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Supporting Information

Copper is a Cofactor of the Formylglycine-Generating Enzyme

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Instruments. HPLC analysis was carried out on a Shimadzu system, using a Gemini-NX C18 column (5 µm, 110 Å, 250 x 4.6mm). UV/vis measurements were performed on a Cary 300Bio UV-Visible Spectrophotometer from Varian at T = RT. LC/MS-TOF experiments were performed on a Agilent 1100 Series instrument coupled to a micrOTOF ESI-TOF from Bruker, using a Jupiter C4 300A column (50 x 2 mm, 5 Microns, Phenomex). Protein concentrations were determined using a NanoDrop2000 from Thermo Scientific. Protein purification via SEC was performed on a GE Healthcare Äkta prime FPLC instrument using a Superdex^{TM} 200 5/150 GL column.

Mutagenesis. Genes for FGE_{S266A}, FGE_{C269S}, FGE_{C274S} and FGE_{S290K} were constructed by primer extension PCR using the primers listed in Tab. S1. The gel-purified fragments were digested using NdeI and XhoI restriction enzymes and ligated into a pET28a expression vector (→ pET28a_FGE_{XXX}).

Table S1: List of primers used to construct FGE variants

Primer Name	Sequence
tFGE-S266As	AAGGGCGGCGCTTTCCTGTGCCACGAGT
tFGE-S266Aa	GGCACAGGAAAGCGCCGCCCTTGGTGAC
tFGE-S290Ks	CCGACTCCAAAGCCGCCCA
tFGE-S290Ka	TGGGCGGCTTTGGAGTCGG
tFGE-C269Ss	GTTCCTGTCCCACGAGTC
tFGE-C269Sa	GACTCGTGGGACAGGAAC
tFGE-C274Ss	AGTCGTACTCCAACCGCTA
tFGE-C274Sa	TAGCGGTTGGAGTACGACT

Protein production. All FGE variants were produced in BL21 pLys cells. The cells were cultured at 37 °C in LB medium in shaker flasks. Gene expression was induced by addition of 0.1 mM IPTG. Protein production was carried out at 37 °C for 3 – 4 h. The proteins were purified following standard NTA-agarose affinity chromatography protocols. The final protein samples were then dialyzed into 50 mM Tris-HCl pH 8.0, 50 mM NaCl and stored at -80 °C. Reduced FGE (Figure S1) was produced by treatment with 5 mM DTT at 0°C for 20 min, followed by size exclusion chromatography to remove DTT, immediately before use in the Cu (I) binding assay.

Determination of *in vitro* FGE activity. We described this assay in detail in a previous paper.[2] Briefly, the activity of FGE was determined in 50 mM Tris buffer containing 2 µM $CuSO_4$, 200 µM substrate (peptide sequence: Abz-SAL-Cys-SPTRA-NH_2, Abz: o-amino benzoic acid), 50 mM EDTA, 50 mM NaCl at pH 8 as well as 2 mM of DTT or other thiol reducing agents (Figure S2). Reactions were initiated by addition of the enzyme. Reqction aliquots were quenched by addition of 1 volume equivalent of 1 % TFA in 4 M urea. Product formation was quantified by RP-HPLC.

BCS Cu(I) competition assay. This method used to determine apparent Cu (I) binding affinities was adapted with minor modifications from Ref. [1]. To limit autooxidation of cysteine residues or low LMW thiols all

samples were prepared using N_2 bubbled buffers and solutions. Protein samples were diluted in a 50 mM Tris buffer pH 8 containing 20 µM $CuSO_4$, 200 µM bathocuproinedisulfonic acid (BCS), 2 mM cysteamine, 4 mM $Na_2S_2O_4$. The concentration of BCS was determined using a molar extinction coefficient of $\varepsilon_{BCS,\,483nm}$ = 13000 $M^{-1}cm^{-1}$. Dissociation constants were estimated for Cu (I) complexes according to Eq. 1.[1] In order to fit the measured UV absorbance data this equation was linearized to Eq. 2. From the slope of this plot (m) a K_D value was calculated using $\beta_2 = 10^{19.8}$ M^{-2}.[1] For measurements using reduced FGE, the protein was incubated with 5 mM DTT on ice for 20 min and the purified by size exclusion chromatography immediately before the measurements. The Cu (I) affinity for FGE_{4C} and FGE_{S266A} was also determined in the presence of 200 µM of Ser-containing substrate analog (peptide sequence: Abz-SAL-Ser-SPTRA-NH_2). The presence of this peptide did not significantly change the Cu (I) affinities.

