Structural basis for copper-oxygen mediated C-H bond activation by the formylglycine-generating enzyme

Marcel Meury[a], Matthias Knop[a] and Florian P. Seebeck[a]*

Abstract: The formylglycine-generating enzyme (FGE) is a unique copper-protein that catalyzes oxygen-dependent C-H activation. We describe 1.66 Å- and 1.28 Å-resolution crystal structures of FGE from *Thermomonospora curvata* in complex with either Ag (I) or Cd (II) providing definitive evidence for a high-affinity metal-binding site in this enzyme. The structures reveal a bis-cysteine linear coordination of the monovalent metal, and tetrahedral coordination of the bivalent metal. Similar coordinational change may occur in the active enzyme as a result of Cu (I)/(II) redox cycling. Complexation of copper by two cysteines is common among copper-trafficking proteins, but is unprecedented for redox-active copper-enzymes or synthetic copper catalysts.

Copper is a versatile catalyst for the transfer of electrons from organic matter to molecular oxygen,[1] and some copper-oxygen adducts can cleave very strong C-H bonds.[2] Understanding the nature of these catalytic species and developing means to control their specific activities by proteins or synthetic ligands, is a major scientific objective. Enzymes that catalyze copper-dependent oxidations must provide metal-binding sites that allow redox cycling of the metal cofactor. In addition, the enzyme must bind all redox states with high affinity, because the free copper concentration in biological media is usually very low.[3] Mononuclear copper enzymes such as lytic polysaccharide monooxygenases (LPMO), peptidylglycine-α-hydroxylating monooxygenase (PHM) or the related dopamine-β-hydroxylase meet these requirements by histidine-dominated coordination spheres (Figure 1).[4]

In contrast, proteins involved in cellular copper trafficking such as the chaperone CopZ, the efflux pump CopA or the transcriptional activator CueR contain two cysteine residues that coordinate Cu (I) in a near-linear coordination geometry (Figure 1).[5] This motif provides exceedingly high affinities (K_D = 10^{17} – 10^{21} M^{-1}),[5d] and yet enables rapid metal transfer from one copper-protein to another, through an associative mechanism.[5d, 6] In general, these copper binding proteins suppress the redox activity of their metal cargo.

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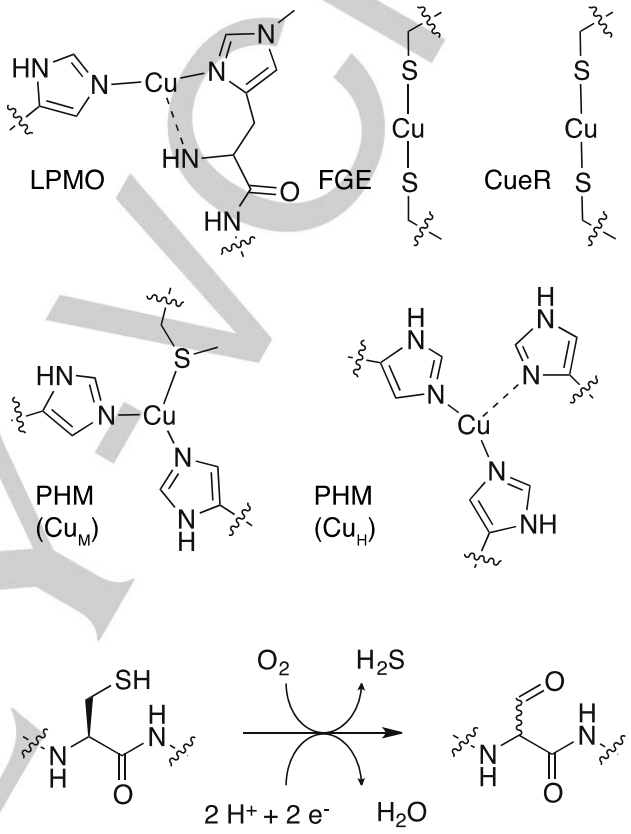

Figure 1. Top: Metal sites in Cu (I) binding proteins: LPMO (PDB:4ALT),[4b] PHM (Cu$_M$ site, PDB:3PHM);[4a, 7] CueR (PDB:1Q05);[5d] Bottom: FGE catalyzed transformation of peptidyl-cysteine to formylglycine (fGly).

