Copper is a cofactor of the formylglycine generating enzyme

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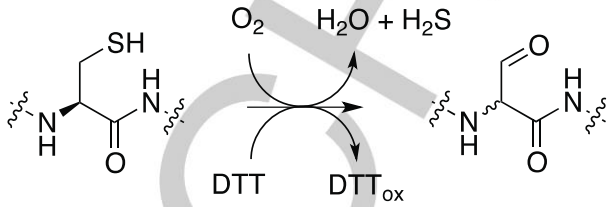
Abstract: The formylglycine generating enzyme (FGE) is an O_2 utilizing oxidase that converts specific cysteine residues of client proteins to formylglycine. In this report we show that Cu(I) is an integral cofactor of this enzyme and binds with high affinity (K_D = of 10^{-17} M) to a pair of active site cysteines. These findings establish FGE as a novel type of copper enzyme.

Formylglycine generating enzymes (FGE) catalyze O_2 dependent conversion of specific cysteine residues on client proteins to formylglycine (fGly) (Scheme 1). This posttranslational modification is essential for the catalytic activity of phosphatases and sulfatases.[1] FGE activity in human cells lead to sulfatase deficiencies.[2] In addition, FGE has emerged as a versatile tool for protein engineering, because it can introduce unique aldehyde functions into recombinant proteins. [1a, 3] Initial biochemical and structural characterization of this enzyme raised an interesting mechanistic question: How does this enzyme activate O_2? None of the published crystal structures of this catalyst revealed any known redox cofactor.[4] The only redox active features in the active site are two conserved cysteine residues which by themselves can hardly activate O_2.[5],[6] One electron transfers between thiols and O_2 are prohibited by mismatched redox potentials, and ionic mechanisms are spin forbidden.

Recently we and others found that copper salts increase the *in vitro* activity of FGE by up to 20-fold.[7] Although copper is a plausible agent in O_2 activation,[8] the interaction between FGE and transition metals remained mysterious.

The general absence of copper in published crystal structures suggested that a potential FGE:copper complex may not be very stable.[4] On the other hand, Cu(I) dependent FGE activity is not affected by millimolar concentrations of EDTA,[7b] which is a strong ligand for Cu(II) (pK_D = 18.8) or DTT, which is a strong ligand for Cu(I) (pK_D = 15.3).[9] Hence, FGE is either an even stronger copper ligand, or does not require direct metallation. In this later scenario copper may serve as an artificial source of electrons or activated oxygen species in the *in vitro* assay.[10]

In this report we address this puzzle. We show that FGE from *Thermomonospora curvata* ($FGE_{curvata}$) binds Cu(I) with an affinity reminiscent of known high-affinity copper proteins.[9] We found that Cu(I) binds to both active site cysteines and



Scheme 1. FGE catalyzes O_2-dependent conversion of cysteine residues to formylglycine (fGly), H_2S and water. To complete the four electron reduction of O_2, the enzyme requires an auxiliary reducing agent such as DTT.

remains bound throughout multiple catalytic cycles. We have previously shown that the active site cysteines of $FGE_{curvata}$ readily form a disulfide bond under standard aerobic conditions.[7b] To allow unambiguous determination of the redox state of these two residues, we constructed an FGE variant in which all cysteines outside the active site are mutated to either serine or alanine (FGE_{4C}).[7b] This variant proved seven-fold more active than wild type,[7b] and therefore was used for most of our subsequent experiments. Using FGE_{4C} as a model we could show that the two active site cysteines form a disulfide bond when the purified enzyme is stored in the absence of a reducing agent. [7b]

To estimate Cu(I) affinities of $FGE_{curvata}$ and FGE_{4C} we used a published titration assay.[9] A solution containing the 1:2 complex between Cu(I) and bathocuproine disulfonate (Cu(I):$(BCS)_2$) was titrated with FGE_{4C} (Figure S1). Transfer of Cu(I) from the BCS complex to FGE was monitored by measuring the decreasing absorption of the Cu(I):$(BCS)_2$ complex at 483 nm. This qualitative experiment revealed that oxidized FGE_{4C} has no greater affinity for Cu(I) than the control protein bovine serum albumin (BSA). By contrast, FGE_{4C} purified in reduced form showed significant Cu(I) affinity (Figure S1). Because the oxidized and reduced form of FGE_{4C} only differ by the redox state of the two active site cysteines, we concluded that these thiols must be essential for Cu(I) binding.

