Spectroscopic and computational insight into the activation of O₂ by the mononuclear Cu center in polysaccharide monooxygenases

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Strategies for O₂ activation by copper enzymes were recently expanded to include mononuclear Cu sites, with the discovery of the copper-dependent polysaccharide monooxygenases, also classified as auxiliary-activity enzymes 9–11 (AA9–11). These enzymes are finding considerable use in industrial biofuel production. Crystal structures of polysaccharide monooxygenases have emerged, but experimental studies are yet to determine the solution structure of the Cu site and how this relates to reactivity. From X-ray absorption near edge structure and extended X-ray absorption fine structure spectroscopies, we observed a change from four-coordinate Cu(II) to three-coordinate Cu(I) of the active site in solution, where three protein-derived nitrogen ligands coordinate the Cu in both redox states, and a labile hydroxide ligand is lost upon reduction. The spectroscopic data allowed for density functional theory calculations of an enzyme active site model, where the optimized Cu(I) and (II) structures were consistent with the experimental data. The O₂ reactivity of the Cu(I) site was probed by EPR and stopped-flow absorption spectroscopies, and a rapid one-electron reduction of O₂ and regeneration of the resting Cu(II) enzyme were observed. This reactivity was evaluated computationally, and by calibration to Cu-superoxide model complexes, formation of an end-on Cu-AA9-superoxide species was found to be thermodynamically favored. We discuss how this thermodynamically difficult one-electron reduction of O₂ is enabled by the unique protein structure where two nitrogen ligands from His1 dictate formation of a T-shaped Cu(I) site, which provides an open coordination position for strong O₂ binding with very little reorganization energy.

X-ray absorption spectroscopy | DFT | dioxygen activation | biofuels

Cu is an important metal cofactor in a number of enzymes that activate dioxygen for reactivity. Several classes with either binuclear or trinuclear copper active sites have been identified and their different strategies for O₂ activation have been elucidated (1). These include the multicopper oxidases that use three Cu ions to reduce O₂ to water with very little overpotential (2, 3), the coupled binuclear Cu enzymes that are involved in dioxygen transport and monooxygenase reactivity (4), and the noncoupled binuclear Cu monooxygenases that activate O₂ for hydroxylation of peptides and hormones (5). Recently, a class of oxygen activating enzymes with a single copper center has been identified, the polysaccharide monooxygenases [PMOs; often termed lytic polysaccharide monooxygenases (LPMOs), reflecting their ability to break polysaccharides chains and loosen crystalline structure] (6–8), or AA9 to 11 enzymes (AA = auxiliary activity) in the Carbohydrate-Active enZymes (CAZy) database (9). AA9 enzymes [formerly glycoside hydrolases family 61 (GH61s)] are fungal enzymes that can enhance major cellulases' enzymatic degradation of cellulose (hence "auxiliary activity"), whereas AA10 enzymes [formerly carbohydrate binding module family 33 (CBM33)] are predominantly bacterial enzymes that can enhance

major chitinases' degradation of chitin (6, 7, 10–13). Enzymes in the latest discovered subclass, AA11, are fungal enzymes, where the currently lone characterized example uses chitin as a substrate (8). Studies by Vaaje-Kolstad et al. (6) and Beeson et al. (14) have shown that AA10 and AA9 introduce a single oxygen atom originating from dioxygen into the respective chitin and cellulose degradation products. Although little is known about the reaction mechanism of these enzymes, it is believed that this does not simply involve Fenton-type chemistry producing reactive oxygen species but rather a more controlled reaction involving a Cu-oxygen intermediate (14–16). Cu-loaded crystal structures have been solved for all three subclasses of AA9–11 enzymes (7, 8, 17–19), highlighting interesting similarities and differences. Common for the three classes is a flat protein surface that harbors the Cu active site, as illustrated for an AA9 enzyme in Fig. 1, *Left*. In AA9 PMOs, the flat surface is proposed to interact with the long-chained substrates via a number of primarily aromatic amino acids (18, 19). In the active Cu site, two histidines provide three nitrogen ligands, two from *N*-His and one from the terminal amine (Fig. 1, *Right*), in a configuration that has been termed a histidine brace (7). An axially oriented tyrosine (Tyr) is highly conserved in the AA9 and AA11 enzymes ~3 Å from the Cu, and hydrogen bonded to an oxygen from a glutamine or glutamate side chain (Fig. 1, *Right*). In the AA10 enzymes, the

Significance

Activation of the O–O bond in dioxygen is difficult but fundamental in biology. Nature has evolved several strategies to achieve this, often including copper as an enzyme cofactor. Copper-dependent enzymes usually use more than one metal to activate O₂ by multielectron reduction, but recently it was discovered that cellulose and chitin degrading polysaccharide monooxygenase enzymes use only a single Cu center for catalysis, in a reaction that is of great interest to the biofuel industries. To understand this reactivity, we have determined the solution structures of both the reduced and oxidized Cu site, and determined experimentally and computationally how this site is capable of facile O₂ activation by a thermodynamically difficult one-electron reduction, via an inner-sphere Cu-superoxide intermediate.

