reoxidation of the reduced LPMO ([ECu(I)]_\text{red}) is 10-fold faster compared with reduction by \(R\). In calculations for C-E, the \(k_{\text{red}}\) was kept at 1 mM, and \(k_{\text{red}}^{\text{app}}\) was varied between 0.01 and 10 s^{-1}. \(k_{\text{cat}}^{\text{app}}/K_{\text{m}}^{\text{app}}\) represents the maximum value of \(k_{\text{cat}}^{\text{app}}\) (i.e., it reflects the hypothetical situation when all enzyme is in its active form). \([ECu(I)]_\text{red} = E_{\text{red}}\). For the calculations in C and E, the value of \(k_{\text{cat}}^{\text{app}}/K_{\text{m}}^{\text{app}}\) was set to 10 s^{-1}. and 0.1 mM AsCA as reductant. As a control experiment, we measured the time curves of the degradation of CNWs at different concentrations of SmLPMO10A (Fig. 8A).

The time curves for reactions with lower concentration of SmLPMO10A (below 25 nM) leveled off below the theoretical \([\text{NAG eq}]^{\text{max}}\) value (80 ± 6 \mu M) that can be released in experiments with 20 \mu M H_{2}O_{2}. As pointed out before, the \([\text{NAG eq}]^{\text{max}}\) value is a product of two competing processes, catalysis and inactivation. The inactivation is first-order with enzyme, and changing the concentration of SmLPMO10A has no influence on its half-life. At the same time, the rate of chitin oxidation is linear with the concentration of SmLPMO10A. Therefore, it is likely that at low concentration of SmLPMO10A, the enzyme is inactivated before it can perform enough oxidative cleavages to reach the theoretical \([\text{NAG eq}]^{\text{max}}\) value. Fig. 8B shows a linear correlation between the initial rate of the LPMO reaction and the total concentration of SmLPMO10A. Of note, such a linear relationship has never been reported for O_{2}-driven reactions, in line with the notion that in such reactions, other factors, such as H_{2}O_{2} generation, are rate-deter-
mining. Besides providing the control for the linearity between the initial rate and the enzyme concentration, this result indicates that the binding sites on CNWs were in excess at all enzyme concentrations used (up to 84 nM). The latter condition is a prerequisite for the applicability of steady-state kinetic models, but it is not often easy to achieve in the case of heterogeneous interfacial catalysis (46).

The time curves for the formation of NAGeq by nonbound SmLPMO10A present in the supernatants of binding experiments are shown in Fig. S4. Analysis of the dependence of the ratio of initial rates measured in the presence (\(v_{\text{CNW}}\)) and absence (\(v_{\text{CNW} = 0}\)) of chitin in the binding experiment on the concentration of CNWs in binding experiments (Fig. 8C) according to Equation 4 results in a half-saturating concentration for CNWs of 3.0 ± 0.7 mg ml\(^{-1}\). This concentration is 4.5-fold higher than the corresponding concentration measured in the presence of 0.1 mM AsCA (25), which is well in line with the recent results by Kracher et al. (45), who demonstrated that the binding of the Cu(I) form of *Neurospora crassa* LPMO9C to cellulose is stronger compared with binding of the Cu(II) form. This latter study showed that reduction of *N. crassa* LPMO9C increased both the binding strength and binding capacity but that the effect was strongest (4.7-fold increase) at the level of the partition coefficient (initial slope of the Langmuir binding isotherm) (45). Because here we measured LPMO binding in conditions satisfying an excess of binding sites, the effects of reductant on binding (4.5-fold increase) reflect mainly effects on the partition coefficient of the Langmuir binding isotherm. The fact that similar results were obtained with *Neurospora crassa* LPMO9C and SmLPMO10A may be taken to suggest that the effects of the redox state of active site copper on binding of LPMOs to their polysaccharide substrate may be similar for LPMOs belonging to different families, but may also be coincidental because the two LPMOs have different substrates.

**Discussion**

Recent findings showing that H\(_2\)O\(_2\) acts as a cosubstrate of LPMOs (22, 23, 25, 26) call for detailed kinetic studies of H\(_2\)O\(_2\)-driven degradation of polysaccharides. Such studies are complicated by the low \(K_m\) of LPMOs for H\(_2\)O\(_2\), which necessitates the use of H\(_2\)O\(_2\) at low micromolar concentrations. The problem is further amplified by the requirement for the priming reduction of the LPMO, because reducing agents are sensitive toward oxidation by O\(_2\), especially in the presence of metal ions. Importantly, H\(_2\)O\(_2\) is often a product of these oxidations. Oxidation of reducing agents by H\(_2\)O\(_2\) is also plausible. Finally, enzyme inactivation is commonly observed for LPMOs, in both O\(_2\)- and H\(_2\)O\(_2\)-driven reactions (4, 23, 25).

Here, we occasionally encountered significant activity of SmLPMO10A in experiments without added H\(_2\)O\(_2\). This background activity depended on the nature of the reductant and showed strong variation between different working batches of chitin (Fig. 1A). Numerous control experiments (Fig. 1B) suggested that the background activity is related to generation of H\(_2\)O\(_2\) in the divalent metal ion-catalyzed oxidation of the reductant. Therefore, the addition of copper salts to reaction mixtures likely has effects beyond supplementing the LPMO with catalytically essential copper, and great care must be taken when doing so (47).