In this report we describe an enzyme that blurs the structural distinction between copper-dependent oxidases and copper-trafficking proteins. We demonstrate that the copper-dependent formylglycine-generating enzyme (FGE) uses a chaperone-like metal-binding site to catalyze oxidative C-H activation. FGE converts specific cysteine residues of sulfatases and some phosphatases to formylglycine (fGly, Figure 1).[8] Because FGE can also introduce fGly into a broad range of recombinant proteins, this enzyme has been recognized as a powerful tool in protein engineering.[8d, 9]

FGE extracts a hydrogen atom and an electron from its peptidyl substrate and two electrons from a thiol co-substrate to reduce oxygen to water.[10] FGE contains two active site cysteines (Cys 269 and Cys274) that coordinate Cu (I) with high affinity (K_D = 10^{17} M^{-1}).[11] In the absence of reducing equivalents these residues oxidize rapidly to a disulfide causing the enzyme to loose any copper-affinity.[11] This instability may be one reason why this cofactor has escaped

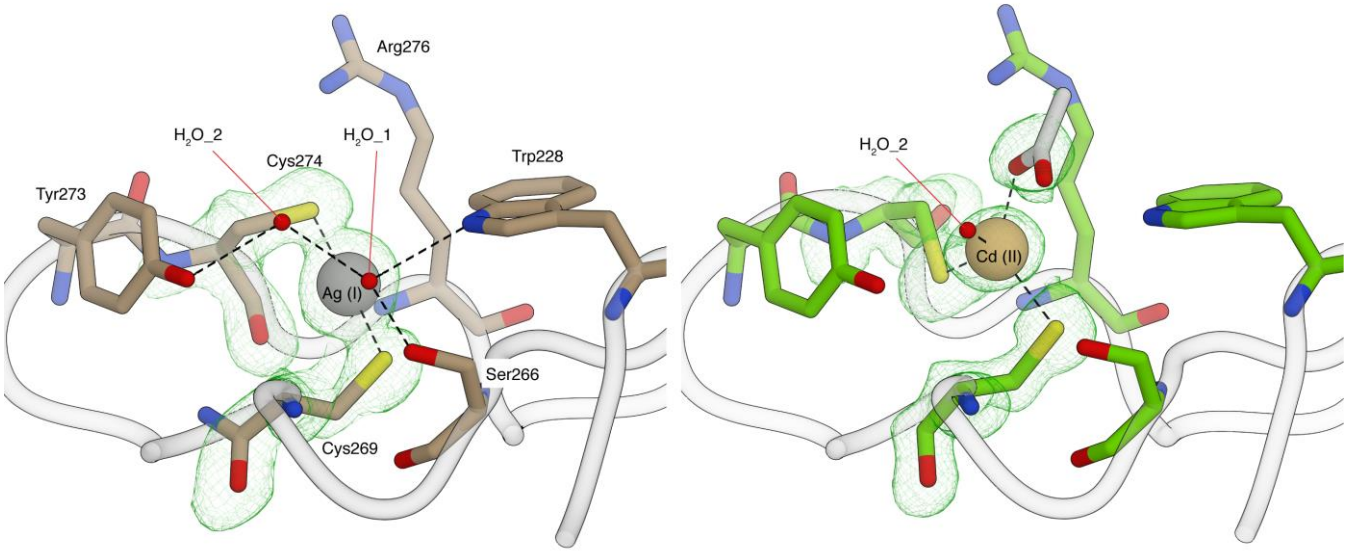

Figure 2. Structure of the active site from $FGE_{curvata}$ in complex with Ag (I) (left, 1.66 A resolution), or Cd (II) (right, 1.28 A resolution). The green mesh depicts electron densities of $m|F_o|-D|F_c|$ omit maps contoured at 3-σ. Stereoviews of the same pictures are shown in Figure S6 & S7.

detection in numerous crystallographic studies on FGE from human (FGE_{human}) and from *Streptomyces coelicolor* ($FGE_{strepto}$).[10a, 12] In fact, the copper-dependence of this catalyst has only been discovered recently using biochemical methods.[10b, 10c]

To avoid this oxidation problem we chose to crystallize FGE from *Thermomonospora curvata* ($FGE_{curvata}$) in complex with Ag (I) instead of Cu (I). Owing to its much higher redox potential ($E°_{Ag(I)/Ag(II)}$: +2.0 V, $E°_{Cu(I)/Cu(II)}$: +0.16 V), silver does not easily reduce oxygen.[13] On the other hand, Ag (I) is also a d^{10} system and has proven an excellent Cu (I) mimic in copper-transporting ATPases,[14] copper-sensing transcription factors,[5d, 15] and copper-chaperones.[16] In keeping with this record, we found that Ag (I) is a potent Cu (I) competitive inhibitor of $FGE_{curvata}$ (Figure S1-S2).