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Conflict of interest statement: F.X. and K.S.J. are employees of Novozymes, which is a commercial supplier of industrial enzymes including (L)PMOs.

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Fig. 1. AA9 from *T. aurantiacus* (modified from PDB: 3ZUD). Surface view (Left) and active site (Right) highlighting six selected residues surrounding the Cu ion (gold sphere).

axial Tyr is replaced with a highly conserved phenylalanine. In addition to these amino acids, water derived molecules are often refined in the vicinity of the Cu ion, but it is not known whether these coordinate to the Cu in solution. Although the crystal structures provide valuable information, they do not determine the detailed environment of the Cu ion in solution, which is critical in elucidating the mechanistic properties of the PMOs. This is partly due to the fact that oxidized Cu readily undergoes photoreduction upon exposure to X-rays, often accompanied by changes in the coordination environment of the metal (17, 20).

The PMO superfamily is of considerable importance in the developing area of second generation (recalcitrant polysaccharide) biofuels, where even preliminary studies have shown twofold to sixfold improvements in biomass conversion using unoptimized mixtures (6, 11) providing further impetus for their detailed characterization. Here we present spectroscopic results on a PMO from *Thermoascus aurantiacus*, denoted Cu-AA9, complemented by density functional theory (DFT) calculations that provide a detailed description of the coordination environment in solution of both the oxidized and reduced Cu active site. Furthermore, the interaction of the reduced Cu site with dioxygen is investigated, focusing on the ability of the PMOs to activate O₂ with a single Cu center.

Results and Analysis

Cu K-Edge X-Ray Absorption Near Edge Structure and Extended X-Ray Absorption Fine Structure. *Oxidized and reduced Cu-AA9.* Cu K-edge X-ray absorption near edge structure (XANES) spectra of reduced and oxidized Cu-AA9 are shown in Fig. 2. In the first fast scan of Cu(II)-AA9 (blue trace) (radiation dose: $\sim 1.7 \times 10^{12}$ photons/mm²), very little intensity at 8984 eV is seen, verifying the presence of the oxidized state of the Cu. However, the oxidized enzyme undergoes photoreduction in the beam as observed in the average of longer scans from four fresh spots (black dashed trace) (radiation dose: $\sim 6.6 \times 10^{12}$ photons/mm² per scan), similar to observations for AA10 PMOs (17). In contrast, the spectrum of the fully reduced state (red trace, Fig. 2) does not change with time (i.e., no further photoreduction) and has an intense 8984-eV feature with normalized intensity of 0.76 originating from a Cu 1s to 4p transition (21). The intensity and shape of the rising edge in the Cu(I) XANES spectrum is consistent with a three-coordinate [either three equidistant ligands or two short plus one longer distance ligands (i.e., T-shaped)] Cu(I) based on results from previously studied model Cu(I) complexes (22).

Extended X-ray absorption fine structure (EXAFS) was performed on the oxidized and reduced enzyme forms to determine the number and type of coordinating ligands to the Cu ion in Cu-AA9. As seen in Fig. 3 *A* and *B*, the beat pattern and the Fourier transform of the Cu(II) enzyme can be simulated with a first sphere of four O/N ligands at an average distance to the Cu(II) ion of 1.98 Å (Table 1). Alternatively, the reduced enzyme requires two ligand sets to fit the first coordination sphere and is simulated with three first-sphere O/N ligands, two at 1.90 Å and one at 2.25 Å (Fig. 3 *C* and *D* and Table 1). This reveals that upon reduction in solution, the AA9 enzyme changes from

a four-coordinate Cu(II) to a three-coordinate T-shaped Cu(I) site structure. We note that the beat pattern and Fourier transform of Cu(II)-AA9 closely resemble that of the oxidized form of copper particulate methane monooxygenase (pMMO) (23, 24), the only other Cu enzyme known to use the histidine brace (6, 19). In contrast to Cu-pMMO, however, the simulation for Cu(II)-AA9 does not require a second Cu ion to be included in the set of nearby scattering atoms.

Cryoreduction of Cu(II)-AA9. To probe the temperature dependence of the reduction-induced change in the coordination environment of Cu-AA9, cryoreduction (at 77 K) of Cu(II)-AA9, followed by XANES and EXAFS evaluation, was performed. A Cu(II)-AA9 sample was prepared in a cryocell, frozen in liquid nitrogen, and subsequently exposed to a γ -emitting ^{137}Cs source that produces free electrons in the cryosolvent. The extent of cryoreduction of Cu(II)-AA9 was monitored by the disappearance of the characteristic Cu(II)-AA9 EPR spectrum (7) (*SI Appendix, Figs. S14 and S2*). After 110 h of exposure time (~ 5.9 -Mrad total dose), the enzyme was $\sim 75\%$ reduced. The XANES spectrum of the $\sim 75\%$ reduced Cu-AA9 (Fig. 4, green trace) has a prominent 8984-eV feature that further increases by exposure to the synchrotron beam with the final spectrum (black trace) estimated to be $\sim 90\%$ reduced, compared with the solution reduced enzyme (red trace, from Fig. 2). The shape of the rising edge in the $\sim 90\%$ cryoreduced sample is similar to that of the solution-reduced peak. Further, the EXAFS data and simulation (*SI Appendix, Fig. S3 and Table S1*) of the cryoreduced Cu-AA9 sample are consistent with a mixture of the oxidized and reduced forms, with the reduced form dominant. Therefore, the observed change in coordination environment of the Cu-ion upon reduction at room temperature, from four-coordinate to three-coordinate, also occurs in the frozen enzyme at 77 K. The fact that this geometry change also occurs at low temperature is indicative of limited rearrangement in the protein structure, consistent with the three protein-derived nitrogen ligands identified in crystal structures [two N-His, one N-terminal amine (Fig. 1)] coordinating the Cu in both the reduced and oxidized state, whereas the water derived ligand is labile and only coordinates to the oxidized Cu center.