Regarding our studies on background activities, the effect of catalase is worth a short discussion. In some studies, it has been noticed that the addition of catalase improves the efficiency of LPMO-containing enzyme mixtures, which was ascribed to the potentially damaging effect of radical formation when H\(_2\)O\(_2\) reacts with transition metals in the reaction mixture (48). For a similar reason, catalase is added in experimental set-ups where LPMO reactions are supplied with excess copper (49). Considering the now well-established beneficial effect of H\(_2\)O\(_2\) on LPMO activity (22, 23, 25, 26), one might expect that catalase inhibits LPMO activity in such enzyme mixtures, which is not compatible with the observed overall beneficial effect of catalase (48, 50). Moreover, neither Bissaro et al. (23) nor Möllers et al. (51) observed an effect of catalase on LPMO activity, except in situations with very high H\(_2\)O\(_2\) concentrations (22). Bearing in mind the high affinity of LPMOs for H\(_2\)O\(_2\) (23), being as low as 2.8 \(\mu\)M for SmLPMO10A (25), and the very low affinity of catalase to H\(_2\)O\(_2\) (52, 53), special care must be taken in dosing catalase and interpreting potential effects. The \(K_m\) for H\(_2\)O\(_2\) of the bovine liver catalase used in this study is around 100 \(\mu\)M (52). Based on the rate of NAGeq formation in the background reaction (0.36 \(\mu\)M NAGeq min\(^{-1}\); Fig. 1B) and the values of
kinetic parameters of SmLPMO10A published before (25), one can estimate a steady-state concentration of 0.025 μM for H₂O₂ in the experiment with 0.1 mM AscA. As defined by the commercial suppliers, the unit of catalase activity (μmol of H₂O₂ min⁻¹) is measured at an H₂O₂ concentration around 10 mM. Therefore, the activity of catalase in our experiment is expected to be 10,000 μmol/0.025 μM = 4 × 10⁶-fold lower than the activity that could be estimated from the information provided by the supplier. This may be the reason why an apparently huge dose of catalase (200 units ml⁻¹) did not completely inhibit the background activity of SmLPMO10A (Fig. 1B).

As pointed out before, the optimal harnessing of the catalytic potential of LPMO takes an experimental set-up with controlled in situ production (e.g. using oxidases) or continuous external supply of H₂O₂ (23, 25, 54). Provided that enzyme inactivation is avoided, the effect of reductant in these set-ups is expected to be similar to the effects of reductant on initial rates measured here (Fig. 5 and Table 1). Note that whereas the necessary reductants under some conditions may contribute to H₂O₂ generation, in the present study, conditions were such that the observed efficiency of reductants reflects the efficiency in priming of the LPMO. The stoichiometry of 18 ± 5 oxidative cleavages made per molecule of reductant, as measured in reactions with low concentrations of AscA (Fig. 3), supports the use of reductant in priming reduction and not as substrate in our experiments. This result is in accordance with conclusions drawn from reactions in which cellulose was degraded by an LPMO-containing commercial cellulase mixture, which showed that, when controlling the supply of H₂O₂, ~200 μM AscA was needed to produce ~3000 μM oxidized products, leading to an approximate stoichiometry of 15 (23).

In accordance with previous studies (10, 12), the present data show that different reductants have very different efficiencies (k_{app}/K_{mR}) in supporting SmLPMO10A catalysis. Although different reductants have somewhat different k_{app} values, we show that the differences in efficiency primarily relate to largely different apparent half-saturating concentration (K_{app}) (Table 1), which again is strongly correlated with the rate constant for LPMO reduction (k_{red}) (Fig. 6D; k_{app} decreases sharply as k_{red} increases). Of note, measuring the true electron transfer efficiency of the reductant (i.e. the values of k_{red} and K_{mR}) implies measurements of electron transfer in the pre-steady state regime. Stopped-flow experiments with cellulose dehydrogenase indicate a second-order rate constant (cf. k_{red}/K_{mR}) of 6.4 × 10⁵ M⁻¹ s⁻¹ for electron transfer between SmLPMO10A and the cellulose dehydrogenase of Myriococcum thermophilum (4).

The different efficiencies of the reductants are likely related to their varying midpoint potentials, as demonstrated in an earlier study by Kracher et al. (10). Kracher et al. (10) showed that MHQ is a moderately effective reductant, which coincides with its high k_{app} (and, thus, low k_{red}) value reported here. MHQ undergoes two successive one-electron and one-proton transfers, leading to the semiquinone and quinone form, with redox potentials of 23 and 460 mV versus NHE, respectively. The standard potential for the direct transfer of two electrons and two protons to reduce the quinone to MHQ is 230 mV versus NHE (55). SmLPMO10A has a midpoint potential of 275 mV versus NHE (42). Ascorbic acid and gallic acid, two of the most commonly used reductants in LPMO research, are oxidized reversibly (56, 57), which prevents the determination of standard midpoint potentials, and which, as such, may affect their efficiency. Comparative studies of oxygen reduction by Kracher et al. (10) indicated that ascorbic acid is a much better reductant than gallic acid and MHQ, which is compatible with the differences observed here.