For a more quantitative estimation of $K_{D,Cu(I)}$ we recorded the absorption at 483 nm as a function of FGE_{4C} or $FGE_{curvata}$ concentration and fitted the resulting curves to an equation describing the equilibrium between the Cu(I):$(BCS)_2$ complex and the FGE:Cu(I) complex (see supporting information).[9] All titration buffers contained 2 mM cysteamine to keep the enzymes in reduced form. Because cysteamine is a comparably weak Cu(I) binder (K_D = $10^{-14.1}$ M) (Table 1), its presence should not affect the apparent Cu (I) affinity of FGE. As a test of this assumption we determined the apparent Cu(I) affinity of DTT in the absence (K_D = $10^{-15.1}$ M) and in the presence ($K_{D,cysteamine}$ = $10^{-15.6}$ M) of cysteamine. Both values are in fair agreement with the published value ($K_{D,lit}$ = $10^{-15.3}$ M).[9] With this assay we determined an apparent dissociation constant (K_D, Table 1) of the Cu(I) complexes with FGE_{4C} or

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Table 1. Kinetic parameters and Cu(I) affinities of FGE variants and auxiliary thiols.[a]

	k_{cat} [min^{-1}]	K_M [μM]	k_{cat}/K_M [$min^{-1}M^{-1}$]	$pK_{D,Cu(I)}$
$FGE_{curvata}$	1.6 ± 0.1	580 ± 40	2900 ± 50	17.1 [i]
FGE_{4C}	4.2 ± 0.5	230 ± 40	20 000 ± 4000	17.1 [i]
FGE_{S266A}	0.006	520 ± 240	49 ± 8	17.7 [i]
FGE_{S290K}	n.a.	n.a.	6.6 ± 0.2	16.7 [i]
FGE_{C269S}	n.a.	n.a.	≤ 1	n.a.
FGE_{C274S}	n.a.	n.a.	≤ 1	n.a.
Cysteamine				14.1[ii]
DTT				15.1[ii],15.6 [i]
DTBA				15.8[ii]

a) Michaelis Menten parameters for FGE catalysed oxidation of a Cys containing substrate peptide to the fGly-containing product were determined as described in the supporting information. n.a.) low specific activities prevented accurate determination of these parameters. Apparent dissociation constants (K_D) of Cu (I) complexes with FGE variants or low molecular weight thiols were determined using a published titration assay.[9] i) these values were determined in the presence of 2 mM cysteamine. ii) in the absence of additional thiols.

$FGE_{curvata}$. Since both proteins bind Cu(I) with similar strength we concluded that none of the Cys residues outside the active site contributes to copper binding. Similar complex stabilities have been reported for copper chaperones from humans (Atox1, $K_D = 10^{-17}$ M)[9], *Saccharomyces cerevisiae* (Atx1, $K_D = 10^{-17}$ M)[9] or *Bacillus subtilis* (CopZ, $K_D = 10^{-17}$ M),[11] suggesting that FGE should be well equipped to procure copper in a cellular context.[12]

On the other hand, it remains puzzling that FGE is fully active in a millimolar DTT solution. The apparent Cu(I) affinity of DTT is 10^2-fold lower than that of FGE. A 10^3-fold excess of DTT should therefore destabilize the FGE:Cu(I) complex. This is not what we observed. Reactions containing 2 μM FGE, 2 μM Cu(I) and 2 mM of either cysteamine ($K_D = 10^{-14.0}$ M), DTT ($K_D = 10^{-15.1}$ M) or dithiobutylamine (DTBA, $K_D = 10^{-15.8}$ M)[13] displayed approximately the same rate of product formation (Figure S2), showing that the Cu(I) affinity of the redox buffer does not influence catalytic activity.