Reactivity of Reduced Cu(I)-AA9 with Dioxygen. Studies have reported the activity of PMOs on cellulose or chitin to be oxidative in nature with dioxygen activation by Cu(I) followed by insertion of one oxygen atom into the substrate (6, 14). We investigated the first step in this proposed reaction sequence by monitoring the reactivity of ascorbate-reduced Cu-AA9 with dioxygen by EPR and stopped-flow absorption spectroscopies. Reduced enzyme was mixed with oxygenated buffer in an EPR tube followed by immediate freezing in liquid nitrogen. Within the freezing time of the reaction mixture (<10 s), the resting Cu(II)-AA9 was fully regenerated as evaluated by EPR spin

Fig. 2. Normalized Cu K-edge XANES spectra of Cu-loaded AA9, Cu(II)-AA9 first short, fast scan (blue), Cu(II)-AA9 average of first scans from four fresh spots (black), and Cu(I)-AA9 (red).

Fig. 3. Cu K-edge EXAFS data (A) and non-phase-shift-corrected Fourier transform (B) of Cu(II)-AA9. Cu K-edge EXAFS data (C) and non-phase-shift-corrected Fourier transform (D) of Cu(I)-AA9. Phase shifts in the first shells are ~ 0.4 Å. Data (black) and fits (red).

integration, consistent with a minimum rate of Cu(I) reoxidation of $>0.15 \text{ s}^{-1}$ (*SI Appendix*, Fig. S4). This reaction was further performed in a stopped-flow absorption spectrometer, and in agreement with the EPR result, low-intensity Cu d-to-d transitions (at 600–700 nm) appeared within 10 s (*SI Appendix*, Fig. S5). The reoxidation of Cu(I)-AA9 by O_2 may occur either through inner- or outer-sphere pathways as shown in Scheme 1. In the outer-sphere mechanism (A), O_2 would oxidize Cu(I) and regenerate the resting Cu(II)-AA9 site in a single reaction step, whereas an inner-sphere reaction would involve the formation of a Cu-superoxide intermediate (B) followed by rapid release of superoxide and regeneration of the resting enzyme state (C).

Outer-sphere reoxidation can be evaluated by the Marcus equation (25, 26). While no redox potential has been reported for AA9, the redox potential of AA10 enzymes has been reported at ~275 mV vs. the normal hydrogen electrode (NHE) (15, 17). Using this potential, and the potential of the one-electron

Table 1. EXAFS least-squares fitting results for $k = 2\text{--}12.8 \text{ \AA}^{-1}$ for Cu(II)-AA9 and Cu(I)-AA9

Coordinate no. and path	$R, \text{\AA}^*$	$\sigma^2, \text{\AA}^{2+}$	$\Delta E_0, \text{eV}^+$	$F^{\S}$
Cu(II)-AA9				
4 Cu-N/O	1.98	500	-8.59	0.28
5 Cu-C	2.94	761		
8 Cu-N-C	3.13	801		
4 Cu-N/C	3.87	965		
16 Cu-N-C	4.11	965		
8 Cu-N-C	4.73	603		
Cu(I)-AA9				
2 Cu-N/O	1.90	308	-9.75	0.06
1 Cu-N/O	2.25	665		
4 Cu-C	2.92	275		
8 Cu-N-C	3.06	582		
4 Cu-N/C	3.90	932		
16 Cu-N-C	4.07	864		

*The estimated SD in R for each fit is ± 0.02 Å.

[†]The σ^2 values are multiplied by 10^5 .

* E_0 is the threshold energy, the point at which the photoelectron wave vector $k = 0$.

⁵The error, F , is given by $\sum[(\chi_{\text{obsd}} - \chi_{\text{calcd}})^2 k^6] / \sum[(\chi_{\text{obsd}})^2 k^6]$. The error in coordination number is 25%, and that in the identity of the scatterer Z is ± 1 .

reduction of O_2 to superoxide (-165 mV vs. NHE) (27), the outer-sphere electron transfer rate from Cu(I) to O_2 is estimated to be $\sim 4.5 \times 10^{-4} \text{ s}^{-1}$ (see [SI Appendix](#)) which is $\sim 10^3$ slower than the experimental rate of Cu(I) reoxidation from the EPR and stopped-flow data. Based on this, the single-electron transfer from Cu(I)-AA9 to O_2 likely proceeds via an inner-sphere pathway involving rapid formation of Cu(II)-superoxide, where binding of the $O_2^{\cdot-}$ to the Cu(II) drives the reaction. The fact that only the resting state is observed in the resulting EPR spectrum indicates that the superoxide that has formed is rapidly displaced from the Cu(II) by H_2O . The inner-sphere Cu(II)-superoxide formation followed by water displacement of the superoxide to give the resting Cu(II) state, are computationally evaluated below.

