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Recent insights into copper-containing *lytic polysaccharide mono-oxygenases*

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Recently the role of oxidative enzymes in the degradation of polysaccharides by saprophytic bacteria and fungi was uncovered, challenging the classical model of polysaccharide degradation of being solely via a hydrolytic pathway. 3D structural analyses of *lytic polysaccharide mono-oxygenases* of both bacterial AA10 (formerly CBM33) and fungal AA9 (formerly GH61) enzymes revealed structures with β-sandwich folds containing an active site with a metal coordinated by an N-terminal histidine. Following some initial confusion about the identity of the metal ion it has now been shown that these enzymes are copper-dependent oxygenases. Here we assess recent developments in the academic literature, focussing on the structures of the copper active sites. We provide critical comparisons with known small-molecules studies of copper–oxygen complexes and with *copper methane monoxygenase*, another of nature's powerful copper oxygenases.

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Introduction

Cellulose is the world's most abundant biopolymer. Global annual production is estimated to be ca. 80×10^{12} kg. It is unrivalled in its capacity to make a contribution to biofuel production [1•,2]. This capacity, however, is limited by a single factor, cellulose's remarkable chemical stability which has so far thwarted or seriously compromised attempts to use it as a sustainable biofuel source. This stability stems principally from cellulose's solid-state structure which contains an extended network of hydrogen-bonds that exist between long chains (up to 10,000 units) of β-1,4-linked glucosidic units (Figure 1). The result is that cellulose is recalcitrant, making it resistant to both chemical and mechanical degradation, a feature it shares with some other polysaccharides such as chitin.

In the context of biofuels therefore, the effective degradation of recalcitrant polysaccharides is a major research objective. Of the methods available, those using enzymatic means to effect the degradation have attracted attention, not least because recent months have seen strides towards a much fuller understanding of the consortium of enzymes deployed by saprophytes in the degradation of biomass. In truly landmark papers published in 2010 [3••,4••] it was demonstrated that certain fungal and bacterial metallo-enzymes (originally classified as GH61 and CBM33 in the CAZy database, but as of March 2013 reclassified as AA9 and AA10, respectively; see Table 1) [5••,6•] disrupt the structures of recalcitrant polysaccharides using an oxidative mechanism of action, thus providing an answer to the long-sought-for question about how the initial attack on cellulose or chitin was carried out by saprophytes. Working in concert with both canonical polysaccharide hydrolases and other electron transfer components, these enzymes significantly accelerate the degradation of polysaccharides into oligosaccharides. As such they hold major potential for enhancing the production of biofuels from sustainable sources [3••,4••,7•,8–10,11••]. However, despite the very significant potential of these enzymes, confusion as to the correct metal-ion at the active site obscured initial mechanistic insight. This was until 2011, which saw the publication of the complete structures and activities of several AA9 (formerly GH61) enzymes [12••,13•,14] which, for the first time, established these enzymes to be copper-containing oxygenases. This observation had further ramifications for the related bacterial AA10 enzymes (formerly CBM33) [7•,15•,16,17] which were subsequently also re-evaluated to be copper-dependent oxygenases.

The unique features of these enzymes' active sites are now challenging the long-held views of polysaccharide degradation by the hydrolytic action of *exo*-glycosidases and *endo*-glycosidases [18,19] and also of the types of copper–oxygen species which nature employs in oxidative reactions [20,21]. Herein, we précis recent advances in our knowledge of the structures and functions of these remarkable and important enzymes.

Overall structures of AA9 (formerly GH61) and AA10 (formerly CBM33) enzymes

Structures of both AA9 and AA10 enzymes had been published before 2011, although none had correctly identified the required metal as copper. Indeed the literature was confused about the identity of the metal, with Na,

