indicate that all three ZnF motifs contain a binding site for adenine at the first cysteine residue. With longer polyadenosine oligonucleotides, the central ZnF 6 motif does not show significant shifts for surface residues, but may play an important structural role in orienting the neighboring ZnF motifs for polyadenosine RNA recognition (Brockmann et al., 2012).

Functional assays using *nab2* variants to suppress a cold sensitive allele of the *dbp5* revealed that the structural integrity of ZnF 6 is critical for proper mRNA export and that polyadenosine RNA binding affinity does not solely dictate proper assembly of the mRNP (Brockmann et al., 2012). Previous studies have shown that the RGG domain of Nab2 is responsible for interactions with the adaptor protein, Yra1, and the carrier protein, Mex67 (Iglesias et al., 2010). The *nab2* variant suppression assays with the *dbp5* allele and other assays with a *yra1* mutant allele suggest that the CCCH

region and in particular ZnF 6 may play a more active role in the assembly of an export competent mRNP (Brockmann et al., 2012). These results raise some important questions regarding the role of Nab2 in mRNA export that we anticipate being addressed in the near future. Does the CCCH region of Nab2 participate directly in Yra1 interactions and recruiting Mex67? Do the inter-domain interactions between ZnF motifs in Nab2 provide a mechanism for packaging polyadenosine RNA around a multimeric core of Nab2 molecules? If the ZnF 5–7 region of Nab2 is sufficient for polyadeonsine binding and mRNP assembly, then what is the role of the first four tandem ZnF motifs in Nab2?

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Polysaccharide Monoxygenases: Giving a Boost to Biofuel Production

Carrie M. Wilmot[1],*
[1]Department of Biochemistry, Molecular Biology and Biophysics, 6-155 Jackson Hall, 321 Church St. SE, University of Minnesota, Minneapolis, MN 55455, USA
*Correspondence: wilmo004@umn.edu

In this issue of *Structure,* Li et al. present high resolution crystal structures of two fungal polysaccharide monoxygenases. Unexpectedly, they observe oxygen species bound at the active sites that inform on the chemistry that can be supported by these enzymes. Additionally, the organization of aromatic amino acids and glycosylation on the carbohydrate binding surfaces suggests that regiospecificity and cellulose orientation can be predicted.

The ready availability of lignocellulosic biomass makes this a key target substrate for the production of biofuels. Organisms in the environment can utilize this biomass as a carbon source, but breakdown is slow, taking months. A two-phase strategy is being pursued within the biofuel community to give high-yield conversion of cellulose to glucose on a rapid time scale. Much work has been done on the pretreatment phase required to enable

physical access to the cellulose, which is crystalline and embedded in a lignin and hemicellulose matrix within the plant cell wall (Agbor et al., 2011). Chemical, physical, physicochemical, and biological methods have been pursued, but none are perfect, and work continues to improve efficiency and cost effectiveness. The second phase, and the one relevant to the Li et al. (2012) study in this issue of *Structure*, involves the use of enzymes

to complete breakdown of cellulose to smaller soluble saccharides that can be fed into biofuel production (Li et al., 2012). Methodology in the biorefinery phase has thus far focused on the use of fungal cellulases that degrade the polysaccharide chains hydrolytically to their ultimate end-product of glucose (Chandel et al., 2011). Unfortunately, cellulases suffer from requiring the polysaccharide chain to be accessible enough to bind in

an active site groove, which means they must be free of the cellulose crystalline state. They also are relatively slow enzymes and so have been the subject of bioengineering efforts that have met with limited success. Therefore, they are used at high concentration, making current biorefinery expensive.

Relatively recently it was shown that polysaccharide monoxygenases (PMOs, also known as GH61 enzymes), which are copper-dependent enzymes, can enhance the effectiveness of cellulose cocktails by up to 2-fold (Harris et al., 2010; Phillips et al., 2011; Quinlan et al., 2011). Genomic and proteomic studies have shown that many genomes contain numerous PMO genes that can outnumber the cellulases (Tian et al., 2009). The research into PMOs is in its infancy, but the work of Li et al. (2012) adds immeasurably to our understanding of oxygen activation, PMO chemistry, and regiospecificity.

The study includes two crystal structures of *Neurospora crassa* PMOs from different subclasses (types-2 and -3) that have different regioselectivity. Only three other crystal structures are currently available, with one shown to be type-3 and another type-1 (Harris et al., 2010; Karkehabadi et al., 2008; Quinlan et al., 2011). The synergistic nature of PMO action arises from its ability to bind to cellulose that is still crystalline, oxidatively cleaving glycosidic bonds, and increasing accessibility to cellulases. To allow this, the carbohydrate binding surface is flat, with the copper-containing active site lying at the surface. With the addition of these two new structures, the authors are able to hypothesize how cellulose might bind across the surface (Li et al., 2012). They base this on the regular spacing of posttranslational glycosylation and tyrosine residues across the PMO carbohydrate binding surface. Aromatic residues are a known hallmark of carbohydrate binding in numerous proteins, where they form favorable stacking interactions with the polysaccharide sugars (Boraston et al., 2004). Looking at the spacing of the sugar units in cellulose, the authors are able to predict how the cellulose will orient on the surface, and in both cases, it aligns the substrate such that the appropriate saccharide unit carbon that becomes hydroxylated lies next to the copper. The structures