Experimentally Calibrated DFT Calculations. Optimized structures of Cu(I)-AA9 and Cu(II)-AA9. Determination of the Cu(I)-AA9 and Cu(II)-AA9 coordination environments by Cu K-edge XANES and EXAFS (*vide supra*) allowed for the optimization of experimentally calibrated DFT structures. As the starting point, a truncated model of the high-resolution (1.25 Å) crystal structure of *T. aurantiacus* AA9 (3ZUD; available on the Protein Data Bank, <http://www.rcsb.org/pdb/explore/explore.do?structureId=3zud>) was used, with a total of six amino acid residues, two providing the three Cu ligands as described above, and two water/hydroxide molecules that are in equatorial and axial orientations with respect to the Cu ion (*SI Appendix, Fig. S6 and Table S2*). The redox state of the Cu ion in the crystal structure is unknown, and the same starting structure was therefore used for optimization of both the oxidized and reduced sites.

Optimization of Cu(I)-AA9 using the B3LYP functional with the 6-311g* basis set on Cu, 6-31g* on the six nearest atoms to Cu, and 3-21g* on the rest, resulted in a three-coordinate Cu(I) site with the two N-His ligands at 1.91 Å from the Cu, and the terminal amine-N providing the third ligand at 2.26 Å, in an overall T-shaped geometry (Fig. S4 and *SI Appendix, Fig. S7A and Table S2*). This is in agreement with the experimental XANES and EXAFS results presented above. To further validate the optimized structure, we used this structure to simulate its EXAFS spectrum, and, as shown in *SI Appendix, Fig. S8*, the simulated EXAFS spectrum is very similar to the experimental Cu(I)-AA9 spectrum. In addition to the first sphere ligands, the equatorial water ligand, which in the starting geometry is at 2.00 Å from the Cu(I), moves out of the inner coordination sphere and is found at a 3.11-Å distance to the Cu, hydrogen bonded to the conserved Gln-173 residue. Finally, the distances to the axially positioned Tyr-O and water-O (2.9 Å and 2.9 Å from the Cu in the crystal structure) increased slightly to 3.23 Å and 3.32 Å, respectively, in the model (*SI Appendix, Fig. S7A and Table S2*). A recent computational paper on AA9 enzymes by Kim et al. (28) optimized to a four-coordinate Cu(I)-AA9 structure, inconsistent

Fig. 4. Normalized Cu K-edge XANES spectra of ~75% cryoreduced Cu-AA9, first scan (green) and ~90% cryoreduced Cu-AA9 (black). Spectra of Cu(I) (red scan) and Cu(II)-AA9 (blue scan) from Fig. 2.

Scheme 1. Possible reaction pathways for reoxidation of Cu(I)-AA9 by O₂.

with our experimental data and optimized structure presented here. Possible reasons for this discrepancy are that Kim et al. used a lower-resolution crystal structure of AA9 (2YET; available on the Protein Data Bank, <http://www.rcsb.org/pdb/explore/explore.do?structureId=2yet>) as the starting structure and used the double-zeta 6-31g* basis set on Cu. If we optimize the 3ZUD-based structure used here with the 6-31g* basis set on Cu we also obtain a four-coordinate Cu(I) structure, and, additionally, if we reoptimize the Cu(I) structure obtained by Kim et al. using the larger triple-zeta 6-311g* basis set on Cu, we obtain a 2 + 1 coordinate DFT structure, in agreement with the experimental data and our calculations.

For the optimization of the resting Cu(II)-AA9, the starting structure was similar to the starting structure for Cu(I)-AA9, with the only difference being the replacement of the equatorial water by a hydroxide. (The equatorial water-derived ligand is modeled as a hydroxide based on the computational results presented in *Release of superoxide in Cu-AA9*.) An optimized structure fully consistent with the experimental data was again obtained, i.e., a four-coordinate tetragonal Cu(II) site with an average Cu-N/O distance of 1.98 Å (Fig. 5B and *SI Appendix, Fig. S7B and Table S2*). To further validate this structure, the simulated EXAFS spectrum of the optimized Cu(II)-AA9 structure with bound OH[−] is found to be in reasonable agreement with the experimental spectrum (*SI Appendix, Fig. S8*).