One explanation for this behavior could be that the Cu(I) affinity of FGE in the presence of substrate is at least two orders of magnitude higher than that determined for the resting enzyme. We could not directly measure the Cu(I) affinity of the enzyme:substrate complex because the substrate peptide (sequence: Abz-SAL**C**SPTRA-NH_2) is a proficient Cu(I) binder in its own right, meaning that saturating concentrations of this peptide are incompatible with the titration assay. A substrate analog containing a Ser instead of the Cys residue (sequence: Abz-SAL**S**SPTRA-NH_2) did not interfere with this assay, but also proved a poor FGE ligand (Figure S3). Consequently, the presence of this peptide did not change the Cu (I) affinity of either FGE_{4C} or FGE_{S266A}.

As an alternative strategy to gauge the influence of the substrate on Cu (I) binding by FGE we analyzed the ability of FGE_{4C} and inactive FGE variants to exchange Cu (I) during catalysis. To conduct this experiment, we designed four variants of FGE_{4C} using a structural model based on the crystal structure of human FGE (Figure 1). We produced two

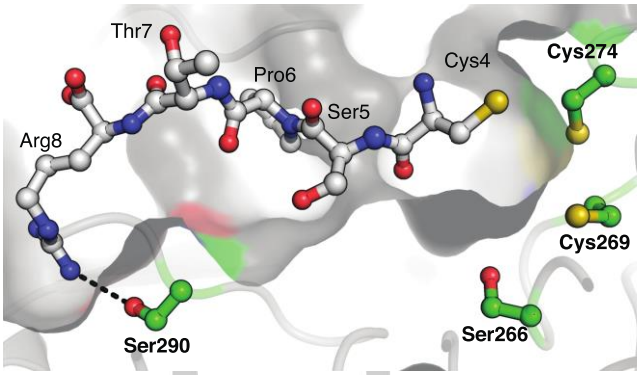


Figure 1. Structural model of $FGE_{curvata}$ based on the structure of human FGE (PDB code: 2AIJ).[4c, 14] Residues 4 – 8 of the substrate were modeled according to a similar substrate bound to the human enzyme. This model suggests that Ser290 makes a 3.0Å hydrogen bond to Arg8 on the substrate (dashed line).

variants that each has one of the active site cysteines mutated to serine (FGE_{C269S} and FGE_{C274S}). Both proteins proved essentially inactive and devoid of measurable Cu(I) affinity (Table 1). Apparently both thiols are important for Cu(I) binding and also for catalysis. The third variant has a conserved active site serine at position 266 mutated to alanine (FGE_{S266A}). This mutation reduced k_{cat} by 270-fold, but did not affect K_M, and did not interfere with copper binding ($K_D = 10^{-17.6}$ M). The mutation in the fourth variant (FGE_{S290K}) was designed to impair substrate binding. Ser290 is located at the bottom of the substrate binding groove, more than 15 Å away from the catalytic site (Figure 1).[4c] We mutated this Ser residue to Lys in order to block substrate binding through steric and coulombic repulsion. As expected, the corresponding protein can not be saturated with substrate and the catalytic efficiency (k_{cat}/K_M) is reduced by 440-fold. At the same time Cu(I) affinity was only reduced by 2.5 fold ($K_D = 10^{-16.7}$ M) (Table 1).

We tested the ability of these variants to compete with FGE_{4C} for Cu(I) during catalysis in reactions containing 0.5 μM FGE_{4C}, 0.5 μM $CuSO_4$, 2 mM DTT, 50 mM EDTA and 200 μM substrate (Figure 2). One minute after these reactions were initiated by addition of FGE_{4C}, we added a 9-fold excess of FGE_{C269S}, FGE_{C274S}, FGE_{S266A}, FGE_{S290K} or BSA (Figure 2). Addition of FGE_{S266A} reduced FGE_{4C} activity by approximately 15-fold ($\mathbf{m_2}/\mathbf{m_1}$, Figure 2a), consistent with redistribution of limiting Cu(I) among 0.5 μM FGE_{4C} and 4.5 μM FGE_{S266A}. Addition of more Cu (I) to this inhibited reaction immediately restored full activity to FGE_{4C} (Figure S4), confirming Cu (I) as the limiting factor.

