Reconstitution of Formylglycine-generating Enzyme with Copper(II) for Aldehyde Tag Conversion

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Background: Aerobic formylglycine-generating enzyme (FGE) converts cysteine to formylglycine in vivo. Purified FGE requires preactivation with copper to convert cysteine to formylglycine in vitro. FGE is a metalloenzyme. It is also a useful biocatalyst for the production of proteins that contain aldehyde tags.

Significance: Understanding FGE biochemistry informs research on sulfatases and enables expanded biotechnology applications of the aldehyde tag.

To further our aim of synthesizing aldehyde-tagged proteins for research and biotechnology applications, we developed methods for recombinant production of aerobic formylglycine-generating enzyme (FGE) in good yield. We then optimized the FGE biocatalytic reaction conditions for conversion of cysteine to formylglycine in aldehyde tags on intact monoclonal antibodies. During the development of these conditions, we discovered that pretreating FGE with copper(II) is required for high turnover rates and yields. After further investigation, we confirmed that both aerobic prokaryotic (Streptomyces coelicolor) and eukaryotic (Homo sapiens) FGEs contain a copper cofactor. The complete kinetic parameters for both forms of FGE are described, along with a proposed mechanism for FGE catalysis that accounts for the copper-dependent activity.

The aerobic class of formylglycine-generating enzyme (FGE) is an oxidase that catalyzes the conversion of cysteine to formylglycine (fGly). FGE recognizes a five-amino acid consensus sequence (CXPXR), and converts Cys within that sequence to fGly. In nature, FGE is required for the endogenous activation of sulfatases. Sulfatases contain an active site fGly that participates in sulfate ester hydrolysis. Inhibitory mutations to FGE in humans result in a debilitating condition known as multiple sulfatase deficiency. FGE is expressed broadly across cell types and organisms, and the FGE consensus sequence is highly conserved across all taxonomic kingdoms, including eukaryotes and prokaryotes. Although the sequence determinants for fGly formation are understood, the mechanism through which Cys is converted to fGly remains unclear.

Beyond its role in the active site of sulfatases, the aldehyde functional group in fGly is a particularly useful site for protein bioconjugation. It is an electrophilic carbonyl, which is not found among the 20 proteogenic amino acids, and aldehydes rarely occur as post-translational modifications; oxidized glycans are a notable exception. Accordingly, fGly stands out as an optimal choice for bioorthogonal conjugation. A variety of bioconjugation reactions are available to target carbonyls. It is possible to use hydrazines and alkoxyamines to form hydrazones and oximes, but these are susceptible to hydrolysis. Newer methods anchor payloads with stable chemistries that form C-C bonds, including Pictet-Spengler and trapped-Knoevenagel ligations.

To leverage these exquisitely selective reactions for synthetic purposes, fGly must be installed into the protein backbone. Fortunately, the method for its incorporation requires only genetic encoding of the consensus sequence (dubbed the aldehyde tag). During translation of the substrate protein in the ER, FGE converts Cys to fGly (Fig. 1a). In previous work, we described methods for the overexpression of FGE with substrate proteins modified with the aldehyde tag (11). This approach endows the resulting protein with fGly during production in cell culture.

In parallel work described here, we explored the value of performing conversion of Cys to fGly in vitro with purified recombinant FGE (Fig. 1b). This goal requires an efficient enzyme that is stable during the course of a biocatalytic reaction. It was not known whether FGE would meet these requirements, and so our first priority was to optimize FGE in vitro conversion efficiency and protein stability. Our initial experiments employed a 14-amino acid peptide substrate, with which we determined the optimal conditions for in vitro conversion. This model system also allowed us to explore the mechanism through which FGE converts Cys to fGly.

Researchers currently propose a mechanism for FGE that does not require an exogenous cofactor for oxygen activation or catalysis. In a variety of experiments to date, x-ray crystallography and biochemical investigations have only revealed the need for the addition of calcium for structural...
Reconstitution of Formylglycine-generating Enzyme with Copper

![Diagram of FGE converting Cys to fGly in vivo and in vitro](image)

**Figure 1.** FGE converts Cys to fGly in vivo and in vitro. a, FGE recognizes the five-amino acid consensus sequence CXPXR. It catalyzes the oxidation of the Cys thiol to an aldehyde to generate fGly. In eukaryotes, conversion occurs in the ER. b, in this work, we demonstrate that FGE is also an efficient biocatalyst in vitro. Treatment of an aldehyde-tagged substrate protein with FGE and a reducing agent generates fGly on folded proteins in high yield.

Support of protein folding. Mechanically, it is suggested that Cys$^{336}$ directly activates molecular oxygen to form a transiently oxidized form of Cys, which is thought to perform substrate oxidation (6, 7, 9). If true, this mechanism of catalysis would be unique in all of biology.

However, during the process of optimizing FGE reaction conditions, we discovered that both the *Streptomyces coelicolor* (Sc-FGE) and *Homo sapiens* (Hs-FGE) forms of FGE are many times more active when they contain a copper cofactor. This result is in clear contrast to the existing hypothesis, and yet high activation numbers for a sample size ($n$) for data displaying error bars are indicated in each figure caption. Errors reported for the enzyme kinetic parameters represent the estimate of error calculated from the minimized sum of squares found during nonlinear regression of activity data to the Michaelis-Menten equation,

$$ y = \frac{E_t \times k_{cat} \times x}{(K_{m} + x)} $$

(Eq. 2)

where $E_t$ is the total enzyme in solution, and $k_{cat}$ and $K_m$ are the standard enzymatic parameters specified by the STRENDa commission (24). Units of enzyme specific activity are the katal, where 1 katal = 1 mol s$^{-1}$. Kinetic parameters were determined from nonlinear regression of [substrate] versus initial velocity using Prism® 6.0 (GraphPad).

**RP-HPLC**—Reversed-phase high performance liquid chromatography was performed on an 1100/1200 series instrument (Agilent Technologies). Chromatography was achieved on an Aeries™ core-shell 250 × 2.1-mm XB-C18 Widepore column (Phenomenex, Inc.) and area under the curve was calculated with Chemstation (Agilent).