$$K_D\beta_2 = \frac{\frac{[P]_{total}}{[MP]} - 1}{\left(\frac{[L]_{total}}{[ML_2]} - 2\right)^2 [ML_2]} \qquad \text{Eq. (1)}$$

$$\left(\frac{[L]_{total}}{[ML_2]} - 2\right)^2 [ML_2]([M]_0 - [ML_2]) = \frac{1}{K_D\beta_2}([P]_{total} - ([M]_0 - [ML_2])) \qquad \text{Eq. (2)}$$

with

$$y = \left(\frac{[L]_{total}}{[ML_2]} - 2\right)^2 [ML_2]([M]_0 - [ML_2])$$

$$x = [P]_{total} - ([M]_0 - [ML_2])$$

and

$$m = \frac{1}{K_D\beta_2}$$

$$y = \frac{1}{K_D\beta_2} \cdot x$$

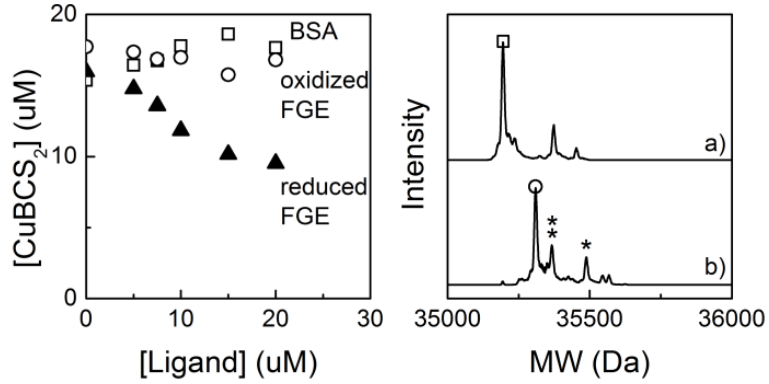

Figure. S1: Left: Qualitative assessment of the Cu(I) binding ability of reduced vs. oxidized FGE_{4C}. A 50 mM Tris buffer at pH 8 containing 200 µM BCS, 20 µM $CuSO_4$, and 4 mM $Na_2S_2O_4$ was titrated with reduced and oxidized FGE_{4C} and BSA. The concentration of the Cu(I):$(BCS)_2$ complex was monitored at 483 nm. **Right:** The oxidized form of FGE_{4C} has been characterized in our previous paper.[2] To confirm that the reduced form of FGE_{4C} contains two reduced active site cysteines the protein treated with the thiol-specific electrophile iodoacetamide (IAA). The reduced and the alkylated protein were then characterized by HRMS-ESI: a) reduced FGE_{4C} (□: MW_{calc} = 35195.5 Da, MW_{obs}= 35194.5 Da); b) reduced and alkylated FGE_{4C} (O: MW_{obs} = 35309.7 Da),

indicating that reduced FGE_{4C} was alkylated twice. Additional signals correspond to His-tag glyconylated FGE_{4C} (*),[3] and to tris-alkylated FGE_{4C} (**).