From the analysis of the reaction scheme in Fig. 6, it follows that there is a linear relationship between the value of K_{cat}/k_{mR} for chitin degradation and the value of the efficiency constant for LPMO priming by the reductant (k_{red}/K_{mR}) according to Equation 3.

\[
\frac{k_{app}}{K_{mR}} = \frac{k_{app}}{k_{cat,max}} + \frac{[O_2]}{K_{O_2}} + \frac{[H_2O_2]}{K_{H_2O_2}}
\]

(Eq 3)

The rate constants and binding constants of Equation 3 are defined in the legend to Fig. 6. To what extent the value of k_{app}/K_{mR} (Table 1) reflects the value of (k_{red}/K_{mR}) depends on the rates of LPMO reoxidation (i.e. the terms in the denominator of Equation 3; note that these are all terms referring to a situation without bound substrate, which is the most common, if not the only, situation in which reduction occurs). To decide which route, O₂- or H₂O₂-driven, governs LPMO reoxidation,
one needs numerical estimates for the corresponding terms in the denominator of Equation 3. The $K_{\text{H}_2\text{O}_2}$ in this denominator reflects the binding of $\text{H}_2\text{O}_2$ to free reduced LPMO (Fig. 6A). This binding mode has been proposed to be responsible for the inactivation of $\text{Sm}LPMO10A$ and has low affinity ($K_{\text{H}_2\text{O}_2} > 100 \mu\text{M}$ as estimated from the data in Ref. 25). The $k_{\text{cat}\text{H}_2\text{O}_2}$ is the catalytic constant for the oxidation of ECu(I) by $\text{H}_2\text{O}_2$. This $\text{H}_2\text{O}_2$-dependent oxidative route can lead to irreversible inactivation of LPMO but also to reoxidation of LPMO to its ECu(II) form without inactivation (as in Fig. 6A). The second-order rate constant for inactivation of $\text{Sm}LPMO10A$ is on the order of $10^3 \text{M}^{-1} \text{s}^{-1}$ (25). Using this number, one can estimate the rate of reoxidation ($k_{\text{redox}\text{H}_2\text{O}_2}$) of $\text{Sm}LPMO10A$ to be around $0.02 \text{s}^{-1}$ ($\text{H}_2\text{O}_2$ at $20 \mu\text{M}$). Note that the true reoxidation rate by $\text{H}_2\text{O}_2$ is expected to be $1/p_i$-fold higher, where $p_i$ is the probability that enzyme is inactivated upon reacting with $\text{H}_2\text{O}_2$ in the absence of chitin (Fig. 6A). The rate of $\text{O}_2$-mediated reoxidation of $\text{Sm}LPMO10A$ is not known, but similar initial rates measured under normal air and anaerobic conditions (Fig. 7) suggest that under the conditions used in our study, reoxidation is governed by $\text{H}_2\text{O}_2$. Regarding other LPMOs, the data for the rate of $\text{O}_2$-mediated reoxidation of nonsubstrate-bound LPMOs vary a lot. Some studies support rate constants below $1 \text{min}^{-1}$ (10, 31, 34, 58), whereas others support values above $10 \text{min}^{-1}$ (59, 60). Recently, Breslmayr et al. (61) showed that the oxidation of 2,6-dimethoxy phenol by $N$. crassa NcLPMO9C is driven by $\text{H}_2\text{O}_2$ and does not depend on the presence of $\text{O}_2$, suggesting that in the absence of a polysaccharide substrate, $\text{O}_2$-driven reoxidation of the LPMO is much slower than $\text{H}_2\text{O}_2$-driven reoxidation, although this of course would depend on the concentrations of the two co-substrates. More studies are needed to make general conclusions about the contribution of possible oxidase/peroxidase reactions in reoxidation of substrate-free LPMOs.

The simplest mechanism of $\text{H}_2\text{O}_2$-driven degradation of CNWs by $\text{Sm}LPMO10A$ that can account for the observations and considerations described here is depicted in Fig. 9A. For simplicity, binding of the Cu(II) form of the LPMO to chitin was omitted. This simplification is, at least to some extent, justified by the experimentally observed weaker binding of the Cu(II) form of $\text{Sm}LPMO10A$ compared with the Cu(I) form (Fig. 8C and data in Ref. 25). Weaker binding of the Cu(II) form is also supported by computational studies of $\text{Sm}LPMO10A$ (44). It has been proposed that $\text{H}_2\text{O}_2$-driven catalysis by $\text{Sm}LPMO10A$ follows a compulsory order ternary complex mechanism, with chitin being the first substrate to bind to the reduced enzyme (25, 44). Simulations suggest that when $\text{Sm}LPMO10A$ is bound to chitin, a channel connecting the bulk solvent to the active site would regulate access of reagents to the latter (44). These considerations imply that the binding modes of $\text{H}_2\text{O}_2$ to free $\text{Sm}LPMO10A$ and to the complex of $\text{Sm}LPMO10A$ with CNWs are different. Therefore, binding of the LPMOCu(I)–$\text{H}_2\text{O}_2$ complex to CNWs is omitted in the scheme of Fig. 9A. Because the $\text{O}_2$-driven mechanism has been shown to follow a random-order mechanism (60) we do not make a similar assumption for binding of $\text{O}_2$ (Fig. 9A). Because in the experimental conditions used to determine the initial rates, the oxidation of chitin without external $\text{H}_2\text{O}_2$ supply was not significant, the possible $\text{O}_2$-driven route of chitin oxidation is omitted in the scheme of Fig. 9A.