**LC-MS/MS**—Mass Spectrometry data were collected on a 4000 QTRAP® mass spectrometer (AB Sciex) with an 1100 series HPLC (Agilent). Chromatography was performed on a Jupiter™ 150 × 1.0-mm C18 column (Phenomenex) enclosed in a butterfly column heater set to 65 °C with a PST-CHC controller (Phoenix S&T). Calculation of LC-MRM (multiple reaction monitoring)/MS transition masses and integration of the resulting data were performed with Skyline 2.6 (25).

**FPLC and Gel Filtration**—Fast performance liquid chromatography was performed on a GE Healthcare Äkta Protein Purification System. Nickel affinity chromatography was performed with a HisTrap Excel 5-ml column (GE Healthcare). Gel filtration was performed by hand with disposable Sephadex® G-25 columns (GE Healthcare).

**ICP-MS**—Inductively coupled plasmon mass spectrometry (ICP-MS) was performed by the Catalent Center for Excellence in Analytical Services (Morrisville, NC). The ratio of copper and calcium were calculated as a mole ratio based upon protein concentrations measured from 280 nm absorption intensity of enzyme stock solutions.

Reagents—Water used was deionized (18 mΩ) with a Milli-Q® Integral 5 system (Merck KGaA). Other commercially available chemicals were reagent grade or higher. Peptide synthesis was performed by New England Peptide, Inc. on solid phase and purified to ≥95%.

**Recombinant Expression and Purification of Prokaryotic FGE from Escherichia coli**—Recombinant expression and purification of N terminally His$_{6}$-tagged FGE from *S. coelicolor* (Sc-FGE, UniProt Q9F3C7) was carried out by modifying a previous method (9). Two significant changes were employed. First, residual DNA was removed from lysed cells using 1% (w/v) streptomycin sulfate for 15 min at 4 °C; then, the lysate was clarified by centrifugation at 25,000 relative centrifugal force for 25 min. Second, the enzyme purification and storage buffer included triethanolamine in place of Tris. The primary amine of Tris buffer was found to react with the aldehyde product of FGE catalysis. Additionally, it is worth noting that high ionic strength conditions, we discovered that both the reducing cytosol (prokaryotes) and the oxidizing body (mAb).

FGE is found across a wide range of organisms, including prokaryotes and eukaryotes. Revealing that copper is a required cofactor for FGE catalysis explains how the enzyme is active in both the reducing cytosol (prokaryotes) and the oxidizing endoplasmic reticulum (eukaryotes); turnover requires copper and oxygen, and not an active site disulfide. In addition, by defining a biocatalytic protocol for in vitro conversion of Cys to fGly on an intact monoclonal antibody (mAb).

FGE converts Cys to fGly in vivo and in vitro. a, FGE recognizes the five-amino acid consensus sequence CXPXR. It catalyzes the oxidation of the Cys thiol to an aldehyde to generate fGly. In eukaryotes, conversion occurs in the ER. b, in this work, we demonstrate that FGE is also an efficient biocatalyst in vitro. Treatment of an aldehyde-tagged substrate protein with FGE and a reducing agent generates fGly on folded proteins in high yield.

**Experimental Procedures**

**Estimation of Uncertainty**—Measurements of uncertainty represent 95% confidence intervals calculated from the mean ± S.E. for a sample size ($n$) using the following equation,

$$ \text{Error} = 1.96 \times \left( \frac{s}{\sqrt{n}} \right) $$

(Eq. 1)

where $s$ is the measured standard deviation for $n$ samples. Data without error bars represent individual experiments. The sample size $n$ for data displaying error bars are indicated in each figure caption. Errors reported for the enzyme kinetic parameters represent the estimate of error calculated from the minimized sum of squares found during nonlinear regression of activity data to the Michaelis-Menten equation,
Reconstitution of Formylglycine-generating Enzyme with Copper

strength buffers (e.g. phosphate and sulfonate-containing Good’s buffers) caused unactivated Sc-FGE to precipitate, and should be avoided.

After DNA precipitation, the cell lysate was loaded onto NTA Superflow (Qiagen) by gravity flow. The resin was washed with 10 column volumes of wash buffer (25 mM triethanolamine (TEAM), 250 mM NaCl, 5 mM calcium acetate, 10 mM imidazole, pH 8). The enzyme was then removed from the column with elution buffer (25 mM TEAM, 5 mM calcium acetate, 300 mM imidazole, pH 8). Fractions containing protein as determined by a protein assay (Bio-Rad) were pooled and loaded onto a Sephadex® G-25 column equilibrated with storage buffer (25 mM TEAM, 8% (v/v) glycerol, pH 7.4). Then, the protein was eluted with storage buffer, concentrated with a 10-kDa Amicon® ultrafiltration membrane (Merck KGaA), and flash frozen in liquid nitrogen. The typical yield of isolated enzyme was 50–75 mg/liter of media. Purified enzyme was flash frozen in liquid nitrogen. The typical yield of isolated enzyme was 10–20 mg/liter of media.

Production of Recombinant Baculovirus Encoding for the Core of Human FGE—DNA encoding the catalytic “core” of H. sapiens FGE followed by a C-terminal His tag for purification (Hs-cFGE) was amplified from Hs-FGE (Uniprot Q8NBK3) by PCR using the following primers: forward, 5′-GAGGCTATACGCCTCCGGCC-3′; reverse, 5′-GTCCATAGTTGGGCA-GGCCTGTC-3′. Preparation of the Hs-cFGE-encoding baculovirus was performed using the Bac-to-Bac baculovirus expression system (Invitrogen).

