In vitro reconstitution of formylglycine generating enzymes requires copper (I)

Matthias Knop,[a] Pascal Engi,[a] Roxana Lemnaru[a] and Florian P. Seebeck[a]*

Abstract: Formylglycine generating enzymes (FGE) catalyze O_2-dependent conversion of specific cysteine residues of arylsulfatases and alkaline phosphatases to formylglycine. The ability to introduce unique aldehyde functions also into recombinant proteins makes FGE a powerful tool for protein engineering. One limitation of this technology is poor *in vitro* activity of reconstituted FGE. Although FGE has been characterized as a cofactor-free enzyme we report that one equivalent of Cu (I) increases catalytic efficiency by more than 20-fold and unmasks stereoselective C-H bond cleavage at the substrate as the rate-limiting step. These findings remove previous limitations of FGE-based protein engineering and also pose new questions about the catalytic mechanism of this O_2-utilizing enzyme.

The formylglycine generating enzyme (FGE) catalyzes O_2-dependent conversion of cysteine residues within a specific peptide motif to formylglycine (fGly, Figure 1).[1] This post-translational modification occurs in the maturation of arylsulfatases and some alkaline phosphatases that use the hydrated form of fGly as catalytic nucleophile.[2] Insufficient FGE activity in human cells causes multiple sulfatase deficiency, which is a rare but fatal disease.[2a, 3] FGE has also become a promising tool in biotechnology,[4] allowing fGly introduction in virtually any recombinant protein provided that a FGE recognition motif is accessible.[5] Genetically encoded aldehyde tags provide excellent handles for selective protein modification.[1, 4a-d, 6] Published protocols for the production of aldehyde bearing proteins recommend co-production of FGE with the protein of interest to achieve efficient insertion of fGly *in vivo*.[4b] The possibility to modify purified proteins *in vitro* would certainly broaden the scope of this technology, but was so far limited by the modest activity of reconstituted FGEs.[5]

Possible reasons for low *in vitro* activity could be incomplete reconstitution of FGE caused by a missing partner enzyme, a missing cofactor or a missing posttranslational modification. These possibilities are difficult to evaluate because the catalytic activity of FGE is poorly understood. Several crystal structures of human and bacterial FGEs provide a detailed picture of the active site consisting of a peptide binding groove, a likely O_2 binding site, and a pair of cysteine residues that are essential for catalysis.[1a, 3a, 7] Surprisingly, none of the structures contained any cofactor that would explain ability of FGE to utilize O_2 as electron acceptor. Although cofactor-independent oxidases are known,

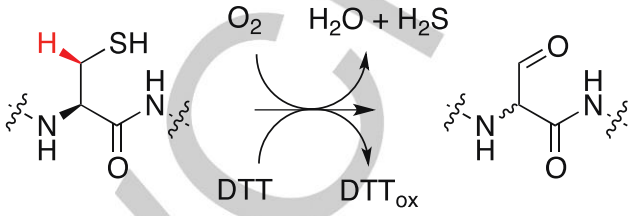

Figure 1. Reaction scheme for FGE catalyzed O_2-dependent formation of formylglycine (fGly). FGE removes the (R)-β-hydrogen atom (red) from the substrate, oxidizes one equivalent of DTT and produces one equivalent of H_2S.

they usually exploit their electron rich substrates to activate O_2 by one-electron transfer.[8] In contrast, O_2 activation by naked polypeptides such as FGE and its peptide substrate would be unprecedented. Therefor we suspected that the crystallized FGEs may not present the complete catalytic principle.

In this report we show that addition of one equivalent of Cu (I) to FGE from *Thermomonospora curvata* or *Mycobacterium smegmatis* increases catalytic efficiency by 20-fold. In presence of copper FGE displays a significant substrate kinetic isotope effect (KIE), suggesting that stereoselective C-H bond cleavage from the substrate is at least partially rate limiting. Four point mutations increase this activity by an additional 10-fold. The overall 200-fold improvement should eliminate previous limitations of FGE as a tool for *in vitro* protein engineering.

To establish a robust study system, we cloned and produced FGE from *T. curvata* (FGE_{wt}). Production in *Escherichia coli* yielded up to 10 mg of purified FGE per culture liter. Owing to its thermophilic origin, this enzyme shows no sign of thermal unfolding up to 50°C (Figure S1).[3a, 3b] We quantified FGE activity using a nonapeptide (sequence: Abz-SALCSPTRA-NH_2) that includes the FGE substrate motif and a N-terminal ortho-amino benzoic acid (Abz) as chromophore. The fGly-containing product was identified by ESI-MS and quantified by HPLC (Figure S2). In a reaction buffer containing 50 mM EDTA, 2 mM DTT, 50 mM NaCl and 50 mM Tris-HCl at pH 8.0 the catalytic efficiency (k_{cat}/K_M) of FGE amounted to 100 $M^{-1}min^{-1}$. This corresponds to less than one turnover per hour in presence of 100 uM substrate. We could not estimate the maximal velocity because no saturation occurred up to a substrate concentration of 1 mM (Figure S3).