The steady-state solution for the mechanism in Fig. 9A was found using the King–Altman procedure (62) and analyzed numerically. In general, the numerical solutions for the dependence of chitin degradation on the efficiency of reductant (Fig. 9) are in qualitative accordance with the analytical solutions found using the scheme that does not consider chitin (Fig. 6). The dependence of initial rates on the concentration of reductant followed Michaelis–Menten saturation kinetics regardless of the $k_{\text{cat}}$ (Fig. 9B), similar to the dependence of the concentration of the ECu(I) form of LPMO on the concentration of reductant found by analytical solutions to the scheme without CNWs (Fig. 6B). The $k_{\text{app}}$ increased and the $k_{\text{pp}}$ decreased with increasing efficiency of reduction (data not shown), similarly to predictions derived from the model without explicit inclusion of chitin (Fig. 6, C and D). Both reaction schemes yield a linear dependence of $k_{\text{app}}^{\text{red}}/k_{\text{pp}}^{\text{red}}$ on the true efficiency of reductant $(k_{\text{red}}/K_{\text{mk}})$, but the slope found in the scheme with the explicit presence of chitin (Fig. 9A) was higher than that predicted in the absence of chitin (Fig. 9C). This can be accounted for by the reduced rate of LPMO reoxidation in the presence of chitin, as only the reoxidation of chitin-free enzyme is considered in Fig. 9A. Numerical solutions to the scheme in Fig. 9A also predicted inhibition by $\text{H}_2\text{O}_2$ (Fig. 9D), as indeed observed when using GA as reductant (Fig. 5B). Of note, this hitherto nonobserved phenomenon refers to true inhibition, reflected in reduced initial rates, and should not be confused with irreversible inactivation caused by $\text{H}_2\text{O}_2$.

Regarding the application of LPMOs, it is important to note that the requirements for the reductant (e.g., its half-saturating concentration) in supporting polysaccharide degradation depend not only on the catalytic efficiency of the reductant $(k_{\text{red}}/K_{\text{mk}})$ (Figs. 6 (C–E) and 9C) but also on the rate of LPMO reoxidation (Fig. 9, F–G). The increase of $k_{\text{pp}}^{\text{mk}}$ (Fig. 9F) and decrease of $k_{\text{cat}}^{\text{pp}}$ (Fig. 9E) with increasing rate of LPMO reoxidation is caused by the competition between the possible polysaccharide monooxygenase/peroxygenase and reductant oxidase/peroxidase activities of the LPMO. Increasing the polysaccharide concentration favors the monooxygenase/peroxygenase reactions, at the expense of the oxidase/peroxidase reactions that take place with free LPMOs, and this is reflected in decreasing $k_{\text{pp}}^{\text{mk}}$ (Fig. 9F) and increasing $k_{\text{cat}}^{\text{pp}}$ (Fig. 9E). Because the irreversible inactivation of LPMO is also related to the reoxidation of free LPMO by $\text{H}_2\text{O}_2$ (23, 25, 26), the high dry matter conditions used in industrial scale degradation of lignocellulosic biomass (63) should reduce the needed amounts of the reductant for the priming of LPMO as well as reduce the inactivation of LPMO. Obviously, careful control of the amount of available $\text{H}_2\text{O}_2$ is also of importance, because this will avoid unproductive oxidation of the reductant as well as LPMO inactivation (54).