Expression and Purification of Hs-cFGE from Hi5 Cells—Hs-cFGE baculovirus was transfected into Hi5 cells (Invitrogen) at a multiplicity of infection of 0.1. After 72 h in culture, the conditioned media was clarified by centrifugation and filtration (0.45 μm), and loaded by FPLC onto HisTrap® Excel resin (GE Healthcare) at 5 ml/min. The resin was washed with 10 column volumes of wash buffer (25 mM TEAM, 250 mM NaCl, 5 mM calcium acetate, 10 mM imidazole, pH 8). The enzyme was then removed from the column with elution buffer (25 mM TEAM, 5 mM calcium acetate, 300 mM imidazole, pH 8). Fractions containing protein (as determined by UV detection of the FPLC elution) were pooled and loaded onto a Sephadex® G-25 column (GE Healthcare) equilibrated with storage buffer (25 mM TEAM, 8% (v/v) glycerol, pH 7.4). Then, the protein was eluted with storage buffer, concentrated with a 10-kDa Amicon® ultrafiltration membrane, and flash frozen in liquid nitrogen. The typical yield of isolated enzyme was 10–20 mg/liter of media.

Purified enzyme was characterized by SDS-PAGE electrophoresis, reversed-phase HPLC, LC/MS of the intact protein, tryptic digestion followed by LC-MS/MS, absorption spectroscopy, and specific activity (as described below).

Activation of FGE with Copper(II) Sulfate—Copper sulfate (50 μM, 5 molar eq) was added to a solution of Sc-FGE (10 μM) in buffered aqueous solution (25 mM TEAM, pH 7.4, 50 mM NaCl) and product (fGly) peptides on reversed-phase HPLC. The identities of product (and side product) peaks were determined by RP-HPLC MS/MS (see below). Each aqueous reaction solution (120 μl) contained C_sub (100 μM), FGE (0.1–1 μM), DTT (1 mM), and buffer (25 mM TEAM, pH 9). A single time course consisted of 5 data points collected at evenly spaced intervals. The reaction was initiated upon addition of FGE stock solution and vortexing for 3 s. The reaction vial was vortexed at 1500 rpm and 25 °C in a thermomixer (Eppendorf). Time points were hand quenched by adding 20 μl of reaction mixture to 2 μl of 1 M HCl. After completion of the reaction, each time point was analyzed by RP-HPLC (see below).

Identification of FGE Peptide Intermediates by LC-MS/MS and RP-HPLC—To identify the intermediates and products of in vitro conversion by FGE on C_sub, reaction mixtures from the FGE activity assay were quenched and analyzed by LC/MS. The reaction mixture consisted of 0.5 mM peptide, 0.5 mM FGE, 5 mM βME, and 25 mM TEAM, pH 9, in a total volume of 200 μl. Time points were prepared by adding 20 μl of reaction mixture to 2 μl of 1 M HCl. The gradient was 5–25% MeCN in water with 0.1% formic acid over 10 min. The relative intensities and retention times of the four peptide peaks detected at 215 nm on RP-HPLC were replicated in the LC-MS/MS data, allowing assignment of each peak by mass.

Quantification of FGE Substrate and Product Peptides by HPLC—Substrate, product, and side products of the FGE substrate peptide ALCTPSRGSLFTGR were separated on RP-HPLC over 7 min with isocratic 18% MeCN in water containing 0.1% TFA. The integrated areas of the fGly (2.1 min), C_sub (3.3 min), the C_sub/βME disulfide (C_sub/βME, 3.6 min), and substrate/substrate disulfide (C_sub/C_sub, 6.1 min) forms of the substrate were used to calculate the total area and each fraction thereof.

Thiol and Disulfide Mapping of FGE—To determine the oxidation state of thiol residues on either Sc-FGE or Hs-FGE, a procedure was developed for mapping thiols and disulfides by LC-MRM/MS using sequential labeling, digestion, and orthogonal labeling. Step 1 was initial labeling and tryptic digestion: the reaction mixture was composed of FGE (7.5 μg), NH4HCO3 (50 mM), iodoacetamide (20 mM), and trypsin (0.75 μg) in a total volume of 30 μl. The tube containing the sample was incubated at 37 °C for 16 h. Step 2 was quenching: water (6 μl) and DTT (4.8 μl of 0.5 M, 50 mM final concentration) were added to the reaction mixture from step 1. The tube containing the sample was incubated at 37 °C and 1500 rpm for 1 h. Step 3 was a second labeling, N-ethylmaleimide (7.2 μl of 0.5 M, 150 mM final concentration) was added to the reaction mixture from step 2. The tube containing the sample was then incubated at 37 °C and 1500 rpm for 1 h. After step 3, the reaction mixture was quenched with HCl (4.8 μl of 1 M, 100 mM final concentration). Digested peptides were separated with a gradient of 0–50% MeCN in water with 0.1% formic acid over 15 min. Cysteine-containing peptides were detected by MRM/MS monitoring iodoacetamide, N-ethylmaleimide, and oxidative modifications of Cys residues.
JUNE 19, 2015 • VOLUME 290 • NUMBER 25

Reconstitution of Formylglycine-generating Enzyme with Copper

A typical reaction yielded a final fGly content in the antibody of 95–100%, corresponding to a reaction conversion yield of 90–100%. To date, the reaction has been performed across a range of scales from 20 μg to 0.6 g.

Competitive ELISA—Competitive ELISA plates (Maxisorp) were coated with 1 μg/ml of His-tagged antigen (Sino Biological) at 1 μg/ml in PBS for 16 h at 4 °C. The plate was then washed four times with PBS, 0.1% Tween 20 (PBS-T), and then blocked with 200 μl of ELISA blocker (Thermo). In a second plate, an 11-point standard curve was prepared from serial 3-fold dilutions (50 μl + 100 μl) of a stock solution of the competitor antibody at 20 μg/ml into ELISA blocker. Next, biotin-tagged antigen (100 μl of 10 ng/ml) was added to all of the wells. Finally, the mixture of competitor and biotin-tagged antigen was transferred to the coated ELISA plate (100 μl/well), which was incubated at room temperature for 1–2 h with shaking. The final concentration of biotin-tagged antigen was 5 ng/ml and the highest concentration of the competitor was 10 μg/ml. The plate was washed four times with PBS-T; then, 100 μl of a 1:10,000 dilution (into blocking buffer) of streptavidin-HRP (Thermo) was added and the plate was incubated at room temperature with shaking for 1–2 h. Next, the plate was washed four times with PBS-T, and developed with 1-Step Ultra TMB (100 μl/well). The reaction was quenched with 50 μl of 2 M sulfuric acid, and absorbance was measured at 450 nm. EC50 values were determined by nonlinear regression.