For crystallization we produced the 20 – 303 residue fragment of $FGE_{curvata}$ fused to an N-terminal His-tag (see supporting information). This protein was crystallized in the presence or absence of $AgNO_3$. The resulting crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions of *a* = 41.39 Å, *b* = 67.51 Å and *c* = 100.19 Å or *a* = 40.95 Å, *b* = 65.57 Å and *c* = 99.5 Å, respectively. In both structures, the asymmetric unit includes one molecule per asymmetric unit. The electron-density of the Ag (I) containing crystal reveals a continuous polypeptide chain between the N-terminal His-tag and Pro302, with the exception of one missing segment between Val92 and Asp100. The residues are numbered according to the gene locus for FGE from *T. curvata* (Tcur_4811). The overall structure of $FGE_{curvata}$ is very similar to FGE_{human} (58 % sequence identity, PDB: 1Y1I, rmsd: 0.55 Å, 255 atoms),[10a] and $FGE_{strepto}$ (64 % sequence identity, PDB: 2Q17, chain C, rmsd: 0.63 Å, 258 atoms)(Figure S4).[12b] The residues that contribute to the active site surface are nearly identical and adopt similar conformations in all three FGE homologs. Most importantly, the five-residue loop between the two active site Cys adopts indistinguishable conformations, indicating that metal binding requires minimal reorganization of the active site.

The two Cys residues ligate Ag (I) with an angle of 178°, and a sulfur-to-sulfur distance of 4.6 Å (Figure 2). This geometry is reminiscent of several high-affinity copper-binding proteins in complex with Ag (I) (4WLW,[15a] 1Q06,[5d] 5F0W). The metal makes no further contacts with FGE. The nearest neighbor is a crystallographic water/hydroxide (3.6 Å, H_2O_1) that hydrogen bonds to Trp228 (2.9 Å), Ser266 (2.7 Å) and a second water molecule (2.6 Å, H_2O_2). H_2O_2 also hydrogen bonds to Tyr273 (2.8 Å), and also makes no direct contact to the metal (4.3 Å). In a crystal structure of FGE_{human} (PDB:2AIJ),[12a] a chloride occupies the equivalent position of H_2O_1, indicating that this pocket can stabilize negative charge. Therefore it is possible that H_2O_1 is in fact a hydroxide.

The similarity between the metal binding sites in $FGE_{curvata}$ and Cu (I) chaperones provides a convincing explanation for the very high Cu (I) affinity of FGE. For a redox catalyst, on the other hand, bis-cysteine coordination of Cu (I) is unprecedented. Therefore we find this structure more difficult to interpret in terms of the catalytic mechanism. However, a comparison with the copper-binding sites in PHM and LPMO is revealing. In both enzymes redox-cycling of the cofactor is accompanied by substantial change of the coordination geometry.[4b, 7]