In pursuit of a more active catalyst, we examined three potential problems that may limit the activity of reconstituted FGE. First, we tested whether the enzyme may have been damaged during purification. We were particularly concerned about the two active site cysteines which may be sensitive to irreversible oxidation.[7]

[a] M. Knop, P. Engi, R. Lemnaru, F.P. Seebeck*
Department for Chemistry
University of Basel
St. Johanns-Ring 19, 4056, Basel, Switzerland
E-mail: florian.seebeck@unibas.ch

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Table 1. Michaelis-Menten parameters of FGE catalysis[a]

enzyme	k_{cat} [min^{-1}]	k_{cat}/K_M [$M^{-1}min^{-1}$]	KIE
FGE_{wt}	n.a	100 ± 50	0.8 ± 0.3
FGE_{wt} + Cu	1.6 ± 0.1	2900 ± 50	3.7 ± 0.1
FGE_{4C}	0.6 ± 0.1	1000 ± 200	1.2 ± 0.2
FGE_{4C} + Cu	4.2 ± 0.5	20000 ± 4000	3.0 ± 0.2

[a] FGE activities were assayed at 25°C in reactions containing 2 uM enzyme, 5 mM DTT, 50 mM EDTA, 50 mM NaCl and 50 mM Tris-HCl pH 8.0, in presence or absence of 2 µM of $CuSO_4$. Product formation was monitored by HPLC.

To determine the redox status of these two cysteine residues we constructed a FGE variant in which all cysteines outside the active site were mutated to either Ala or Ser ($FGE_{C187A,C231A,C284S,\ C298A}$ = FGE_{4C}) (Figure S1). The resulting variant is still a stable protein (Figure S1B). More surprisingly, this enzyme is a 10-fold more efficient catalyst than the wild type enzyme and displays saturation behavior, characterized by a K_M of 0.6 mM (Table 1). Evidently, only the two active site cysteines are essential for catalysis. Three of the mutated cysteines are either strictly (Cys298) or highly conserved (Cys187 and Cys231) among FGEs (Figure S1), suggesting that these residues might enhance or regulate activity *in vivo*, but are obsolete *in vitro*.

Using two complementary assay based on the Ellman's reagent[9] we determined that the two remaining cysteine are predominantly oxidized to a disulfide in purified FGE_{4C} (Table S1). Consistently, the mass of FGE_{4C} as inferred by ESI-MS analysis proved 2 Da lighter than the calculated mass (Table S2). Furthermore, FGE_{4C} resisted alkylation by the thiol specific electrophile iodoacetamide, but in presence of 2 mM DTT the enzyme readily reacted to the bis-alkylated form (Table S2). Drawn together these observations suggest that purified FGE_{4C} contains a DTT reducible disulfide bond in the active site and that oxidative damage is not limiting *in vitro* activity of recombinant FGE_{4C}.

A second explanation for low *in vitro* activity could be the absence of a required co-substrate. While FGE definitely uses O_2 to remove two electrons from the substrate,[7b] it is not entirely clear what happens to the resulting partially reduced oxygen species.[1a] The enzyme may either release hydrogen peroxide, or may further reduce the oxygen species to water using two additional electrons from an auxiliary reducing agent. The reported observation that *in vitro* activity of FGE_{human} depends on DTT is consistent with the second scenario. [3a, 7a, 10] To address this question on a quantitative basis we measured the rates of FGE catalyzed peptide modification, DTT oxidation, and H_2S formation (Figure S4 & S5). This analysis revealed that the three reactions are coupled indicating that FGE depends on an external electron donor such as DTT and that the sulfur atom on the substrate is not oxidized in the reaction cycle.[1a]

In absence of DTT FGE catalysis is 50-fold slower, indicating that the thiol containing substrate is a poor electron source

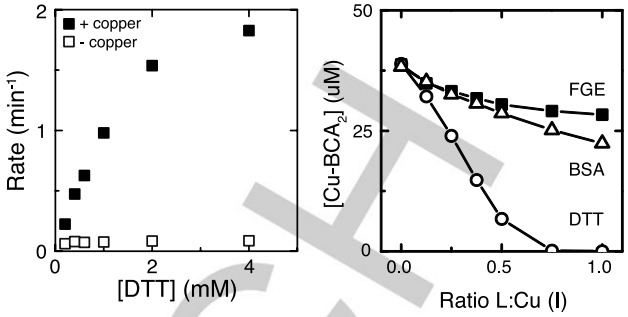

Figure 2. Left: FGE_{4C} activity as a function of [DTT] in presence (■) and absence (□) of Cu (I). Reactions contained 200 µM substrate, 0.2 – 4 mM DTT, 50 mM EDTA, 50 mM NaCl and 50 mM Tris-HCl pH 8.0. Product formation was monitored by HPLC. Right: A 50 uM solution of a chromogenic complex (ε_{562} = 7900 $cm^{-1}M^{-1}$) containing Cu (I) and bicinchoninic acid (BCA) was titrated with DTT, FGE_{4C} or bovine serum albumin (BSA). The competition for Cu (I) between BCA and the titrant was monitored by UV/Vis-spectroscopy.