Results

Sc-FGE and Hs-cFGE Have Modest Catalytic Activity as Isolated from Cell Culture—To develop a highly efficient, soluble form of FGE for use as a reagent in a biocatalytic reaction, we first developed reliable methods for its recombinant expression. FGEs from two species, S. coelicolor and H. sapiens, were selected for study because they had been previously characterized to some extent (7–9), and because they represented both prokaryotic and eukaryotic forms of the enzyme. In eukaryotes, FGE resides in the ER, an oxidizing environment; in prokaryotes, it resides in the cytosol, a reducing environment. These two forms of the enzyme are highly homologous in primary sequence and structure. We hoped to determine how highly similar FGEs are active in both of these different redox environments.

Sc-FGE was prepared in a similar manner to previous reports, by transformation of E. coli and purification from clarified cell lysates. Hs-cFGE was prepared in high purity and good yield by viral transduction of insect cells. We elected to produce the human form in insect cells because it expressed poorly in E. coli, perhaps because it bears both structural disulfides and an N-linked glycan.

Initial results indicated that FGEs from both species were prone to precipitation depending on buffer conditions. We eventually optimized the buffer to minimize this problem. However, with respect to the human enzyme, we also removed a noncatalytic portion of the enzyme that appeared to worsen precipitation (described in detail later). With soluble enzyme in hand, we proceeded to evaluate the activity of FGEs as produced from cell culture, and the reaction conditions required for catalysis.
To measure the specific activity of FGE, we sought to develop an assay that could continuously read out product formation during turnover. We tested a variety of detection methods hoping to find an assay to monitor the quantity of substrate(s) and/or product(s). Notably, we attempted secondary conjugation to fGly, but found that the rate of turnover far exceeded the rate of any carbonyl condensation reaction. In addition, fluorescence detection of H₂S (the hypothesized sulfurous leaving group) was unsuccessful because none of the sensors attempted were selective for H₂S in the presence of other thiol reducing agents, which are, by necessity, added to the FGE reaction mixture.

In the end, a discontinuous assay was the most reliable and informative. We adapted a previously reported method that used MALDI-MS to quantify the starting material and product of quenched reaction mixtures (5). In our assay, reversed-phase HPLC was used to separate the Cys and fGly forms of a peptide containing the FGE consensus sequence. The 14-amino acid peptide ALCTPSRGSLFTGR (derived from a bacterial sulfatase sequence) was used as a substrate for both the Sc- and Hs-forms of the enzyme.

Individual FGE reactions were initiated by rapidly mixing FGE into a premixed buffered solution of Csub and reducing agent. It was assumed that all buffered solutions contained oxygen at ~270 μM at 25 °C (based upon Henry’s law). Time points were chemically quenched by hand with HCl. The integrated peak areas of substrate and product (Fig. 2a) were used to calculate the initial velocity (Fig. 2b) of FGE using the method described by Boeker (30, 31). The identities of both the Cys- and fGly-containing peptides were confirmed by LC-MS/MS (Fig. 2c and d).

**FIGURE 2.** The specific activities of FGEs are measured using a discontinuous activity assay. *a,* a 14-amino acid peptide substrate was used to measure the kinetics of FGE catalysis. The Cys- and fGly-containing peptides were separated by RP-HPLC, and their quantities were determined by integrating the peak areas at 215 nm. The asterisk indicates an impurity formed during peptide synthesis. *b,* initial velocity of the reaction (v₀) was determined by extrapolating a plot of the instantaneous reaction velocity (p/t) versus time (t) to the y axis (t = 0). The identities of the product (c, retention t = 2.1 min) and starting material (d, t = 3.3 min) were verified by LC-MS/MS.
As will be described below, the specific activity of FGE can vary dramatically as a function of the chemical state of its active site, and so enzyme concentrations in this assay spanned the range of 0.1–10 \( \text{mol} \% \) (0.1–10 mol %) enzyme. The conversion of Cys to fGly requires a 2 \( \text{H}^+ \)/2 \( \text{e}^- \) oxidation of the substrate Cys thiol as well as exchange of sulfur for oxygen. Current data support a mechanism in which molecular oxygen is the terminal electron acceptor (9). However, because \( \text{O}_2 \) is a 4 \( \text{H}^+ \)/4 \( \text{e}^- \) acceptor, a reducing agent is required to provide a second equivalent of 2 \( \text{H}^+ \) and 2 \( \text{e}^- \), completing the reduction from \( \text{O}_2 \) to 2 mol of \( \text{H}_2\text{O} \). We hypothesized that one might be able to bypass the requirement for a reductant by adding a 2 \( \text{H}^+ \)/2 \( \text{e}^- \) oxidant directly to the \textit{in vitro} reaction. Unfortunately, we found that addition of the oxidant \( \text{H}_2\text{O}_2 \) served only to dimerize the substrate by generating a disulfide (\( \text{C}_{\text{sub}}-\text{C}_{\text{sub}} \), Fig. 3a), which halted the reaction (Fig. 3, b and c). We returned to using a mixture of reductant (DTT) and \( \text{O}_2 \) for routine activity measurements. We now had the ability to produce active FGEs in good yield and high purity from both species, and we turned to developing methods that would enhance the activity of FGE. To do so, we investigated the mechanism by which FGE performs catalysis.