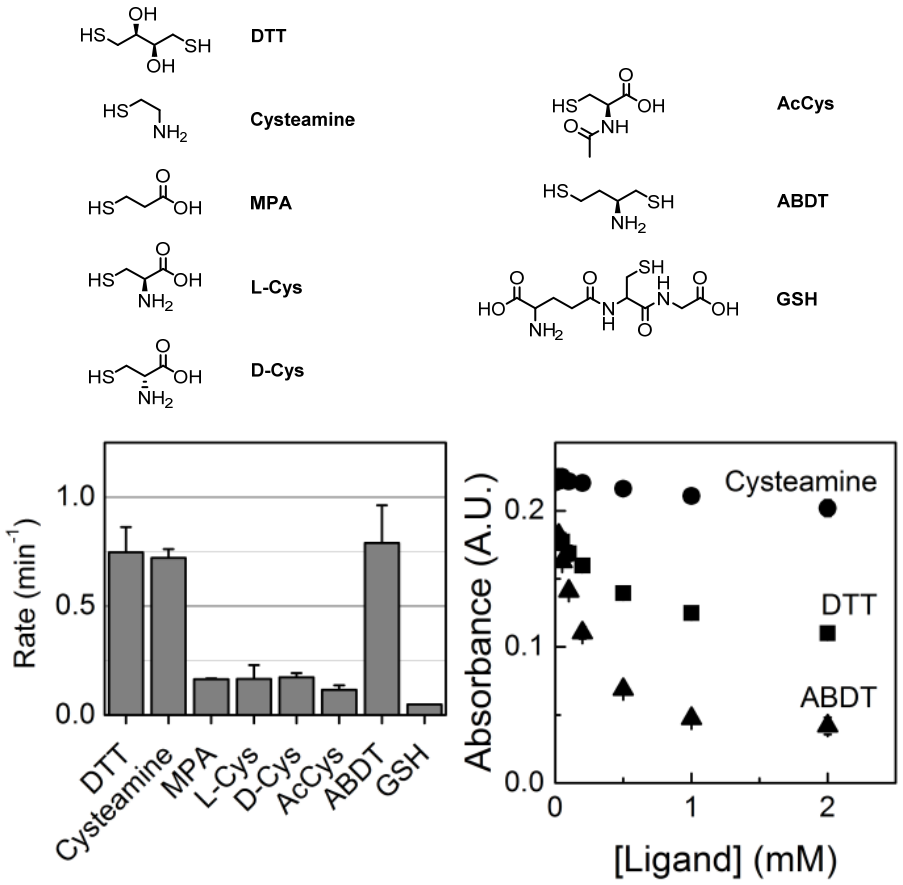

Figure S2. Top: Selected reducing agents for the analysis of the dependency of activity on the structure of the reducing agent. **Left:** The ability of different thiols to support FGE catalysis was determined using the titration assay described above, substitution DTT with other thiols. **Right:** Thiols that support FGE activity were tested for their Cu(I) binding abilities. Absorbance of $CuBCS_2$ complex at $\lambda = 483$ nm in the presence of different reducing agents acting as ligands. Samples have been prepared by diluting the reducing agent to a final concentration of 0 – 2 mM in a Tris buffer pH 8 containing 20 µM $CuSO_4$, 200 µM BCS, 4 mM $Na_2S_2O_4$. K_D values were determined as described above.

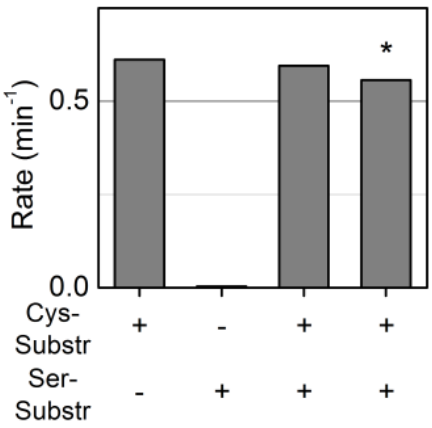

Figure. S3: FGE_{4C} catalyzed substrate turnover in the presence of a substrate analogue containing serine instead of cysteine (peptide sequence: Abz-SAL-**Ser**-SPTRA-NH_2). Reactions containing 2 μM FGE 2 μM $CuSO_4$, 2 mM DTT, 50 mM EDTA, 50 mM NaCl and 50 mM Tris pH8 were supplemented with a) 200 μM substrate, b) 200 μM substrate analogue, c) 200 μM of each substrate and substrate analogue or d) with 200 μM substrate and 2000 μM substrate analogue. Product formation in these reactions was monitored by RP-HPLC. This analysis showed that even a ten-fold excess of substrate analogue does not significantly inhibit substrate turnover. Evidently the substrate analogue is a poor competitive inhibitor. The thiol function on the substrate plays an important role in stabilizing the ternary enzyme:copper:substrate complex. **Peptide synthesis.** The substrate analogue was synthesized using standard protocols for Fmoc solid phase peptide synthesis. SPPS building blocks were purchased from Bachem. The peptide was purified to homogeneity by C_{18} RP-HPLC and analyzed by ESI-MS (m/z = 1007.5, calc.: 1006.52 ($+H^+$)).

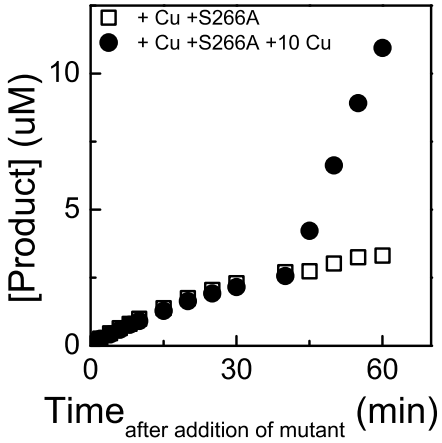

Figure S4: Reactivation of FGE_{S266A} inhibited FGE_{4C} by addition of excess Cu (I). Reaction containing 200 μM substrate, 0.5 μM $CuSO_4$, 2 mM DTT, 50 mM EDTA, 50 mM NaCl and 50 mM Tris pH 8 were initiated by addition of 0.5 μM FGE_{4C}. After one minute incubation we added a 4.5 μM FGE_{S266A}. After 40 min we added 4.5 μM $CuSO_4$ to one reaction (black spheres). Addition of Cu (I) immediately increased the rate of product formation, suggesting that in presence of 4.5 μM FGE_{S266A} 0.5 μM Cu (I) is the limiting factor. Product formation was monitored by RP-HPLC.

References.

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2. M. Knop, P. Engi, R. Lemnaru and F. P. Seebeck, *Chembiochem*, 2015, **16**, 2147 - 2150.
3. K. F. Geoghegan, H. B. Dixon, P. J. Rosner, L. Hoth, R,, A. J. Lanzetti, K. A. Borzilleri, E. S. Marr, L. H. Pezzullo, L. B. Martin, P. K. LeMotte, A. S. McColl, A. V. Kamath and J. G. Stroh, *Anal. Biochem.*, 1999, **267**, 169 - 184.