O₂-binding to Cu(I)-AA9. From the fast rate of O₂ reduction by the Cu(I) state that regenerates the resting Cu(II)-AA9 (*vide supra*), dioxygen appears to undergo the thermodynamically difficult superoxide formation by inner-sphere reduction by the Cu(I) active site. This was evaluated computationally by adding an O₂ molecule to the optimized Cu(I)-AA9 structure followed by reoptimization (Fig. 6A and *SI Appendix, Fig. S9 and Table S3*). [Note that the O₂ in the Cu-O₂ structure displaces the non-coordinating equatorial water which is hydrogen-bonded to Gln-173 in the Cu(I) optimized structure, whereas the axial water moves to a position immediately above the nitrogen N1.] In the most stable O₂-bound structure obtained, superoxide binds equatorially to the Cu(II) ion in an end-on fashion. The lowest energy spin state has an *S* = 1 (for molecular orbitals, see *SI Appendix, Fig. S9 B and C*), whereas the *S* = 0 open shell singlet (broken symmetry corrected) is ~4.5 kcal/mol less stable. It was not possible to stabilize a side-on bound Cu-O₂ structure in the AA9 site. The thermodynamics of O₂ binding to the Cu(I)-AA9 site (from the energies of the Cu-O₂ + H₂O relative to the Cu(I) + O₂) were calculated to be thermoneutral ($\Delta G^\circ = -0.5$ kcal/mol and $\Delta E^\circ = -0.7$ kcal/mol) (Table 2).

To evaluate the effect of the included water molecules on the reaction energy and geometric structures, the Cu(II)-superoxide formation was also calculated by optimizing Cu(I) and Cu(II)-O₂-AA9 structures with no waters present (Fig. 6B). This resulted in optimized structures of the reduced and the O₂-bound Cu-AA9 that were very similar to those obtained with the water molecules included (Fig. 6A and B and *SI Appendix, Table S3*). In terms of binding energy, Cu-superoxide formation in the absence of the water molecules is highly favorable ($\Delta E^\circ = -14.7$ kcal/mol) compared with the calculation with water molecules included ($\Delta E^\circ = -0.7$ kcal/mol). However, this favorable binding energy is countered by a decrease in entropy (Table 2) and results in a small decrease in ΔG° from -0.5 kcal/mol with water molecules included to -2.6 kcal/mol in the absence of H₂O

displacement. Therefore, the slightly favorable free energy of superoxide formation in Cu-AA9 is a property of the protein derived ligation (two N-His and one N-terminal amine) and not simply a consequence of solvent exposure of the Cu active site.

Comparison of O₂-bound Cu-AA9 to characterized Cu-superoxide model complexes. Cu-superoxide species have been proposed as reactive intermediates in H-atom abstraction in the noncoupled binuclear Cu enzymes (1, 29, 30), although a Cu(II)-superoxide enzyme species has yet to be spectroscopically characterized. Alternatively, a variety of inorganic Cu(II)-superoxide model complexes have been trapped at low temperature (31–35). These include a diazacyclooctane supported complex (1) (32) (*SI Appendix, Fig. S10A and Table S3*) and a TMG₃tren supported complex (2) (34) (*SI Appendix, Fig. S10B and Table S3*), both of which have neutral nitrogen ligands to the copper, similar to Cu-AA9. Experimental energies for superoxide formation from reduced **1** (36) and **2** (37) have been reported in acetone (**1**) and dimethylformamide (DMF) (**2**), respectively. In both systems, the reaction free energy is close to thermoneutral with **1** being slightly uphill by 1.6 ± 0.2 kcal/mol and **2** being favorable by 1.5 ± 1.7 kcal/mol (Table 2). These experimental values allowed us to evaluate the DFT calculations of reductive O₂ binding by Cu(I)-AA9 presented above.

To compare the O₂ binding to Cu-AA9 relative to the model complexes, we optimized reduced and O₂-bound structures of both **1** and **2** (*SI Appendix, Fig. S10 and Table S3*) using the same basis set and functional used above for Cu-AA9. The ΔG° for O₂ bonding to reduced **1** is calculated to be +5.3 kcal/mol, which is in reasonable agreement with the experimental value of +1.6 kcal/mol (Table 2). While the calculated entropy is similar to the experimental value, the O₂ binding energy is slightly less favorable, consistent with the B3LYP functional that tends toward low bond strengths. Interestingly, the calculated reduced and O₂-bound structures for **1** have very similar geometries compared with the DFT optimized Cu(I)-AA9 and Cu(II)-AA9-superoxide structures (Fig. 6 and *SI Appendix, Fig. S10A and Table S3*). For **2**, the ΔG° is calculated to be -4.2 kcal/mol, again consistent with the experimental value of -1.5 kcal/mol. Furthermore, the geometry optimized O₂-bound structure of **2** is very close to the reported crystal structure (*SI Appendix, Fig. S10B and Table S3*) (34). Interestingly, the entropy is less negative in the calculations compared with experiment, suggesting solvent interaction in the reduced state that would also influence the ΔE calculation. However, since there is no structural information available on reduced **2**, this is not included in the model. Overall, the agreement between experiments and calculations of **1** and **2** validates our calculations on the truncated Cu-AA9 enzyme models presented above, supporting the argument for inner-sphere Cu(II)-superoxide formation in the AA9 enzyme.

Release of superoxide in Cu-AA9. To complete the correspondence between our observed experimental reactivity of reduced Cu-AA9 with dioxygen in the absence of polysaccharide substrate,

Fig. 5. DFT structures of Cu-AA9. Optimized structure of Cu(I)-AA9 (A) and optimized structure of Cu(II)-AA9 with OH[−] as equatorial ligand (B). For complete structures, see *Fig. S7 A and B*.