The Activity of FGE Depends Upon the Presence of a Copper Cofactor, and Not the Redox State of Active Site Cysteine Residues—Hs-FGE contains three primary domains: a signal peptide, an N-terminal extension (NTE), and a core that binds substrate and performs turnover (Fig. 4a). After transport to the ER and proteolytic removal of the signal peptide, the “full-length” wild-type enzyme (Hs-FGE) contains both the NTE and core. Previous reports provide evidence that the NTE is required for ER retention through disulfide bond formation between Cys\(^{30} \) and/or Cys\(^{52} \) with ERp44 (32). The border between the NTE and the core is a proteolytic cleavage site for proprotein convertases such as furin and PACE (33). When FGE encounters these enzymes in the secretory pathway, the NTE is removed and the truncated core can be secreted. We discovered that \textit{in vitro}, the full-length FGE had a propensity to aggregate at high concentrations, which led us to prepare the truncated core (cFGE). We were unsure whether this approach would be successful because previous results showed that the NTE is required for FGE activity \textit{in vitro} (33). In our hands, the specific activity of Hs-cFGE (1,143 pkatal mg\(^{-1} \)) was similar to Hs-FGE that contained the NTE (850 pkatal mg\(^{-1} \)). In addition, we found that Sc-FGE expressed in \textit{E. coli} had a significantly lower specific activity (214 pkatal mg\(^{-1} \)), relative to the human enzyme expressed in insect cells (Fig. 4b).

Our analysis of FGE catalysis then turned to testing the previously reported hypotheses of its mechanism. Foremost among these is that the enzyme requires the presence of an active site disulfide residue (8). Therefore, we prepared reduced and oxidized forms of FGE and measured their specific activities. Formation of the reduced active site Cys residues was accomplished by pretreatment of Sc-FGE with an excess (100 molar eq) of a strong reducing agent (DTT) for 1 h at 25 °C, after which the reagent was removed by gel filtration. Formation of the oxidized active site was accomplished by pretreatment of the enzyme either with an excess (100 molar eq) of \( \text{H}_2\text{O}_2 \) (Fig. 4b).
Reconstitution of Formylglycine-generating Enzyme with Copper

The specific activity of FGE increases significantly upon treatment with stoichiometric amounts of copper(II). a, wild-type Hs-FGE contains three primary domains: an ER-directing signal, an NTE that interacts with ERP44 for ER retention, and a catalytic core. Most strikingly, both the copper-treated “activated” forms of FGE contained just above 1 mol of copper/FGE. Furthermore, the specific activity of each of these enzyme preparations correlated with the ratio of copper/FGE, suggesting that copper is an integral component of the active form of FGE (Fig. 4d). Any amount less than 1 molar eq resulted in proportionally lower activity, an observation that is inconsistent with the catalytic activation of active site thiols.

After optimization, we found that both Sc-FGE and Hs-cFGE could be efficiently activated with 5 molar eq of CuSO4 for 1 h at 25 °C followed by removal of excess copper. The specific activity of Sc-FGE increased by more than an order of magnitude, from 214 ± 34 pkatal mg⁻¹ (n = 9) to 3579 ± 215 pkatal mg⁻¹ (n = 7); Hs-cFGE specific activity increased by a factor of 3, from 1,143 ± 84 pkatal mg⁻¹ (n = 10) to 3,050 ± 115 pkatal mg⁻¹ (n = 5, Fig. 4e). Next, we tested whether FGE that was treated with copper contained the metal after purification. To answer this question, we measured the quantities of copper and calcium in both the unactivated and the activated FGE samples using inductively coupled plasmon mass spectrometry. The Sc-FGE produced in E. coli contained almost no copper above the level of the noise of the measurement. By contrast, Hs-cFGE, as isolated from insect cells, contained 0.35 mol of copper per mol of FGE. Most strikingly, both the copper-treated “activated” forms of FGE contained just above 1 mol of copper/FGE. Furthermore, the specific activity of each of these enzyme preparations correlated with the ratio of copper/FGE, suggesting that copper is an integral component of the active form of FGE (Fig. 4e and Table 1). All of the FGE preparations also contained ~1-2 calcium atoms per FGE.

We then considered whether it is possible to enhance the FGE reaction rate by adding copper directly to the reaction mixture. Instead, our data confirmed the previous report (7) that product formation was significantly inhibited by the inclusion of copper in the reaction. Specifically, we observed very rapid formation of the oxidized form of DTT as well as Csub as a result of copper-catalyzed disulfide formation (Fig. 5).

To confirm that catalytic activity in the activated form of FGE does not depend upon an active site disulfide, we tested whether addition of strong reducing agents to the activated enzyme would decrease the specific activity of the resulting material. If the integrity of the active site disulfide is indeed important for turnover, then strong reducing agents should have decreased FGE turnover rates. Hs-cFGE has the potential to form the active site disulfide (Cys2356-Cys2341) as well as two...
structural disulfides (Cys\(^{346-345}\) and Cys\(^{365-364}\)) elsewhere in the protein (Fig. 6a). Treatment of Hs-cFGE with 20 mM DTT (or 20 mM TCEP, not shown) produced an FGE with unchanged specific activity (Fig. 6b). To confirm that the relevant Cys residues changed redox state as a result of this treatment, we developed an LC-MRM/MS assay to detect solution-accessible Cys residues in the active site. With this method, we successfully monitored both the Cys\(^{341}\) and Cys\(^{235}\)-containing peptides. As expected, treatment with DTT increased the proportion of accessible active site Cys\(^{341}\) from 28 to 93%. Notably, Cys\(^{235}\), which participates in a buried structural disulfide, was inaccessible, and thus, in a disulfide independent of DTT treatment. Taken together, these data confirm that reductive treatment generated almost quantitatively reduced active site thiols, did not perturb other structural disulfides, but had no effect on the catalytic activity of FGE.