Regarding the natural environment of LPMOs and potential sources of $\text{H}_2\text{O}_2$, a plethora of $\text{H}_2\text{O}_2$ suppliers as potential candidate partner enzymes have been identified for fungal LPMOs involved in lignocellulose conversion, although most connections remain to be clearly established (24). In the case of bacte-
Figure 9. Theoretical analysis of the effects of reductant on H₂O₂–driven degradation of polysaccharides by an LPMO. A, the reaction scheme is constructed by adding the chitin (CNW) to the scheme shown in Fig. 6A. The routes shown with gray are not included in the analyses. Numerical solutions for the steady-state are shown in B–G. If not stated otherwise, the values of k₁, k₂, k₃, k₄, and k₅ were set to 10⁸ m⁻¹ s⁻¹. The values of k₆, k₇, and k₈ were set to 100 s⁻¹, whereas the value of k₉ was 10³ s⁻¹. Therefore, the k₆, k₇, and k₈ (for definitions, see the legend to Fig. 6) had values of 1, 0.1, and 0.1 mm, respectively. The binding constants for CNWs were adjusted to 0.5 mg ml⁻¹ by setting k₂ = k₆ = 100 mm mg⁻¹ s⁻¹ and k₇ = k₈ = 50 s⁻¹. For the dissociation of H₂O₂ from its Cu(I)–CNW complex, the value of k₋₁ was set to 2 s⁻¹. The value of k₆ was set to 10 s⁻¹. Concentrations of O₂, H₂O₂, and CNWs were set to 0.2 mM, 0.02 mM, and 1.0 mg ml⁻¹, respectively. B, dependence of the initial rate of NAGeq formation (v) on the concentration of reductant at different k₆ values as shown in the plot. The concentration of enzyme was set to 0.05 mm, and four NAGeq are released per molecule of H₂O₂. The values of k₆ and k₈ were set to 0.01 and 0.1 s⁻¹, respectively (i.e. the rates of reoxidation of LPMO by O₂ and H₂O₂ are both 0.02 s⁻¹). The v, based on the scheme with inclusion of chitin (CNW+i) was found from numerical solution of the scheme in A, whereas the v, based on the scheme without explicit inclusion of chitin (CNW−) was calculated from the dependence of the concentration of the Cu(I) form of LPMO on the concentration of the reductant, as shown in Fig. 6B. C, dependence of k₆ on [Cu(I)–CNW] for chiton oxidation on the efficiency of reductant (k₆/k₅) based on reaction schemes with (CNW+i; Fig. 9A) or without (CNW−; Fig. 6A and Equation 3) the inclusion of chitin. Obviously, there is no LPMO activity without chitin, but the calculations without chitin were included as they represent a prediction of what will happen in the situation where chitin does not influence the steady-state (ECu[I]). In the calculations, the K₆ was kept at 1 mm, and k₆ was varied between 0.01 and 0.1 s⁻¹. As in B, the rates of LPMO reoxidation by O₂ and H₂O₂ were both set to 0.02 s⁻¹. D, dependence of initial rates of NAGeq formation (v) on the concentration of reductant at different concentrations of H₂O₂ (as shown in the plot). The concentration of enzyme was set to 0.05 mm, and four NAGeq are released per molecule of H₂O₂. The values of k₆ and k₈ were set to 0.01 and 0.1 s⁻¹, respectively. The binding strength of chitin was varied between 0.01 and 10 s⁻¹. E, dependence of the initial rate of NAGeq formation (v) on the concentration of reductant at different concentrations of H₂O₂ (as shown in the plot). The concentration of enzyme was set to 0.05 mm, and four NAGeq are released per molecule of H₂O₂. The values of k₆ and k₈ were set to 0.01 and 0.1 s⁻¹, respectively. The binding strength of chitin was varied between 0.01 and 10 s⁻¹. F, dependence of the initial rate of NAGeq formation (v) on the concentration of reductant at different concentrations of H₂O₂ (as shown in the plot). The concentration of enzyme was set to 0.05 mm, and four NAGeq are released per molecule of H₂O₂. The values of k₆ and k₈ were set to 0.01 and 0.1 s⁻¹, respectively. The binding strength of chitin was varied between 0.01 and 10 s⁻¹. G, dependence of initial rates of NAGeq formation (v) on the concentration of reductant at different concentrations of H₂O₂ (as shown in the plot). The concentration of enzyme was set to 0.05 mm, and four NAGeq are released per molecule of H₂O₂. The values of k₆ and k₈ were set to 0.01 and 0.1 s⁻¹, respectively. The binding strength of chitin was varied between 0.01 and 10 s⁻¹. The effect of k₆ on apparent parameters of the reductant was assessed using three different scenarios: (i) k₆ = 0 (mimicked by setting k₆ = 0), and (ii) k₆ = 0 (mimicked by setting [O₂] = 0). All scenarios were analyzed at two concentrations of CNWs, 1.0 mg ml⁻¹ (solid lines) and 10 mg ml⁻¹ (dashed lines). Note that the results of the first two scenarios (blue and green lines) overlap. Note also that the scale for k₆/k₅ is logarithmic.

Effects of the reductant on the kinetics of LPMO catalysis

Experimental procedures

Substrates and enzymes

¹⁴C-Labeled CNWs were prepared from α-chitin of crab shells (Sigma C7170) using N-acetylation of free amino groups with 1.¹⁴C acetic anhydride, as described by Kuusk et al. (64). The specific radioactivity of the CNW preparation was 4.18 × 10⁶ dpm mg⁻¹. A mother stock suspension of CNWs (8.5 mg ml⁻¹) was kept at 4 °C in 50 mM NaAc buffer pH 6.1 (0.01% NaN₃). The batches of CNWs were prepared from the mother stock by washing out the NaN₃ through repetitive centrifuga-

rial LPMOs, even less is known because no obvious, conserved redox partner, like cellulobiose dehydrogenase in fungi, has been identified yet. As to the matter of the availability of H₂O₂, reported concentrations in natural ecosystems span several orders of magnitude, but existing data need to be interpreted with caution because H₂O₂ concentrations measured in vivo may reflect the result of multiple, and sometimes unknown, production and consumption fluxes (24). Of note, the very low Km values for H₂O₂ (2.8 μM for SnLPMO10A) indicate that H₂O₂-driven LPMO action should be compatible with most forms of microbial life.

In summary, the optimal concentration and the performance of the reductant depend on the relative activities of the different reactions catalyzed by a particular LPMO, but also on process conditions like the concentrations of O₂, H₂O₂, and the polysaccharide substrate.
Effects of the reductant on the kinetics of LPMO catalysis

tion (5 min, 2,900 × g) and resuspension (in 50 mM NaAc buffer, pH 6.1) steps. Although the water was Milli-Q ultrapure water (>18.2 megohms cm⁻¹), we encountered variation in the background activity between working batches of CNWs when AscA was used as reductant, as described under "Results." Apart from the experiments shown in Fig. 1, only the working batches of CNWs with low background activity were used in the experiments with AscA as reductant. There were no differences in background activities between working batches of CNWs when GA or MHQ were used as reductants, and the results with these reductants represent results obtained using different working batches of CNWs. Nonlabeled CNWs used in the binding experiment were prepared from these reductants except ASCA, which was used as described previously (64). Purified SmLPMO10A was saturated with copper by incubating with CuSO₄ and following removal of unbound copper by ultracentrifugation exactly as described by Kuusk et al. (25). The concentration of SmLPMO10A was determined by absorbance at 280 nm using a theoretical molar extinction coefficient of 35,200 M⁻¹ cm⁻¹. Catalase from bovine liver (Sigma, C9322) and peroxidase from horseradish (Sigma, P8375) were used as purchased.