(Figure S6). Addition of 2 mM of DTT or cysteamine to this slow reaction immediately restored full activity. In contrast, addition of glutathione or tris(2-carboxyethyl)-phosphine (TCEP) recovered less than 2 % of the DTT supported activity suggesting that FGE displays selectivity for the reducing agent which is not necessarily correlated with their redox potential. FGE_{4C} activity saturated at a DTT concentration well below 1 mM (Figure 2), indicating that availability of reducing equivalents should not be a limiting factor for *in vitro* activity.

A third reason for low activity of reconstituted FGE may be the absence of an additional redox catalyst – despite previous characterizations of FGE as a cofactor-independent enzyme.[3a, 7b, 8b] In a screen for possible candidates we observed that addition of $CuSO_4$ or CuBr to FGE_{4C} and FGE_{wt} increases the catalytic efficiency by over 20-fold (Table 1). In reactions containing 0.5-10 uM FGE_{4C} this activating effect depends on precisely one equivalent of copper (Figure S7). Other metal salts containing Mn, Fe, Co, Ni, Zn, Cd, Ag, Pd, Hg or Ca did not accelerate the reaction (Figure S8). The specificity for copper suggests that FGE activation is due to the redox activity rather than the Lewis acidity of this metal. Hence, our search for an efficient *in vitro* FGE catalyst resulted in the surprising proposition that FGE from *T. curvata* may be a copper-dependent enzyme.

To test whether copper-dependence is a capricious property of this particular homolog we also cloned and produced FGE from *Mycobacterium smegmatis* ($FGE_{smegmatis}$). Although the two organisms are both actinobacteria, their FGEs map to opposite branches of an unrooted phylogenetic tree of FGE homologs (Figure S9). In fact FGE_{wt} is more related to FGE_{human} (58 % sequence identity) than to $FGE_{smegmatis}$ (46 %). Still, $FGE_{smegmatis}$ shows similar copper-dependent activation as FGE_{4C} or FGE_{wt} (Figure S10). In this light it seems surprising that FGE_{human} should be inhibited by micromolar copper concentrations as stated in an earlier report.[10]

To understand the mechanism of activation in more detail we characterized the kinetic properties of copper supplemented FGE. Michaelis-Menten kinetics showed that copper increased the catalytic efficiency of FGE_{4C} mainly by

increasing k_{cat} (Table 1, Figure S3). Copper also accelerated catalysis by FGE_{wt} and induced saturation behavior (Table 1). We also assayed FGE_{4C} and FGE_{wt} with a substrate analog in which the (R)-β-proton on the cysteine residue was replaced with a deuteron (in red, Figure 1). The deuterated and non-deuterated substrates were converted to products with identical mass and isotope distribution (Figure S11), indicating that FGE specifically removes the (R)-β-hydrogen atom. In presence of copper both FGE_{4C} and FGE_{wt} show a significantly larger substrate KIE than in absence of copper (Table 1). These KIEs proved independent of substrate concentration (Figure S12). We therefor conclude that copper increases the extent to which C-H bond cleavage is rate limiting.