After observing that FGE contained ~1 copper atom/enzyme, we made an effort to remove the metal from the protein to demonstrate its requirement for catalytic activity. Cyanide is known to be an inhibitor of copper-containing oxidases (36–38). Attempting to extract copper through pretreatment with 5 molar eq of KCN did decrease the activity of Hs-cFGE (but not Sc-FGE) by a modest amount, suggesting that it could gain access to the bound copper (Fig. 7a). We next tested whether FGE turnover could be inhibited competitively by cyanide. Having discovered that Cu(II) generates the FGE holoenzyme, we next determined the standard enzymatic parameters of both Sc-FGE and Hs-cFGE (Fig. 8). Much to our surprise, despite their structural homology, the two species catalytic parameters were different. Sc-FGE did not interact with substrate very strongly (\(K_m = 96 \mu M\)) and exhibited a relatively rapid \(k_{cat}\) (17.3 min\(^{-1}\)), which together resulted in a modest enzyme efficiency with \(k_{cat}/K_m = 3.0 \times 10^4 M^{-1} s^{-1}\) (Fig. 8a). By comparison, Hs-cFGE turned over substrate with a slower catalytic rate constant (\(k_{cat} = 6.06 \text{ min}^{-1}\)) but demonstrated a strong interaction with the substrate peptide (\(K_m = 0.34 \mu M\)).

### Table 1

| Preparation  | [Protein]\(^a\) | Calcium \(\mu M\) | Calcium \(\mu g/\text{liter}\) | Calcium (ratio)\(^a\) | Copper \(\mu M\) | Copper \(\mu g/\text{liter}\) | Copper (ratio)\(^a\) |
|--------------|-----------------|----------------|----------------|-----------------|----------------|----------------|----------------|
| Sc-FGE       | 131.4           | 375            | 373.75        | 2.84            | 111            | 1.74            | 0.01           |
| Hs-cFGE      | 204.3           | 98             | 97.4          | 0.48            | 4368           | 68.73           | 0.34           |
| Sc-FGE + copper | 64.5         | 55             | 54.99         | 0.85            | 4768           | 75.03           | 1.16           |
| Hs-FGE + copper | 69.7         | 106            | 105.59        | 1.51            | 6112           | 96.18           | 1.38           |

\(^a\) Protein concentrations were measured using the absorption at 280 nm of stock protein solutions. Extinction coefficients for protein were calculated from the primary sequence of the enzyme using the analysis tools on the ExPASy bioinformatics server (51). For Hs-cFGE, \(\epsilon = 85,745 \text{ M}^{-1} \text{ cm}^{-1}\) and molecular mass = 33,286 Da; for Sc-FGE, \(\epsilon = 88,000 \text{ M}^{-1} \text{ cm}^{-1}\) and molecular mass = 37,432 Da.

\(^a\) Ratio describes molar equivalents of metal versus FGE.
Taken together, the Hs-cFGE ($k_{\text{cat}}/K_m = 3.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) was ~10-fold more efficient than its bacterial counterpart (Fig. 8b).

In total, our data demonstrate that the modestly active forms of FGE isolated from cell culture are a mixture of the apoenzyme, lacking the copper cofactor, and the holoenzyme, which is highly active and efficiently converts Cys to fGly on model peptide substrates. We then began developing robust conditions for using the FGE holoenzyme in biocatalytic reactions on folded protein substrates.

**FGE Is an Efficient Biocatalyst for in Vitro Conversion of Cys to fGly on Folded Proteins**—To use in vitro conversion for the production of aldehyde-tagged mAbs, we needed to modify the reaction conditions from those used for the kinetic assay. For most substrates, 5–10 molar eq of DTT was sufficient to achieve complete conversion. We found that turnover was also possible with TCEP, lipoic acid, and bis(2-mercaptoethyl)sulfone. However, the presence of even 1 molar eq of a strong reducing agent (e.g. DTT, TCEP, lipoic acid, or bis(2-mercaptoethyl)sulfone) can cleave disulfides that are present in an mAb substrate (40). To avoid substrate disulfide cleavage, we selected an alternate reducing agent for the reaction. We found that weaker reducing agents, e.g. βME or GSH, were tolerated by antibody substrates, and also enabled FGE catalysis. As it was most cost effective, we elected to move forward with βME for our antibody conversion reactions. However, we found that changing from a cyclizing (DTT) to a non-cyclizing reducing agent (βME) resulted in side products that had not previously been observed (discussed below). To reduce this side product formation and increase product yield, we further considered the role(s) of the reducing agent in product formation.

X-ray crystallography of FGE (what we now understand to be the apoenzyme) bound to a substrate peptide shows Cys341 and Csub covalently linked by a disulfide in the active site (6). If the substrate is indeed anchored by this Cys341-Csub disulfide during turnover, an exogenous thiol might be able to react with the enzyme-substrate complex (E+S) by thiol-disulfide exchange to release substrate in the form of a thiol-Csub disulfide. Indeed,
we observed two off-pathway products formed by FGE during turnover. First, when reducing agent was not added to the reaction, we observed C\textsubscript{sub}-C\textsubscript{sub} dimer formation. Second, when we added a monothiol as the reductant (e.g. BME), we observed rapid production of the C\textsubscript{sub}-BME disulfide (Fig. 9a). In control reactions lacking enzyme, neither C\textsubscript{sub}-C\textsubscript{sub} nor C\textsubscript{sub}-BME were generated (not shown).

To limit the formation of C\textsubscript{sub}-C\textsubscript{sub} and C\textsubscript{sub}-BME, we monitored their formation as a function of reaction conditions. First, we varied the concentration of reducing agent in the reaction, and quenched the reactions when they were half-complete (Fig. 9b). At low [BME] versus [C\textsubscript{sub}], the C\textsubscript{sub}-C\textsubscript{sub} predominated. Increasing [BME] resulted in a concurrent decrease in C\textsubscript{sub}-C\textsubscript{sub}, and an increase in C\textsubscript{sub}-BME. Importantly, increasing [BME] also resulted in a higher yield of fGly.