suggest that the orientation of the cellulose polysaccharide chains across PMO surfaces is varied, and the sequences of the other eight expressed *N. crassa* PMO genes indicate that the binding surface residues show weak conservation (Tian et al., 2009). It will be interesting to see whether their hypothesis that aromatic amino acid spacing and glycosylation across the PMO binding surface enables the interacting orientation of cellulose to be deduced will be generally applicable. It is highly likely that other binding modes and subclasses will come to light as the interest builds in characterizing these enzymes.

Prior work from several groups has begun to reveal the underlying chemistry. Type-1 PMOs oxidize the C1 carbon at the reducing end of the glycosidic linkage, while type-2 PMOs oxidize the C4 carbon on the nonreducing end (Beeson et al., 2012; Phillips et al., 2011). The C1 and C4 hydroxylations destabilize the glycosidic bond, with the subsequent elimination of glycan being either spontaneous or possibly PMO catalyzed, to produce an arodonic acid or 4-ketoaldose, respectively. Molecular oxygen is the source of the additional hydroxyl oxygen added to the sugar unit, with the other oxygen atom ending up in H_2O. Oxygen activation requires Cu(I), and so a reductant is necessary. Although small molecule reductants are adequate in vitro, it has been recently shown that for most, if not all PMOs, the electrons come via cellobiose dehydrogenase (CDH) (Phillips et al., 2011). This flavin enzyme oxidizes cellubiose to the 1-5-δ-lactone and then passes the electron to a heme in another domain, but until recently, the biological purpose of reducing the heme was unclear (Beeson et al., 2011). The recent data show that CDHs act as reductases passing an electron from the heme to the PMO copper, reducing it to Cu(I) that can then activate oxygen (Phillips et al., 2011). Although fundamental to aerobic biology, the activation of molecular oxygen is still poorly understood. Therefore, the unexpected observation of oxygen species in the active sites is exciting (Li et al., 2012). In the PMO-2 crystal structure, oxygen was bound end-on to the copper in a bent geometry that is typical of a Cu(II)-superoxo complex. The structure was of sufficient resolution (to 1.10 Å) that they were able

to refine the bond length between the oxygen atoms without restraints, and this fit with the superoxo assignment. In addition, the electron density on the proximal oxygen atom was denser than that of the distal atom, which is also consistent with the assignment of a Cu(II)-superoxo.

The authors had not been attempting to trap oxygen species in the crystal, and no chemical reductant was present. However, X-rays are highly oxidizing and strip electrons from water, which can find their way into redox active sites (Garman and Owen, 2006; Wilmot et al., 2002). This explains the formation of Cu(I) that can activate molecular oxygen. But in crystals cryocooled to 100K for data collection, diffusion is severely limited, and there is a resistance to reorganize structurally. Molecular oxygen is hydrophobic, and thus at low concentration in solution. The high occupancy of the superoxo is therefore surprising considering that the formation of Cu(I) occurred by photoreduction at 100K. This suggests that there may be a molecular oxygen binding site or hydrophobic "sticky" patch very close to the metal, which again would be highly unusual considering how polar and solvent exposed the active site is. Perhaps the unusual methylation of His1, a copper ligand, has a role to play in molecular oxygen capture?

The diatomic observed in the PMO-3 structure is not actually bound to the copper, being 3.4 Å away. Although the crystal structure is at lower resolution (1.37 Å), this was enough to demonstrate that the O-O bond length was most consistent with a peroxide assignment. Although hydrogen peroxide is not a product of the PMO reaction, it is possible that in the absence of cellulose substrate, further photoreduction of the Cu(II)-superoxo leads to a copper-bound peroxo species that further protonates and dissociates from the copper as H_2O_2. A copper-hydroperoxo species has been postulated in the PMO mechanism that results from hydrogen atom abstraction by the Cu(II)-superoxo to give a cellulose-based radical. The PMO-3 structure is also interesting in that the carbohydrate binding surfaces of two PMO-3 molecules come together in a non-physiological crystal contact. This is part of the reason why dissociated H_2O_2

is observable. In addition, the closest amino acid from the adjacent molecule, Tyr24, has been oxidized to 3,4-phenylalanine during data collection. Although non-physiological, this demonstrates the type of reactive oxidative chemistry PMO can catalyze.

The ability of PMOs to oxidize crystalline cellulose when coupled to CHD catalysis means increased access for cellulases to complete the breakdown of cellulose to glucose. Thus, the addition of PMOs and CHD to biorefinery enzyme cocktails holds the promise of reducing cellulase loading and cost. The Li et al. (2012) study brings us one step closer to understanding the action, regio-specificity, and substrate recognition of PMOs, which will enable strategies to be developed for their effective utilization in biorefinery and the production of biofuels.

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