The rate at which Cu (I) redistributed between FGE_{4C} and FGE_{S266A} (0.07 ± 0.02 min^{-1}, Figure 2) provides an estimation of how fast the FGE_{4C}:Cu(I) complex decays ($k_{off,Cu(I)}$) (Figure 2). This rate is eleven-fold slower than catalytic turnover ($\mathbf{m_2}/[FGE_{4C}] = 0.8$ min^{-1}), meaning that metal binding and unbinding cannot be part of the catalytic cycle. The same competition experiment shows that BSA, FGE_{C269S}, FGE_{C274S} or FGE_{S290K} cannot extract Cu(I) from FGE_{4C}. For BSA and the two cysteine variants this result is consistent with their complete lack of Cu(I) affinity (Table 1). FGE_{S290K} on the other

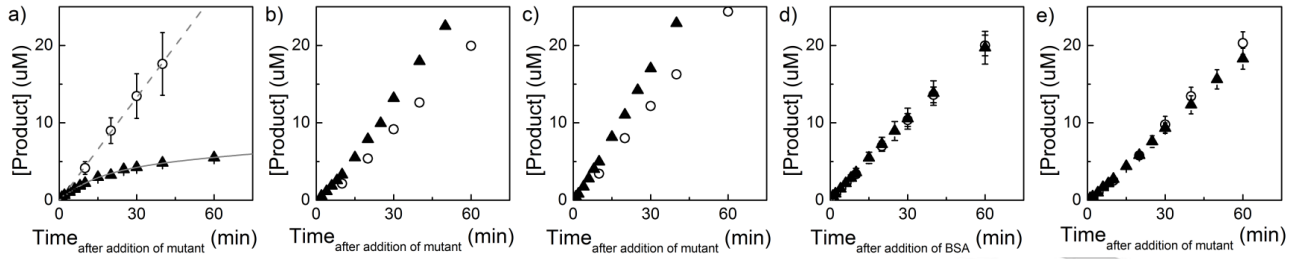


Figure 2: FGE_{4C} catalyzed product formation as a function of time (○) in reactions containing 0.5 µM FGE_{4C}, 200 µM substrate, 0.5 µM $CuSO_4$, 2 mM DTT, 50 mM EDTA, 50 mM NaCl and 50 mM Tris pH8. Identical reactions were supplemented with 5 µM $FGES_{266A}$ (A), FGE_{C274S} (B), FGE_{C269S} (C), BSA (D), $FGES_{290K}$ (E), one minute after initiation with FGE_{4C} (▲). In Figure 2a the ▲-data were fitted to the function $[P] = A\cdot(1-\exp(-k_{off,Cu(I)}\cdot t))+m_1\cdot t$ (solid line), the O-data were fitted to the function $[P] = m_2\cdot t$ (dashed line). A corresponds to the concentration of product formed until Cu(I) redistribution between FGE_{4C} and $FGES_{266A}$ is completed (= 4.0 µM). $k_{off,Cu(I)}$ corresponds to the rate at which the FGE_{4C}:Cu(I) complex decays (= 0.07 min^{-1}). m_1 corresponds to the residual activity after Cu(I) redistribution ($m_1/[FGE_{4C}]$ = 0.05 min^{-1}). m_2 corresponds to the activity of Cu(I) complemented FGE_{4C} ($m_2/[FGE_{4C}]$ = 0.8 min^{-1}). The presented data corresponds to averaged values of two or more independent experiments.