Fig. 6. Representations of DFT optimized structures of reduced and O_2 -bound Cu-AA9. (A) Cu-AA9 reduced with water molecules included (Left) and O_2 -bound with equatorial water displaced by O_2 (Right). (B) Cu-AA9 reduced with water molecules removed (Left) and O_2 -bound (Right). Hydrogen atoms are omitted for clarity, except for water molecules in A.

and the computational results, it is necessary to evaluate the displacement of the superoxide to reform the resting Cu(II)-AA9 site. Displacement of ligands from tetragonal Cu(II) complexes generally involves an associative mechanism. Therefore, an additional water molecule was included at the axial position of the optimized Cu(II)-superoxide enzyme structure (Fig. 6A, Right, and *SI Appendix*, Fig. S11A) and a linear transit calculation was performed by displacing the O_2^- moiety in increments of 0.1 Å from the Cu(II) with reoptimization of the remaining coordinates (Scheme 2).

This allowed the axial water molecule to move closer to the Cu(II) and eventually occupy the vacated equatorial position (Scheme 2 and *SI Appendix*, Fig. S11B and C). At a Cu- O_2 distance between 2.9 Å and 3.0 Å, a proton from the water shifts to the superoxide, at which point a resting Cu(II)-AA9 structure with an equatorial bound hydroxide ligand is generated. This superoxide release was found to be uphill by 10.3 kcal/mol (ΔG°), which is reasonably consistent with the experimentally determined rate of regeneration of the resting Cu(II)-AA9 ($>0.15\text{ s}^{-1}$) (*vide supra*). It is interesting to note, in that context, that in a recent Cu-AA9 crystal structure by Li et al., a superoxide molecule was refined at a distance of ~ 2.9 Å from the Cu-ion (19).

Discussion

A detailed description of the active site properties, in solution, of the newly discovered cellulose and chitin degrading polysaccharide monooxygenases, AA9-11, is a critical step in understanding how these enzymes perform their O_2 activation using only a single Cu center. From the combination of spectroscopic and computational results presented above, the Cu-AA9 enzyme from *T. aurantiacus* is found to have a four-coordinate tetragonal geometry in its oxidized state, whereas the reduced state of the enzyme has a three-coordinate T-shaped structure.

The protein-derived nitrogen ligands identified by crystallography, N-His1, N-His86, and the terminal amine, coordinate to the Cu(I) and the Cu(II), with protonative loss of a hydroxide ligand upon reduction of the Cu center.

As observed by EPR and stopped-flow absorption spectroscopies, the T-shaped Cu(I) in AA9 undergoes rapid reoxidation when reacted with O_2 , with a rate constant of $>0.15\text{ s}^{-1}$, consistent with an inner-sphere mechanism where O_2 coordinates to the vacant equatorial position and is concertedly reduced by one electron by the Cu(I). Thus, the binding of the superoxide to the Cu(II) drives the thermodynamically difficult one-electron reduction of O_2 . This is supported by experimentally calibrated DFT calculations that show O_2 binding as an end-on Cu(II)-superoxide triplet with a slightly favorable free energy.

It is interesting from a structure–function perspective to consider why the single Cu site in PMOs has evolved to incorporate the terminal amine and side chain nitrogen of His1 as ligands. The dual ligation by the His1 residue provides a N-Cu-N angle of $\sim 90^\circ$, and by incorporating a third coordinating nitrogen (from His86) at $\sim 180^\circ$ to N-His1, the protein-derived coordination sphere allows for the formation of the tetragonal Cu(II) geometry (with addition of an exogenous H_2O ligand as OH^-) and the T-shaped Cu(I) geometry, observed experimentally in this study. Both of these structures are favorable for the Cu ion in the respective oxidation states. This ensures that the Cu ion can cycle between the oxidized and reduced state with relatively limited reorganization of the protein-derived ligands, thereby promoting rapid reductive O_2 binding. Also, the T-shaped geometry of the Cu(I) provides excellent σ -overlap with the protein-derived ligands, which will raise the energy of the filled antibonding $d_{x^2-y^2}$ redox active molecular orbital. This, combined with the vacant equatorial coordination position, allows for favorable overlap with one of the π^* -LUMOs of O_2 , resulting in the calculated thermodynamically favorable formation of a Cu-superoxide intermediate.

Finally, the observed (by EPR) regeneration of resting Cu(II)-AA9, in the absence of substrate, indicates rapid release of superoxide. As mentioned in the Introduction, the cleavage of cellulose substrate occurs in a controlled manner, inconsistent with random attack by released oxygen species. While the turnover rate for cellulose cleavage has yet to be determined in AA9 enzymes, it is likely that this is a relatively slow process based on the long time scale used in activity assays (6, 7, 11, 14, 18). Therefore, the observed rapid superoxide release must be limited when substrate is bound to the enzyme. As evaluated by the DFT calculations, the tetragonal Cu-superoxide allows for a water molecule to enter the axial coordinate position, which would result in associative displacement of the superoxide with a relatively low barrier (10.3 kcal/mol). Substrate binding to the enzyme surface (Fig. 1, Left) may block the axial position, which would then lead to stabilization of the Cu-superoxide intermediate, and prevent its decay to the resting form of the enzyme. It is interesting to note that in a recently published study by Isaksen et al. (38), hydrogen peroxide formation, by a Cu-AA9 enzyme with different reductants and O_2 , was detected only in the absence of substrate or with substrates that were not subject to AA9 activity. If the superoxide is indeed stabilized by the substrate, it may be able to directly attack the polysaccharide, or it may be further reduced to a more reactive species by either small molecules or cellobiose dehydrogenase