The dramatic effects of copper on the catalytic properties of FGE_{wt} and FGE_{4C} certainly raise doubts as to whether FGE should be characterized as a cofactor-independent enzyme.[1a, 3a, 7b, 8b] On the other hand we do not think that FGE is bona fide copper-enzyme either. Although copper would provide an excellent cofactor for O_2 activation, we were puzzled by the fact that DTT did not inhibit Cu (I) supported FGE activity. DTT is an strong ligand for Cu (I) ($K_{D,Cu(I)} = 10^{-15}$ M) and only few specialized proteins can form stable copper complexes in presence of this artificial dithiol.[11] A qualitative experiment using a chromogenic Cu (I) complex that was titrated with DTT, FGE_{4C}, bovine serum albumin (BSA) or green fluorescent protein (Figures 2 & S13) showed that the three proteins are a much weaker Cu (I) binder than DTT.[11] Consequently, 1 uM FGE and 1 uM Cu (I) can hardly form a stable complex in presence of 1 mM DTT. The observation that Cu (I) supported FGE catalysis is accelerated and not inhibited by increasing DTT concentration (Figure 2) further discredits the idea of a FGE:Cu (I) complex as the catalytic species. Cysteamine, which is also a strong Cu (I) ligand supports FGE activity to a similar extent as DTT (Figure S14). The requirement for millimolar concentrations of DTT or cysteamine may point an alternative role of Cu (I). The binary complex between Cu (I) and DTT forms at femtomolar concentration.[11] In contrast, ternary Cu (I) complexes comprising two ligands such as DTT or cysteamine are significantly less stable and only form at millimolar ligand concentrations.[12] Once formed these electron rich species $[Cu(I)\text{-}(DTT)_2]^-$ could serve as a single-electron donor to activate FGE bound O_2. The previous observation that Cu (I) in presence of 0.9 mM glutathione can reduce dissolved O_2 to superoxide,[13] provides good precedence for this idea. Although such low molecular weight Cu (I)-thiol complexes are improbable species in a cellular context,[14] it seems possible that *in vivo* FGE activity may depend on single-electron donors such as flavodoxins, cytochromes or iron-sulfur proteins.[15]

Residual *in vitro* FGE activity in absence of Cu (I) is probably caused by the significant level of reactive oxygen species present in any buffered aqueous system. For example, in our standart reaction buffer containing 50 mM Tris-HCl at pH 8.0, 50 mM EDTA, 2 mM DTT and 300 uM peptide substrate, we observed DTT "autooxidation" at a rate of 4 ± 0.5 uM/min. A fraction of this unspecific redox activity could support the observed catalytic activity of 1 uM FGE_{4C} (~ 0.3 uM/min) in absence of Cu (I).

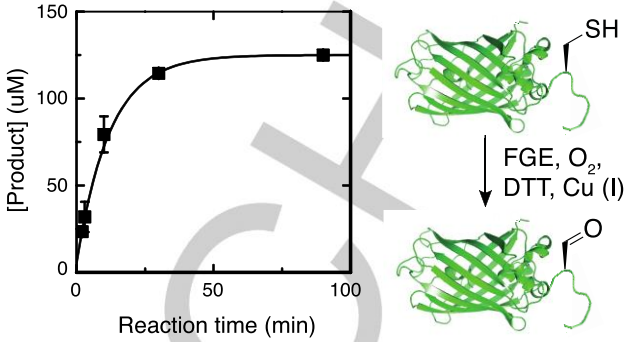

Figure 3. FGE_{4C} catalyzed insertion of fGly into the N-terminal segment of a modified green fluorescent protein (GFP). Product formation was monitored by HPLC analysis of trypsin-digested GFP samples (Figures S15 & S16).

Finally, we tested whether the 200-fold improved activity of FGE_{4C} also applies to protein substrates. To this end we produced a green fluorescent protein (GFP) variant featuring an N-terminal FGE recognition motif. In an example reaction 130 uM of this protein substrate was completely converted to aldehyde containing product by 1.3 uM FGE_{4C} within less than one hour (Figure 3). The observed initial rate (k_{obs} = 7.4 min^{-1}) is consistent with the catalytic parameters determined for the synthetic peptide substrate (Table 1). Given the simplicity of this protocol and many published schemes for aldehyde specific protein modification,[1, 4a-d, 6] we anticipate that FGE mediated *in vitro* protein engineering will become a widely used methodology.

Conclusions. In this report we showed that addition of one equivalent of copper increases the activity of *in vitro* reconstituted FGE by 20-fold and unmasks stereoselective C-H bond cleavage from the substrate as the rate-limiting step. In combination with four point mutations the copper supplemented enzyme catalyzes fGly insertion into peptides and proteins 200-fold more efficiently than the wild type enzymes from *T. curvata* or *M. smegmatis*. These observations raise the possibility that *in vivo* FGE activity may also depend on a one-electron donating cofactor or partner protein that is absent in the purified and crystallized enzymes.

A final note. While this manuscript was under review an independent study was published in the *Journal of Biological Chemisty* describing a similar activating effect of copper on FGE from humans and from *Streptomyces coelicolor*. The two studies demonstrate the generality of copper-dependent activation of *in vitro* FGE activity.[16]

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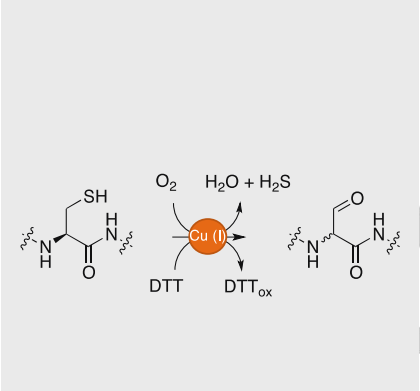

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