Degradation of CNWs by SmLPMO10A

Reactions were prepared essentially as described by Kuusk et al. (25). All reactions were performed in 1.5-ml polypropylene microcentrifuge tubes in NaAc buffer (50 mM, pH 6.1) at 25 °C without stirring, although the suspension was mixed by pipetting before withdrawing samples, at each sampling time point. If not stated otherwise, the reaction mixture contained ¹⁴C-labeled CNW (1.0 mg ml⁻¹), SmLPMO10A (42 nM), H₂O₂, and reductant. The total volume of the reactions was 0.8 ml. SmLPMO10A was added to CNWs, and after 5–10 min of incubation, the reaction was initiated by the addition of AscA, the reaction was diluted 2-fold, meaning that the maximal (i.e. no binding of SmLPMO10A to nonlabeled CNWs) total concentration of SmLPMO10A in the activity measurement was 42 nM. The control experiment ([CNW] = 0 mg ml⁻¹) was undertaken exactly as described above but without the nonlabeled CNWs in the binding experiment. The results of the binding experiments were fitted to Equation 4.

In Equation 4, v_{CNW} and v_{CNW = 0} are the initial rates of CNW degradation by the supernatants from the binding experiment made in the presence and absence of CNWs, respectively. [CNW] is the concentration of CNWs in the binding experiment, and K_{i(CNW)} is the half-saturating concentration of CNWs at which 50% of the SmLPMO10A molecules are bound to CNWs and 50% are free in the solution.
Author contributions—P. V. conceived and coordinated the study. S. K., R. K., and P. V. designed, performed, and analyzed the experiments, interpreted data, and wrote the paper. A. H. assisted with the experiments. P. K. derived the rate equations. B. B., M. S., and V. G. H. E. interpreted data and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

References

1. Meier, K. K., Jones, S. M., Kaper, T., Hansson, H., Koetsier, M. J., Karkehabadi, S., Solomon, E. I., Sandgren, M., and Keleman, B. (2018) Oxygen activation by Cu LPMOs in recalcitrant carbohydrate polysaccharide conversion to monomer sugars. Chem. Rev. 118, 2593–2635 CrossRef Medline

2. Vaaje-Kolstad, G., Horn, S. J., van Aalten, D. M. F., Synstad, B., and Eijsink, V. G. H. (2005) The non-catalytic chitin-binding protein CBP21 from Serratia marcescens is essential for chitin degradation. J. Biol. Chem. 280, 28492–28497 CrossRef Medline

3. Vaaje-Kolstad, G., Westereng, B., Horn, S. J., Liu, Z., Zhai, H., Sorlie, M., and Eijsink, V. G. H. (2016) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 350, 219–222 CrossRef Medline

4. Loose, J. S. M., Forsberg, Z., Kracher, D., Scheiblbrandner, S., Ludwig, R., Eijsink, V. G. H., and Vaaje-Kolstad, G. (2016). Activation of bacterial lytic polysaccharide monooxygenases with cellulbiose dehydrogenase. Protein Sci. 25, 2175–2186 CrossRef Medline

5. Loose, J. S. M., Arntzen, M. Ø., Bissaro, B., Ludwig, R., Eijsink, V. G. H., and Vaaje-Kolstad, G. (2018) Multi-point precision binding of substrate proteolysis of LPMOs from self-destructive off-pathway processes. Biochemistry 57, 4114–4124 CrossRef Medline

6. Frommhagen, M., Westphal, A. H., van Berkel, W. J. H., and Kabel, M. A. (2018) Distinct substrate specificity and electron-donating systems of fungal lytic polysaccharide monooxygenases. Front. Microbiol. 9, 1080 CrossRef Medline

7. Harris, P. V., Welner, D., McFarland, K. C., Re, E., Navarro Poulsen, J.-C., Brown, K., Salbo, R., Ding, H., Vlasenko, E., Merino, S., Xu, F., Cherry, J., Larsen, S., and Lo Leggio, L. (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49, 3305–3316 CrossRef Medline

8. Quinlan, R. J., Sweeney, M. D., Lo Leggio, L., Otten, H., Poulsen, J.-C. N., Johansen, K. S., Krogh, K. B. R. M., Jorgensen, C. I., Tovborg, M., Anthonsen, A., Tryfona, T., Walter, C. P., Dupree, P., Xu, F., Davies, G. J., and Walton, P. H. (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc. Natl. Acad. Sci. U.S.A. 108, 15079–15084 CrossRef Medline

9. Hu, J., Arantes, V., Pribowo, A., Gourlay, K., and Saddler, J. N. (2014) Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass. Energy Environ. Sci. 7, 2308–2315 CrossRef Medline

10. Kracher, D., Scheiblbrandner, S., Felice, A. K. G., Breslmayr, E., Preims, M., Ludwigka, K., Haltrich, D., Eijsink, V. G. H., and Ludwig, R. (2016) Extracellular electron transfer systems fuel cellulose oxidative degradation. Science 352, 1098–1101 CrossRef Medline