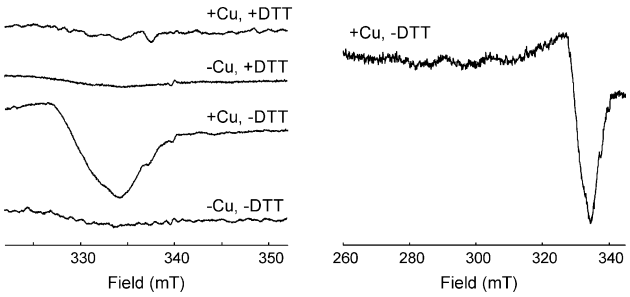
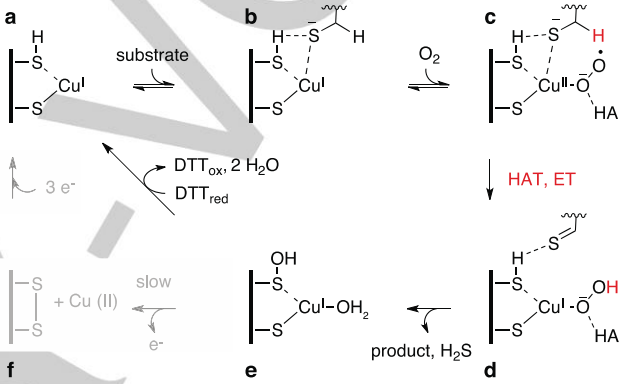


Figure 3. X-band (~9.52 GHz) continuous-wave EPR spectra of 50 µM FGE_{4C} and 0.5 mM substrate in the presence and absence of 50 µM $CuSO_4$ and 5 mM DTT. Temperature: 150 K, modulation amplitude: 1 mT, microwave attenuation: 15 dB (6.346 mW power), 160 scans each. Spectra were acquired on a Bruker Elexsys 500 spectrometer equipped with a super-high Q resonator. **Left:** Detail spectra in the region of the strongest Cu(II) signal component. **Right:** Full spectrum of the Cu(II) species observed in the absence of DTT.



Scheme 2. Plausible catalytic mechanism of FGE catalyzed formylglycine formation.

hand, is a strong Cu (I) binder (Table 1) but its ternary complex with copper and substrate is weak. The observation that $FGES_{290K}$ cannot sequester Cu (I) from the FGE_{4C} catalyzed reaction is consistent with the idea that substrate binding increases the apparent Cu (I) affinity of the enzyme. This finding, in combination with the observation that the Ser containing substrate analog is a poor FGE ligand indicate that the thiol function of the substrate might be the third copper ligand in the active site.

As a final experiment we used EPR spectroscopy to probe the redox state of copper bound to FGE_{4C}. Freeze quenched reactions containing FGE_{4C}, Cu(I), EDTA, DTT and substrate yielded a featureless EPR spectrum, not significantly different from that measured with a control sample without added copper (Figure 3). Apparently, the accumulating copper species during catalysis is EPR silent. By contrast, a sample without DTT showed the clear EPR signal of Cu(II), whereas a control reaction containing no DTT and no added copper again is EPR silent (Figure 3). Accumulation of a Cu(II) species is consistent with the fact that in absence of DTT FGE_{4C} oxidizes to the disulfide form and looses any affinity for copper.

The combination of catalytic and structural properties of FGE described here and elsewhere [4a-c, 7, 15] strongly implicates FGE as a copper-metalloenzyme: The active form of FGE strongly binds one equivalent of Cu (I) in the active site;[7b] the Cu(I):protein complex remains intact throughout the entire catalytic cycle (Figure 2); other transition metals cannot complement FGE,[7] suggesting that the cofactor engages in redox chemistry;[8] FGE reduces O_2 using two electrons form the substrate and two electrons from an auxiliary reducing agent such as DTT;[4a, 7b] in presence of DTT the rate limiting step is hydrogen atom abstraction from the substrate; [7b] the accumulating species during catalysis is an EPR silent species (Figure 3); in absence of an appropriate reducing agent turnover becomes much slower,[4a, 4b, 7] and a Cu(II) containing species accumulates (Figure 3); this oxidized species can slowly turn over using the substrate thiol as an electron source;[7b] and finally, addition of a proper reducing agent to this slow reaction immediately reactivates the enzyme.[7b, 15] In our view these observations are best consolidated in the following mechanistic proposal (Scheme 2): The cuprous state of FGE (**a**) binds substrate (**b**) and O_2 to form a cupric superoxo intermediate (**c**). Hydrogen atom transfer (HAT) and electron transfer (ET) from the substrate reduce this intermediate to a Cu (I) hydroperoxo complex (**d**). The resulting thioaldehyde hydrolyzes to form the fGly containing product and hydrogen sulfide and the hydroperoxo complex collapses into a stable but oxidized form of FGE (**e**). In presence of DTT this species is quickly reduced to the active resting state (**a**). In absence of a proper reducing agent,