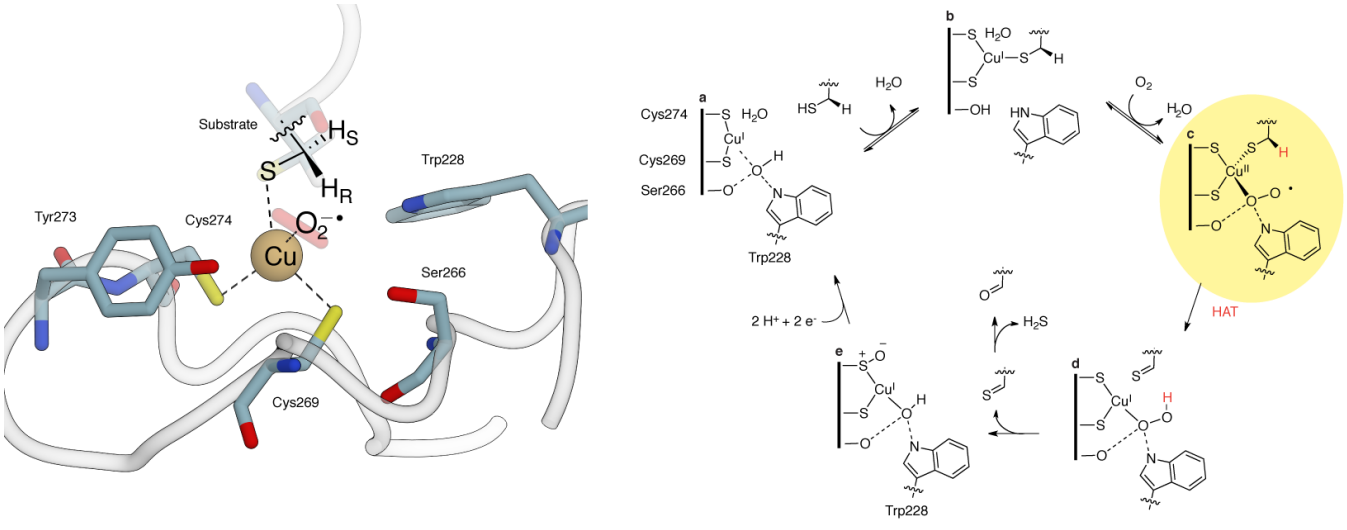

Figure 3. Left: Model of $FGE_{curvata}$ as the Cu (II) superoxo-complex in complex with the peptidyl-cysteine substrate. **Right:** Proposed catalytic mechanism of FGE (see text). The Cu (II) superoxo-complex is highlighted in yellow.

In PHM the Cu_H site changes from bis-histidine coordinated Cu (I)(Figure 1) to tetrahedral coordination of Cu (II) by three histidines and one water ligand. The Cu_M site binds Cu (I) through two histidines and one methionine in trigonal geometry (Figure 1). Oxidation of this copper introduces one or two water ligands to form a tetragonal complex.[7, 17] In LPMO two histidines coordinate Cu (I) in a T-shaped geometry (Figure 1), whereas Cu (II) binds two additional water ligands to form a trigonal bipyramidal complex.[4b]

To examine if FGE undergoes similar conformational change when the oxidation state of the metal increases we also analyzed the crystal structure of $FGE_{curvata}$ in complex with Cd (II). Divalent cadmium is a thiophilic d^{10} system with similar complex chemistry as Cu (II). Indeed, Cadmium has been used as a copper-mimic at sulfur-rich tetrahedral coordination sites in proteins.[5b, 6a] Most importantly, Cd (II) forms a redox-stable complex with FGE, whereas Cu (II) does not.[11]

The 1.28 Å resolution structure of Cd (II) bound $FGE_{curvata}$ is nearly indistinguishable from the Ag (I) structure (Figure 2). However, closer inspection revealed specific changes in the active site. Cd (II) is bound in a tetrahedral coordination sphere consisting of Cys269 (2.5 Å), Cys274 (2.5 Å), an acetate molecule (2.5 Å) and a water molecule (2.4 Å) in the same position as H_2O_2 described in the Ag (I) structure. To accommodate the change from linear to tetrahedral coordination the metal moved 1.8 Å away from the Ag (I) position and towards H_2O_2, which moves slightly towards the metal. In addition, the side chain of Cys274 rotated by 67° along the Cα-Cβ bond to form a S-Cd-S bond angle of 100°. This rotation is coupled to a 135° flip of the amide bond between Cys274 and Asn275, caused by large changes of the adjacent dihedral angles ($\Delta\psi_{Cys274}$ = -139°; $\Delta\phi_{Asn275}$= 154°)(Figure S5). The apo structures from FGE_{human} and $FGE_{strepto}$ adopt the same conformation as the Ag (I) complex, indicating that this conformation of Cys274 is more stable – and that the active site is optimized for binding of monovalent metals by linear coordination. In keeping with this idea, bivalent metals – with the exception of Hg (II) – are poor copper-competitive inhibitors of FGE (Figure S3).