11. Bulakhov, A. G., Gusakov, A. V., Chekushina, A. V., Satruditinov, A. D., Koshcheev, A. V., Matys, V. Y., and Sinitsyn, A. P. (2016) Isolation of homogeneous polysaccharide monooxygenases from fungal sources and investigation of their synergism with cellulases when acting on cellulose. Biochemistry (Moscow) 81, 530–537 CrossRef Medline

12. Frommhagen, M., Koetsier, M. J., Westphal, A. H., Visser, J., Hinz, S. W. A., Vincken, J.-P., van Berkel, W. J. H., Kabel, M. A., and Gruppen, H. (2016) Lytic polysaccharide monooxygenases from Myceliophthora ther- mophila C1 differ in substrate preference and reducing agent specificity. Biotechnol. Biofuels 9, 186 CrossRef Medline

13. Frommhagen, M., Mutte, S. K., Westphal, A. H., Koetsier, M. J., Hinz, S. W. A., Visser, J., Vincken, J.-P., Weijers, D., van Berkel, W. J. H., Gruppen, H., and Kabel, M. A. (2017) Boosting LPMO-driven lignocelluloses degradation by polyphenol oxidase-activated lignin building blocks. Biotechnol. Biofuels 10, 121 CrossRef Medline

14. Sabbadin, F., Hemsworth, G. R., Ciano, L., Henriassit, B., Dupree, P., Tryfona, T., Marques, R. D. S., Sweeney, S. T., Besser, K., Elias, L., Pesante, G., Li, Y., Owle, A. A., Bates, R., Gomez, L. D., Simister, R., et al. (2018) An ancient family of lytic polysaccharide monooxygenases with roles in arthropod development and biomass digestion. Nat. Commun. 9, 756 CrossRef Medline

15. Frommhagen, M., Westphal, A. H., Higlers, R., Koetsier, M. J., Hinz, S. W. A., Visser, J., Gruppen, H., van Berkel, W. J. H., and Kabel, M. A. (2018) Quantification of the catalytic performance of C1-cellulose-specific lytic polysaccharide monooxygenases. Appl. Microbiol. Biotechnol. 102, 1281–1295 CrossRef Medline

16. Bremleni, L., Serna, M. J., Falby, C., and Cannella, D. (2018) Laccase- derived lignin compounds boost cellulose oxidative enzymes AA9. Biotechnol. Biofuels 11, 10 CrossRef Medline

17. Westereng, B., Cannella, D., Wütrup Agger, J. W., Jørgensen, H., Larsen Andersen, M., Eijsink, V. G. H., and Falby, C. (2015) Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer. Sci. Rep. 5, 18561 Medline

18. Rodriguez-Zuniga, U. F., Cannella, D., Giordano, R. C., Giordano, R. L. C., Jørgensen, H., and Falby, C. (2015) Lignocellulose pretreatment technologies affect the level of enzymatic cellulose oxidation by LPMO. Green Chem. 17, 2896–2903 CrossRef Medline

19. Muraleedharan, M. N., Zouraris, A., Karantonis, A., Topakas, E., Sandgren, M., Rova, U., Christakopoulos, P., and Karnaouri, A. (2018) Effect of lignin fractions isolated from different biomass sources on cellulose oxidation by fungal lytic polysaccharide monooxygenases. Biotechnol. Biofuels 11, 296 CrossRef Medline

20. Bissaro, B., Rahr Å. K., Skaugen M., Forsberg, Z., Horn, S. J., Vaaje-Kolstad, G., and Eijsink, V. G. H. (2016) Fenton-type chemistry by a copper enzyme: molecular mechanism of polysaccharide oxidative cleavage. bioRxiv CrossRef Medline

21. Bissaro, B., Rahr Å. K., Müllerg, S., Chylenski, P., Skaugen M., Forsberg, Z., Horn, S. J., Vaaje-Kolstad, G., and Eijsink, V. G. H. (2017) Oxidative cleavage of polysaccharides by mononuclear copper enzymes depends on H2O2. Nat. Chem. Biol. 13, 1123–1128 CrossRef Medline

22. Bissaro, B., Vaara, K., Eijsink, V. G. H. (2018) Oxidoreductases and reactive oxygen species in conversion of lignocellulosic biomass. Microbiol. Mol. Biol. Rev. 82, e00029-18 CrossRef Medline

23. Kuusk, S., Bissaro, B., Kuusk, P., Forsberg, Z., Eijsink, V. G. H., Sørli, M., and Valjamae, P. (2018) Kinetics of H2O2-driven degradation of chitin by a bacterial lytic polysaccharide monooxygenase. J. Biol. Chem. 293, 523–531 CrossRef Medline

24. Hangas, J. A., Iavarone, A. T., and Marletta, M. A. (2018) Reactivity of O2 versus H2O2 with polysaccharide monooxygenases. Proc. Natl. Acad. Sci. U.S.A. 115, 4915–4920 CrossRef Medline

25. Bertini, L., Breglia, R., Lamburghi, M., Fantucci, P., De Gioia, L., Borsari, M., Sola, M., Bortolotti, C. A., and Bruschi, M. (2018) Catalytic mechanism of fungal lytic polysaccharide monooxygenases investigated by first-principles calculations. Inorg. Chem. 57, 86–97 CrossRef Medline