Table 2. DFT calculated thermodynamic values for O_2 binding to Cu(I)-AA9 and model complexes 1 and 2

Structure	ΔE , kcal/mol	ΔH , kcal/mol	ΔS , cal/mol	ΔG , kcal/mol
O_2 -Cu(II)-AA9	−0.7	−2.5	−6.7	−0.5
O_2 -Cu(II)-AA9 no H_2O	−14.7	−13.7	−37.4	−2.6
O_2 -1 (36) (experimental)	—	-9.1 ± 0.3	-35.8 ± 0.7	1.6 ± 0.2
O_2 -1 (calculated)	−6.1	−4.7	−33.7	5.3
O_2 -2 (37) (experimental)	—	-8.4 ± 0.8	-23.0 ± 3.0	-1.5 ± 1.7
O_2 -2 (calculated)	−17.9	−15.7	−38.7	−4.2

Scheme 2. Superoxide release from Cu(II)-AA9. Representations of selected geometry optimized structures with Cu-O1 distances fixed at 2.0 Å, 2.4 Å, 2.6 Å, 2.9 Å, and 3.0 Å, respectively. Note that the model structures are rotated 180° with respect to *SI Appendix, Fig. S11*.

(a known AA9 reducing cofactor) (7, 13, 39). This awaits further experimental investigation.

In summary, we have determined the coordination geometries for Cu(I) and Cu(II)-AA9 in solution, and found that the enzyme active site structure is well configured for rapid inner-sphere reductive activation of O₂ by Cu(II)-superoxide formation. This is an important step toward elucidating the mechanism by which this recently identified class of mononuclear Cu oxygenases functions to activate the inert O₂ molecule by one-electron reduction for subsequent degradation of polysaccharides.

Materials and Methods

For details, see *SI Materials and Methods*. AA9 from *T. aurantiacus* was expressed and purified in accordance with previously published protocols (34). The AA9 enzyme was purified in the Apo form and loaded with Cu(II) (NO₃)₂ to a final Cu concentration of 90–95% compared with the enzyme

concentration. All experiments were conducted in 25 mM Mes buffer, pH 6 unless otherwise stated. DFT calculations were performed with the Gaussian 09 software package (for full reference, see *SI Appendix*).