Second, we investigated the effect of [BME] as the reaction evolved over time. As mentioned above, in the absence of exogenous reducing agent product did form, but a significant proportion of C\textsubscript{sub}-C\textsubscript{sub} was generated (Fig. 9c), suggesting that C\textsubscript{sub} can act in place of the reducing agent during turnover, but at the expense of product yield. At low [BME] (0.25 mM, Fig. 9d), the C\textsubscript{sub}-C\textsubscript{sub} initially grew in at a rate competitive with product formation, but as the reaction proceeded, it reached a maximum, and then decayed away slowly. At this [BME], the C\textsubscript{sub}-C\textsubscript{sub} was consumed, but the C\textsubscript{sub}-BME reached a plateau of 13% of total peptide, where it remained, thereby limiting product yield. In a subsequent reaction with higher [BME] (10 mM, Fig. 9e), C\textsubscript{sub}-C\textsubscript{sub} was almost undetectable. Moreover, C\textsubscript{sub}-BME rose to 10% of the total substrate, but it was consumed concomitantly with product formation. In all cases, the consumption of both C\textsubscript{sub}-C\textsubscript{sub} and C\textsubscript{sub}-BME correlated with a higher yield of product formation.

In contrast to BME, when we used DTT as the stoichiometric reductant neither the intermediate C\textsubscript{sub}-C\textsubscript{sub} nor the C\textsubscript{sub}-DTT disulfides were observed. This is likely because DTT can cyclize to form an intramolecular disulfide and release substrate. However, we found that the rates of product formation in reaction using BME or DTT were not the same (Fig. 9f). Specifically, the rates of product formation were 5.07 or 3.27 min\(^{-1}\) in the presence of BME or DTT, respectively. Because reducing agent was present in large excess versus both the substrate and enzyme, it must be involved in limiting the rate of turnover.

If the reductants are acting by a 1-electron mechanism, their relative reaction rates should depend directly upon their reduction potentials: \(E'_{\text{r}}\) (BME) = -207 mV and \(E'_{\text{r}}\) (DTT) = -323 mV (41). If instead, the reductants are acting by thiol-disulfide exchange, their reaction rate should correlate with their thiol-disulfide exchange rate constants: \(k_{\text{BME}} = 1 \) and \(k_{\text{DTT}} = 6 \times 10^{5}\), as measured versus glutathione disulfide (41). Both of these scenarios predict that reactions performed in the presence of DTT should have a faster rate than those performed in the presence of BME. By contrast, we observed that reactions performed with DTT were slower than those with BME, and so the role of the thiol in the reaction cannot be explained by the reaction rate of the thiol alone.

The covalent disulfide in E:S that was previously observed by crystallography is likely the source of the discrepancy just described (Fig. 10). From this state, E:S can either proceed to product with a rate constant of \(k_{\text{cat}}\) or it can react with an exogenous thiol to form a disulfide with a rate constant of \(k_{DS}\). For any given reducing agent, \(k_{\text{cat}}\) is likely the same based upon the mechanisms of analogous copper-dependent oxidases, in which active site redox chemistry is not rate-determining (42). However, \(k_{DS}\) will depend directly upon the identity of the reducing agent in solution and its reaction rate constant. Said another way, if E:S involves a disulfide, it should dissociate faster in the presence of a stronger reducing agent, in competition with product formation. If the substrate is released as a disulfide that does not decay quickly (as with BME-DS), it could react with the enzyme again to regenerate E:S. Therefore, stronger reductants should slow turnover by dissociating the E:S complex, as observed for DTT and BME.

The predominance of C\textsubscript{sub}-BME also confirms that the reducing agents are reacting with E:S by thiol/disulfide exchange: 1-electron reduction would regenerate free substrate thiol, and not a significant proportion of a substrate disulfide. Interestingly, this aspect of the cycle also explains the faster turnover rate observed at pH 9 relative to pH 7, thiol/disulfide exchange is faster under basic conditions because more of the nucleophilic thiolate is present.

After determining this dual role of reducing agent during catalysis, we were able to finalize reaction conditions that smoothly translated to protein substrates. As we have previously described, the aldehyde tag can be introduced at the N or C terminus or at any solvent accessible internal sequence (11). Here, Cys was converted to fGly in high yield by Hs-cFGE in three independent regions of a mAb (Fig. 11a) and across a wide range of reaction scales (from 0.8 to 200 mg) on three independent mAbs (Fig. 11, b and c). In each case, the \(in vitro\) conversion yield was >90%, as measured by LC-MRM/MS (Fig. 12). Furthermore, the pH 9 reaction conditions did not affect antibody binding affinity (Fig. 13, a and b) or oxidation at Met\(^{292}\) (Fig. 13c), a residue that is important for FcRn binding and mAb circulation \(in vivo\) (43, 44).

**Discussion**

Formylglycine-generating enzyme was produced by recombinant expression for the generation of aldehyde-tagged proteins \(in vitro\). We successfully developed a discontinuous HPLC assay to measure the kinetic parameters of both FGEs. In contrast to previous work, these results indicate that the N-terminal extension of Hs-FGE is not required for catalytic activity \(in vitro\). Surprisingly, when purified from insect cell culture, the human enzyme was much more active than the bacterial form \(E. coli\).

We also designed experiments to explore the mechanism of FGE turnover. The existing mechanistic hypotheses focus on the redox state of the FGE active site cysteine residues as revealed by crystallography. When Hs-cFGE was isolated previously, Cys\(^{336}\) (Cys\(^{272}\) in Sc-FGE) and Cys\(^{341}\) (Cys\(^{277}\)) were present in the enzyme in a mixture of reduced and oxidized forms (8). The oxidation state of Cys\(^{336}\) and Cys\(^{341}\) can be manipulated using chemical reagents in solution, either to form the Cys\(^{336}\)-Cys\(^{341}\) disulfide, or with \(H_2O_2\) over a longer period of time, to form a sulfonic acid at both positions. In addition, a C336S mutation enables the “trapping” of C\textsubscript{sub} as an intermo-
FIGURE 9. FGE can catalyze substrate/reductant disulfide formation, which can be inhibitory to product yield, and confirms that substrate is bound in the active site as a disulfide. a, LC/MS identification of C_sub–βME. b, product formation in FGE reaction mixtures across a range of 0–10 mM βME. Without βME, fGly and C_sub–C_sub form at comparable rates. As [βME] is increased, C_sub–C_sub is replaced by C_sub–βME, and product forms at a higher rate. Product formation over time catalyzed by FGE in the absence of reducing agent (c), with 2.5 mol eq of reducing agent (d), and with 100 mol eq of reducing agent (e). f, the initial velocity of FGE catalysis with βME is higher than with DTT (1 mM each reducing agent).
lecular disulfide with Cys341 (6). This convincing set of evidence prompted the hypothesis that FGE uses a thiol-disulfide interchange to anchor Csub and activate Cys336. This residue would then perform the activation of molecular oxygen to form a higher oxidation state Cys residue (either sulfenic acid or cysteine peroxide) and subsequently oxidize the substrate.