species **e** decays to the disulfide form of FGE (**f**) that does not bind copper. Therefore Cu (I) leaves the active site and oxidizes to Cu (II). A much slower three-electron process restores the reduced FGE:Cu(I) complex (**a**).

The absence of any EPR signature during turnover may be explained by the following scenarios: a) the cupric superoxo species (**c**, Scheme 2) is not formed or does not accumulate to a significant extent, even though the following HAT is rate limiting; b) species **c** does accumulate but has a diamagnetic singlet ground state due to a highly covalent interaction between Cu (II) and superoxide;[16] or **c**) the accumulating Cu (II) species has a triplet ground state hat does not produce an EPR signature in the X-band spectrum.[17] The reactivity of previously characterized Cu (II) superoxo complexes showed that diamagnetic species generally do not cleave C-H bonds,[16, 18] whereas paramagnetic species do.[19] Based on this precedence we predict that FGE forms an EPR-silent paramagnetic Cu (II) superoxo species that mediates homolytic C-H bond cleavage.

A similar sequence of events has been implicated in the catalytic mechanisms of the copper enzymes polysaccharide monooxygenase (PMO)[20] and peptidylglycine □-hydroxylating monooxygenase (PHM).[8a, 21] In PHM a Cu (II) superoxo species has been shown to cleave the C□-H bond of a C-terminal glycine residue. Electron transfer from a neighbouring Cu (I) center forms the Cu (I) hydroperoxo species, that immediately eliminates water to form Cu (II)-oxyl which in turn hydroxylates the substrate radical. In PMOs a Cu (II) superoxo species has been proposed to extract a hydrogen atom from the anomeric carbon (C1) in polysaccharides. Electron transfer from an auxiliary reducing agent forms a Cu (I) hydroperoxo complex, followed by Cu (II)-oxyl formation, followed by hydroxylation of the substrate radical. FGE, PMO and PHM oxidize their substrates by two electrons and therefore depend on a reducing agent to fully reduce oxygen. PHM activity depends on ascorbate,[8a] in vitro activity of FGE depends on thiols, and PMOs seem to accept electron donors such as gallic acid or the reduced form of cellobiose dehydrogenase.[20a] Future investigations will tell to what detail these three reactions follow analogous pathways.

In conclusion, the presented data shows that FGE is a copper-dependent oxygenase. Although the reduced enzyme:Cu(I) complex is very stable, it is highly sensitive to autooxidation. The apparent instability under aerobic conditions may explain the previous difficulties to trace Cu (I) in the active site of FGE by crystallography. This discovery raises novel questions about the *in vivo* copper delivery to FGE, and highlights a potential connection between oxidative stress, copper homeostasis and sulfatase deficiencies in humans.

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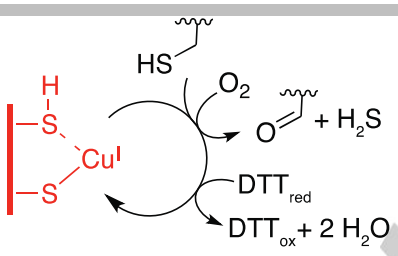
Layout 1:

COMMUNICATION

Biochemical characterization reveals the formylglycine-generating enzyme as a copper-dependent oxidase. The enzyme binds copper with attomolar affinity using two active site cysteine residues as ligands. In the absence of reducing agent, the cysteine residues forms a disulfide bond and the enzyme looses all metal affinity.



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Copper is a cofactor of the formylglycine generating enzyme