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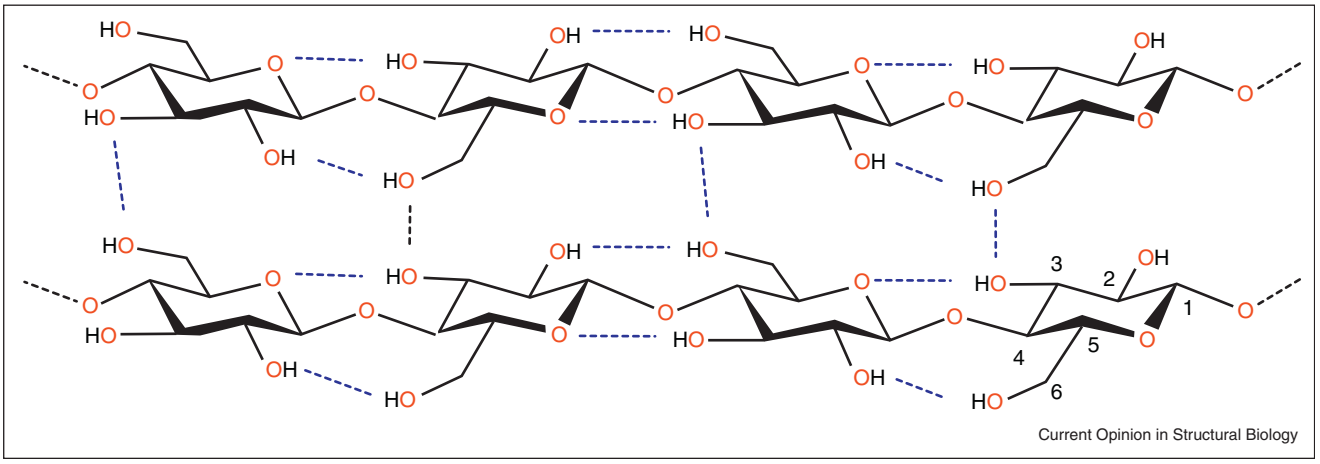
Figure 1



(Partial) structure of cellulose, depicting internal network of hydrogen bonds. Carbon numbering scheme is depicted on one glucosidic unit.

Mg, Ca, Ni, Mn and Zn all suggested, or implied from deposited PDB files, as possibilities [3••,4••,22•]. What was found in these structures, and the subsequent correct copper-containing ones from 2011 onwards, is that the enzymes share a common tertiary structural motif in which the core consists of an immunoglobulin-like β-sandwich sometimes augmented by an alpha-helical loop (Figure 2) accompanied by large areas of variability [23]. The active site is created by an N-terminal histidine which, along with a further histidine, binds to a copper ion.

This active site presents itself in the centre of an extended flat face measuring some 40 Å × 30 Å, quite unlike all other known polysaccharide hydrolases that contain grooves or tunnels into which a single and free polysaccharide chain can bind [24]. The assumption is that the flat face of AA9 and AA10 enzymes is the site of interaction with an extended solid-state substrate such as cellulose or chitin. As seen in deposited structures in the PDB, differences in the structure and nature of this flat face exist between AA9 and AA10 enzymes as well as within different members of each family. Using sequence conservation analysis of the amino acids which appear on the binding face, an AA9 subclassification has been proposed in which three subgroups are formed: PMO1 (Figure 2a), PMO2 (Figure 2b) and PMO3 (Figure 2c) (PMO = polysaccharide mono-oxygenase) according to the nature and number of aromatic amino acid residues which can interact directly with the polysaccharide [25,26•]. This is then correlated with the site of oxidation on the glucosidic unit (Figure 2) suggesting that the positioning of the enzyme on the substrate surface determines the site of oxidation. Interestingly on the same basis, AA10s described thus far find their closest overall structural match with the PMO3 class of AA9 enzymes, but appear to mediate their interaction with substrate not through aromatic residues as in AA9s but through direct hydrogen-bonding interactions of residues (e.g. glutamine, threonine, glutamate) which are immediately adjacent to the copper active site (Figure 2d) [16,27]. The reasons for the differences between AA9 and AA10 are unclear but probably have functional significance [26•].

It is in any case too early to draw many firm conclusions from the types of amino acid residues present on the binding face. For instance, secretome and transcriptome studies of fungal AA9 enzymes show that multiple isoforms are secreted by a single organism during degradation of a substrate, whereas AA10 enzymes are often only present in one or two isoforms within a single bacterial genome [28–34]. Additionally, and more compellingly, a full understanding of the nature of AA9/10 interaction with a polysaccharide substrate can only be

Table 1

Naming and classifications of *lytic polysaccharide mono-oxygenases* (LPMO). In this article we will use the AA9 and AA10 classifications and the general term *lytic polysaccharide mono-oxygenases*

Former CAZy classification	New CAZy classification	Examples
GH61	AA9 (Auxiliary activity 9)	Cel61, PMO, LPMO
CBM33	AA10 (Auxiliary activity 10)	CBP21, Cbp, CelS2, ChbB, CpbD, Gbpa, YucG, Chi

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