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- Solomon EI, et al. (2011) Copper dioxygen (bio)inorganic chemistry. *Faraday Discuss* 148:11–39, and discussion (2011) 148:97–108.
- Solomon EI, Augustine AJ, Yoon J (2008) O₂ reduction to H₂O by the multicopper oxidases. *Dalton Trans* (30):3921–3932.
- Mano N, et al. (2003) Oxygen is electroreduced to water on a “wired” enzyme electrode at a lesser overpotential than on platinum. *J Am Chem Soc* 125(50):15290–15291.
- Ginsbach JW, et al. (2012) Structure/function correlations among coupled binuclear copper proteins through spectroscopic and reactivity studies of NspF. *Proc Natl Acad Sci USA* 109(27):10793–10797.
- Klinman JP (2006) The copper-enzyme family of dopamine beta-monooxygenase and peptidylglycine alpha-hydroxylating monooxygenase: Resolving the chemical pathway for substrate hydroxylation. *J Biol Chem* 281(6):3013–3016.
- Vaaje-Kolstad G, et al. (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330(6001):219–222.
- Quinlan RJ, et al. (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. *Proc Natl Acad Sci USA* 108(37):15079–15084.
- Hemsworth GR, Henrissat B, Davies GJ, Walton PH (2014) Discovery and characterization of a new family of lytic polysaccharide monooxygenases. *Nat Chem Biol* 10(2):122–126.
- Levasseur A, Drula E, Lombard V, Coutinho PM, & Henrissat B (2013) Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol Biofuels* 6(1):41.
- Hemsworth GR, Davies GJ, Walton PH (2013) Recent insights into copper-containing lytic polysaccharide mono-oxygenases. *Curr Opin Struct Biol* 23(5):660–668.
- Harris PV, et al. (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: Structure and function of a large, enigmatic family. *Biochemistry* 49(15):3305–3316.
- Forsberg Z, et al. (2011) Cleavage of cellulose by a CBM33 protein. *Protein Sci* 20(9):1479–1483.
- Phillips CM, Beeson WT, Cate JH, Marletta MA (2011) Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by *Neurospora crassa*. *ACS Chem Biol* 6(12):1399–1406.
- Beeson WT, Phillips CM, Cate JHD, Marletta MA (2012) Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases. *J Am Chem Soc* 134(2):890–892.
- Aachmann FL, Sørleie M, Skjåk-Bræk G, Eijsink VGH, Vaaje-Kolstad G (2012) NMR structure of a lytic polysaccharide monooxygenase provides insight into copper binding, protein dynamics, and substrate interactions. *Proc Natl Acad Sci USA* 109(46):18779–18784.
- Vu VV, Beeson WT, Phillips CM, Cate JHD, Marletta MA (2014) Determinants of regioselective hydroxylation in the fungal polysaccharide monooxygenases. *J Am Chem Soc* 136(2):562–565.
- Hemsworth GR, et al. (2013) The copper active site of CBM33 polysaccharide oxygenases. *J Am Chem Soc* 135(16):6069–6077.
- Wu M, et al. (2013) Crystal structure and computational characterization of the lytic polysaccharide monooxygenase GH61D from the Basidiomycota fungus *Phanerochaete chrysosporium*. *J Biol Chem* 288(18):12828–12839.
- Li X, Beeson WT, 4th, Phillips CM, Marletta MA, Cate JHD (2012) Structural basis for substrate targeting and catalysis by fungal polysaccharide monooxygenases. *Structure* 20(6):1051–1061.
- Macedo S, et al. (2009) Can soaked-in scavengers protect metalloprotein active sites from reduction during data collection? *J Synchrotron Radiat* 16(Pt 2):191–204.
- Smith TA, Penner-Hahn JE, Hodgson KO, Berding MA, Doniach S (1984) Polarized Cu K-edge studies. *EXAFS and Near Edge Structure III. Proceedings of an International Conference* (Springer, New York), pp 58–60.
- Kau LS, Spirasolomon DJ, Pennerhahn JE, Hodgson KO, Solomon EI (1987) X-ray absorption edge determination of the oxidation state and coordination number of copper: Application to the type-3 site in *Rhus vernicifera* laccase and its reaction with oxygen. *J Am Chem Soc* 109(21):6433–6442.
- Balasubramanian R, et al. (2010) Oxidation of methane by a biological dicopper centre. *Nature* 465(7294):115–119.
- Smith SM, et al. (2011) Crystal structure and characterization of particulate methane monooxygenase from methylocystis species strain M. *Biochemistry* 50(47):10231–10240.
- Marcus RA (1964) Chemical and electrochemical electron-transfer theory. *Annu Rev Phys Chem* 15:155–196.
- Marcus RA (1993) Electron-transfer reactions in chemistry: Theory and experiment (Nobel lecture). *Angew Chem Int Ed Engl* 32(8):1111–1121.
- Wood PM (1988) The potential diagram for oxygen at pH 7. *Biochem J* 253(1):287–289.
- Kim S, Ståhlberg J, Sandgren M, Paton RS, Beckham GT (2014) Quantum mechanical calculations suggest that lytic polysaccharide monooxygenases use a copper-oxyl, oxygen-rebound mechanism. *Proc Natl Acad Sci USA* 111(1):149–154.
- Prigge ST, Eipper BA, Mains RE, Amzel LM (2004) Dioxygen binds end-on to mononuclear copper in a precatalytic enzyme complex. *Science* 304(5672):864–867.
- Evans JP, Ahn K, Klinman JP (2003) Evidence that dioxygen and substrate activation are tightly coupled in dopamine β-monooxygenase. Implications for the reactive oxygen species. *J Biol Chem* 278(50):49691–49698.
- Chen P, Root DE, Campochiaro C, Fujisawa K, Solomon EI (2003) Spectroscopic and electronic structure studies of the diamagnetic side-on CuII-superoxo complex Cu(O₂) [HB(3-R-5-iPrpz)₃]: Antiferromagnetic coupling versus covalent delocalization. *J Am Chem Soc* 125(2):466–474.
- Kunishita A, et al. (2009) Mononuclear copper(II)-superoxo complexes that mimic the structure and reactivity of the active centers of PHM and DbetaM. *J Am Chem Soc* 131(8):2788–2789.
- Ginsbach JW, Peterson RL, Cowley RE, Karlin KD, Solomon EI (2013) Correlation of the electronic and geometric structures in mononuclear copper(II) superoxide complexes. *Inorg Chem* 52(22):12872–12874.
- Würtele C, et al. (2006) Crystallographic characterization of a synthetic 1:1 end-on copper dioxygen adduct complex. *Angew Chem Int Ed Engl* 45(23):3867–3869.
- Donoghue PJ, Gupta AK, Boyce DW, Cramer CJ, Tolman WB (2010) An anionic, tetragonal copper(II) superoxide complex. *J Am Chem Soc* 132(45):15869–15871.
- Kunishita A, et al. (2012) Active site models for the Cu(A) site of peptidylglycine α-hydroxylating monooxygenase and dopamine β-monooxygenase. *Inorg Chem* 51(17):9465–9480.
- Lanci MP, et al. (2007) Isotopic probing of molecular oxygen activation at copper(II) sites. *J Am Chem Soc* 129(47):14697–14709.
- Isaksen T, et al. (2014) A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides. *J Biol Chem* 289(5):2632–2642.
- Bey M, et al. (2013) Cello-oligosaccharide oxidation reveals differences between two lytic polysaccharide monooxygenases (family GH61) from *Podospora anserina*. *Appl Environ Microbiol* 79(2):488–496.