26. Wang, B., Johnston, E. M., Li, P., Shaik, S., Davies, G. J., Walton, P. H., and Rovira, C. (2018) QM/MM studies into the H2O2-dependent activity of lytic polysaccharide monooxygenases: evidence for the formation of caged hydroxyl radical intermediate. ACS Catal. 8, 1346–1351 CrossRef Medline

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Effects of the reductant on the kinetics of LPMO catalysis

29. Hedegård, E. D., and Ryde, U. (2018) Molecular mechanism of lytic polysaccharide monoxygenases. Chem. Sci. 9, 3866–3880 CrossRef Medline

30. Walton, P. H., and Davies, G. J. (2016) On the catalytic mechanisms of lytic polysaccharide monoxygenases. Curr. Opin. Chem. Biol. 31, 195–207 CrossRef Medline

31. Kittl, R., Kracher, D., Burgstaller, D., Haltrich, D., and Ludwig, R. (2012) Production of four Neurospora crassa lytic polysaccharide monoxygenases in Pichia pastoris monitored by a fluorimetric assay. Biotechnol. Biofuels 5, 79 CrossRef Medline

32. Holtmann, D., and Hollmann, F. (2016) The oxygen dilemma: a severe challenge for the application of monoxygenases? ChemBioChem 17, 1391–1398 CrossRef Medline

33. Scarpa, M., Stevanato, R., Viglino, P., and Rigo, A. (1983) Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. J. Biol. Chem. 258, 6695–6697 Medline

34. Forsberg, Z., Bissaro, B., Gullese, J., Dalhus, B., Vaaaje-Kolstad, G., and Ejsink, V. G. H. (2018) Structural determinants of bacterial lytic polysaccharide monoxygenase function. J. Biol. Chem. 293, 1397–1412 CrossRef Medline

35. Bradshaw, M. P., Barril, C., Clark, A. C., Prenzler, P. D., and Scollary, G. R. (2011) Ascorbic acid: a review of its chemistry and reactivity in relation to a wine environment. Crit. Rev. Food Sci. Nutr. 51, 479–498 CrossRef Medline

36. Khan, M. M. T., and Martell, A. E. (1967) Metal ion and chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I. Cupric and ferric ion catalyzed oxidation. J. Am. Chem. Soc. 89, 4176–4185 CrossRef Medline

37. Bootright, W. L. (2016) Oxygen dependency of one-electron reactions generating ascorbate radicals and hydrogen peroxide from ascorbic acid. Food Chem. 196, 1361–1367 CrossRef Medline

38. Fränzle, S. (2015) Adsorption to chitin—a viable and organism-protecting method for biomonitoring metals present in different environmental compartments getting contacted with arthropods. Ann. Bot. 5, 79–87

39. Camci-Unal, G., and Pohl, N. L. B. (2010) Quantitative determination of heavy metal contaminant complexation by the carbohydrate polymer chitin. J. Chem. Eng. Data 55, 1117–1121 CrossRef Medline

40. Karlsen, V., Heggeset, E. B., and Sørlie, M. (2010) The use of isothermal titration calorimetry to determine the thermodynamics of metal ion binding to low-cost sorbents. Thermochim. Acta 501, 119–121 CrossRef Medline

41. Khan, M. M. T., and Martell, A. E. (1967) Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. II. Cupric and ferric chelate catalyzed oxidation. J. Am. Chem. Soc. 89, 7104–7111 CrossRef Medline

42. Achmann, F. L., Sørlie, M., Skjåk-Brak, G., Ejsink, V. G. H., and Vaaaje-Kolstad, G. (2012) NMR structure of a lytic polysaccharide monoxygenase provides insight into copper binding, protein dynamics, and substrate interactions. Proc. Natl. Acad. Sci. U.S.A. 109, 18779–18784 CrossRef Medline

43. Chaplin, A. K., Wilson, M. T., Hough, M. A., Svistunenko, D. A., Hemsworth, G. R., Walton, P. H., Vigenboom, E., and Worral, J. A. R. (2016) Heterogeneity in the histidine-brace copper coordination sphere in auxiliary activity family 10 (AA10) lytic polysaccharide monoxygenases. J. Biol. Chem. 291, 12838–12850 CrossRef Medline

44. Bissaro, B., Isaksen, L., Vaaaje-Kolstad, G., Ejsink, V. G. H., and Rørh Å. K. (2018) How a lytic polysaccharide monoxygenase binds crystalline chitin. Biochemistry 57, 1893–1906 CrossRef Medline

45. Kracher, D., Andlar, M., Furtmüller, P. G., and Ludwig, R. (2018) Activesite copper reduction promotes substrate binding of fungal lytic polysaccharide monoxygenase and reduces stability. J. Biol. Chem. 293, 1676–1687 CrossRef Medline

46. Kari, J., Andersen, M., Borch, K., and Westh, P. (2017) An inverse Michaelis-Menten approach for interfacial enzyme kinetics. ACS Catal. 7, 4904–4914 CrossRef

47. Loose, J. S. M., Forsberg, Z., Fraaije, M. W., Ejsink, V. G. H., and Vaaaje-Kolstad, G. (2014) A rapid quantitative activity assay shows that the Vibrio cholerae colonization factor GbpA is an active lytic polysaccharide monoxygenase. FEBS Lett. 588, 3435–3440 CrossRef Medline