The presence of the acetate ligand in the Cd (II) structure is suggestive of a possible substrate-binding mode. This ligand demonstrates that the coordination site can accommodate a third anionic ligand. Hence, it is conceivable that the thiolate of the substrate may directly coordinate to the metal. The binding mode of the substrate has been examined before based on the structure of FGE_{human} in complex with a short substrate analog (PDB:2AIJ).[12a] This protein contained no metal in the active site, one of the active site Cys was mutated to Ser, and the other formed a disulfide bond to the substrate. Clearly, this constellation does not represent a relevant species on the catalytic cycle. However, superposition of this structure with the Cd (II) complex of $FGE_{curvata}$ places the sulfur atom of the substrate exactly on top of the metal-coordinating oxygen atom of acetate. Based on this superposition we constructed a model of $FGE_{curvata}$ in complex with a bivalent metal and the peptide substrate (Figure 3). The positions of H_2O_1 and H_2O_2 were used to approximate the position of metal bound oxygen.

We have to emphasize that the observed Cd (II) structure may not reflect the precise geometry of the catalytically relevant Cu (II) species. However, the model does remarkably well in explaining experimental observations. For example, in this model the *pro*-R-β-hydrogen of the substrate cysteine residue (Figure 3) points towards the metal-coordinated oxygen. This geometry is fully consistent with the observation that FGE abstracts the pro-R hydrogen from the substrate.[10b]

The suggestion that the substrate is a copper ligand is also consistent with the observation that the presence of substrate increases the Cu (I) affinity of FGE. In contrast, a substrate analog that contains a Ser in place of Cys is a poor FGE ligand, and does not increase the Cu (I) affinity of FGE.[11]

Furthermore, the model implicates Trp228, Ser266 and Tyr273 as catalytic residues. In the Ag (I) and Cd (II) structures the three side chains hydrogen bond with H_2O_1 and H_2O_2. The same interactions would provide an ideal environment for oxygen activation.[18] Indeed, in a previous study we did find that the Ser266Ala mutation reduces k_{cat} by 270-fold, but leaves $K_{M,substrate}$ and the affinity for Cu (I) unaffected.[11] These effects are consistent with a role of Ser266 in oxygen activation. In contrast, the Tyr273Phe mutation *increases* k_{cat} by 6-fold and left K_M or Cu (I) affinity unaffected.[19] The same mutation in FGE_{human} produced similar results.[10a] Given the considerable conservation of this residue across bacterial and eukaryotic FGE homologs, we believe that Tyr273 may have an important function outside the catalytic cycle. For example, its stabilizing interaction to H_2O_2 may prevent oxygen activation in the absence of substrate.

The Ag (I) and the Cd (II) structures demonstrate that FGE can accommodate mono- and bivalent metal with very little active site reorganization. This flexibility provides a plausible model for how this chaperone-like copper-binding site can expand the coordination sphere to allow substrate binding and Cu (I)/(II) redox cycling. In view of these structures, and based on published kinetic data we propose the following catalytic mechanism. The copper-containing resting state of FGE (**a**, Figure 3) is represented by the Ag (I) structure, [5d, 15a, 20] although it is possible that the equilibrium S-Cu-S bond angle is somewhat smaller than 180°.[20] Substrate binding ejects a water from the active site and leads to trigonal sulfur-only coordination of Cu (I) (**b**). This electron-rich complex may then bind and reduce oxygen to form a tetrahedral Cu (II) superoxo complex (**c**) depicted by the model shown in Figure 3 (left). This species should be a proficient reagent for abstraction of the *pro*-R-β-hydrogen atom from the substrate.[2a, 2b, 21] Hydrogen atom abstraction is coupled to electron transfer from the substrate to the Cu (II) center to form a Cu (I) hydroperoxo species (**d**) and a thioaldehyde peptide. This product leaves the enzyme and hydrolyzes spontaneously to the fGly-containing peptide and hydrogen sulfide. The remaining complex decays into an oxidized form of FGE that requires two external electrons to return to the resting state (**a**).[10b]

Conclusions. This report describes the metal-binding site of a novel type of mononuclear copper-enzyme. Comparison of FGE in complex with Ag (I) or Cd (II) with published structures of homologous apo-enzymes provides a plausible model for substrate-binding, oxygen-activation and stereoselective hydrogen-atom abstraction. These structures pave the way for kinetic, spectroscopic and computational methods to elucidate the mechanism of this unorthodox metallo-enzyme. These findings provide new perspectives for the design and discovery of novel copper-enzymes or synthetic catalysts. In addition, the realization that the sulfatase-modifying FGE is a copper-dependent enzyme raises novel questions about the genetic and metabolic causes of multiple sulfatase deficiencies.[8a, 8c, 10a]

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Keywords: formylglycine • copper enzyme • copper chaperone • catalysis • C-H activation

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