In this work, we demonstrated that the presence of a disulfide of the active site cysteines does not correlate with enzyme activity. Instead, we made the fortuitous discovery that FGE incorporates copper as a cofactor for catalysis. Our results indicate that recombinant expression of FGE most often results in the apoenzyme, which can be reconstituted as the holoenzyme by the addition of copper(II) sulfate at pH 7. This reconstitution was ineffective with other metal ions commonly found in enzymes, including Mn$^{2+}$, Fe$^{2+}$, and Zn$^{2+}$. Interestingly, the only previous characterization of FGE metal content identified 0.09 copper/human FGE as produced in the mammalian HT1080 cell line (7). We observed that the catalytic rates of both human and bacterial FGE were significantly increased upon activation with stoichiometric amounts of Cu$^{2+}$, and that turnover was inhibited by the addition of cyanide to FGE reaction mixtures. Previous reports have shown that the inhibition of aerobic copper oxidases by CN$^{-}$ results from ligation of CN$^{-}$ at the metal center (in the reduced state of Cu$^{1+}$), which prevents oxygen binding and substrate activation (38). Furthermore, FGE activity was extinguished completely after treating the enzyme with a combination of a chelator and a reductant, reinforcing the highly specific and tight binding interaction between FGE and Cu$^{2+}$.

Taken together, we hypothesize that FGE resides in the well studied class of copper oxidases that perform 2 H$^{+}$/2 e$^{-}$ oxidation, activate molecular oxygen, and require exogenous reductants to complete a catalytic cycle. This also helps explain how FGEs that reside in subcellular compartments with varying redox environments can still perform catalysis, turnover depends upon copper and oxygen, and not the presence of an intact disulfide.

More specifically, FGE most closely parallels the structure and reactivity of the Cu$_{A}$ site in enzymes like peptidylglycine α-hydroxylating monoxygenase, which hydroxylates a C terminally extended peptide (42). In peptidylglycine α-hydroxylating monoxygenase (and other related enzymes), the Cu$_{A}$ is the location of oxygen activation, consumption of a molar eq of reducing agent, and 2 H$^{+}$/2 e$^{-}$ substrate oxidation.
Despite these similarities to existing mononuclear copper oxidases, FGE remains unique. In CuM, His and Met bind the copper atom, but not a Cys (42). The mononuclear copper-binding motif in FGE is likely a combination of the ligands that are highly conserved, and those whose mutation results in decreased or eliminated activity. Specifically, the residues whose mutations are known to change FGE activity are Cys336, Cys341, Ser333, His337, and Tyr340.

With respect to cysteine, other enzymes in which isolated copper centers are ligated by thiols (e.g., blue copper proteins), the copper center typically performs 1-electron redox chemistry, and not oxygen activation or substrate oxidation (45). In addition, the ligand-to-metal charge transfer of the Cys results in a strong absorption band in the visible spectrum of the enzyme. However, reconstituted FGE holoenzyme does not have any absorption bands above 280 nm, and so it is unlikely to contain a type I copper center. Despite this, mutation of Cys336 and Cys341 have a significant effect on FGE activity. Their role in the copper active site is unclear.

What we now believe is the FGE apoenzyme is well characterized by x-ray crystallography (6, 8, 9, 20, 21, 46). With respect to other FGE residues, His337 seems to be a likely candidate for copper ligation because of the prevalence of His in copper.
Reconstitution of Formylglycine-generating Enzyme with Copper

![Diagram of the proposed catalytic mechanism for FGE that accounts for the copper cofactor](image)

**FIGURE 14.** The proposed catalytic mechanism for FGE that accounts for the copper cofactor. **a**, substrate binds enzyme and is covalently attached through Cys341-Csub. This covalent bond formation may be catalyzed by Cu^{2+}, or it could result from thiol-disulfide exchange between an existing Cys341-Csub disulfide bridge, as shown for the other oxygen-activating copper oxidases (23). **b**, oxidation of substrate by the Cu(II)-O2 through proton-coupled electron transfer would generate a disulfide bridge. **c**, H2O2 as a reaction product, but the cupric peroxide Cu(II)-OOH might also undergo further reduction to generate only H2O as a byproduct. We would predict that the resting state ligand on copper is a hydroxide, although we cannot rule out direct involvement of Cys341 through an alternative mechanism. These scenarios will serve as a guide, but it is clear that developing a comprehensive catalytic cycle for FGE will require additional structural and paramagnetic spectroscopic studies of the enzyme.

With the mechanistic knowledge described above, we optimized the reaction conditions for efficient production of fGly without disrupting existing intramolecular disulfides in the substrate protein. Interestingly, we found that FGE was able to catalyze the formation of disulfides at the substrate Cys when monothiol reducing agents were present in solution. Furthermore, when dithiol reagents such as DTT are used, the rate of product formation decreased, suggesting that any intermolecular disulfide is quickly abolished by DTT cyclization. These data confirm the existing proposal that substrate is anchored during turnover, and optimal turnover requires balancing the type and amount of reductant in solution with product formation.

We have further demonstrated that FGE can be produced in good yield and used as an excellent biocatalyst for the production of aldehyde-tagged proteins in vitro. Reactions with FGE can scale across at least 3 orders of magnitude in mass with no decrease in yield, and proceed without perturbing existing disulfides or modifying residues that are important for mAb performance in vivo.

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