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Chemoenzymatic Fc Glycosylation via Engineered Aldehyde Tags

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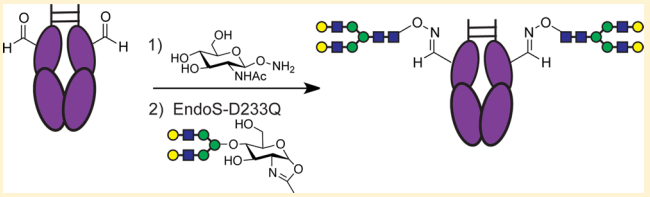
Supporting Information

ABSTRACT: Glycoproteins with chemically defined glycosylation sites and structures are important biopharmaceutical targets and critical tools for glycobiology. One approach toward constructing such molecules involves chemical glycosylation of aldehyde-tagged proteins. Here, we report the installation of a genetically encoded aldehyde tag at the internal glycosylation site of the crystallizable fragment (Fc) of IgG1. We replaced the natural Fc *N*-glycosylation sequon with a five amino-acid sequence that was efficiently converted by recombinant formylglycine generating enzyme *in vitro*, thereby introducing aldehyde groups for subsequent chemical elaboration. Oxime-linked glycoconjugates were synthesized by conjugating aminooxy *N*-acetylglucosamine to the modified Fc followed by enzymatic transfer of complex *N*-glycans from corresponding glycan oxazolines by an EndoS-derived glycosynthase. In this manner we generated specific Fc glycoforms without relying on natural protein glycosylation machineries.



INTRODUCTION

Structurally defined and homogeneous glycoforms are of benefit for applications of glycoproteins as biotherapeutics and tools for biological research.[1−4] Unfortunately, precise structural control of protein glycosylation is difficult to achieve in conventional glycoprotein expression systems. Thus, chemists have sought to contribute new technologies to conjugate chemically defined glycans to either synthetic or recombinant proteins. One approach is to replace natural protein−glycan linkages with conservatively altered structures generated by chemoselective ligation.[5,6] In a typical workflow, the protein is first engineered to display a uniquely reactive functional group that serves as a point of attachment for glycans armed with complementary functionality. Popular chemistries include disulfide conjugation,[7,8] elimination/addition reactions,[9,10] azide/alkyne-based click chemistries,[11−13] and oxime formation.[14−17]

A central challenge of such efforts is to arm the protein for chemical glycosylation only at the desired sites. We recently achieved this goal using the aldehyde tag method.[18−20] We installed a six amino-acid sequence (LCTPSR) recognized by the formylglycine generating enzyme (FGE) near the C-terminus of a recombinant protein; FGE converted the Cys residue to the aldehyde-bearing residue formylglycine (fGly) during coexpression in *E. coli*.[17] Subsequently, the fGly aldehyde was chemically conjugated to synthetic aminooxy glycans, affording homogeneous oxime-linked glycoprotein products. Notably, the oxime-linked products bear resemblance to native *N*-glycans (Figure 1A) and therefore are close structural mimics.

In our previous work, high Cys-to-fGly conversion efficiencies were achieved by overexpressing recombinant FGE alongside the tagged protein of interest.[18−21] An alternative to this *in vivo* enzymatic conversion approach is to express proteins with unconverted Cys residues, and then modify them with recombinant FGE *ex vivo*. X-ray structures of FGE in complex with synthetic substrates show the bound peptides in an extended conformation, consistent with the assumption that FGE modifies the target Cys co-translationally, before folding has occurred.[22,23] Not surprisingly then, FGE readily introduces fGly residues on short synthetic peptide substrates[24,25] and on recombinant proteins wherein the aldehyde tag sequence was added at a C-terminal position.[21]

Whether FGE can modify internal sequences located in relatively structured parts of a folded protein remains an open question. For applications in glycoengineering, this is a critical issue since most protein glycosylation sites reside at internal positions rather than near the termini. The crystallizable fragment (Fc) of IgG, for example, is a homodimer that contains a highly conserved *N*-glycosylation site. The Fc domain typically exists as a mixture of different glycoforms wherein each monomer might be differently glycosylated.[26−28] These glycans modulate the binding of Fc to various immune

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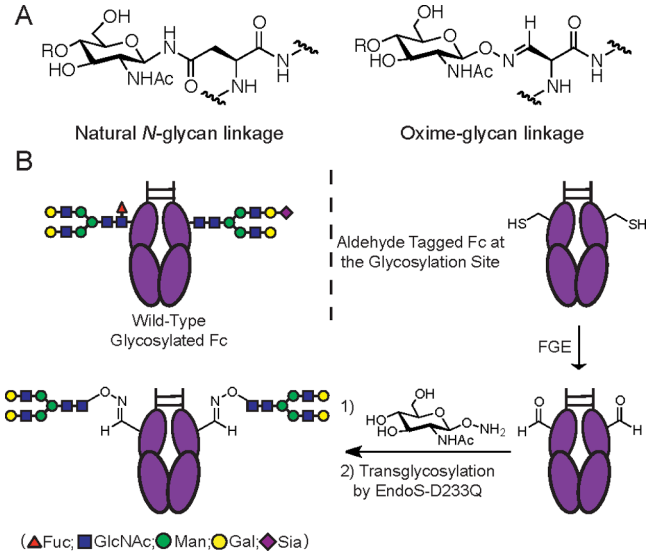

Figure 1. Oxime-linked glycoconjugates. (A) Natural *N*-glycan linkage is structurally similar to oxime-glycan linkage. (B) Schematic representation of incorporation of the aldehyde tag at the glycosyation site of Fc in order to make oxime-linked glycoconjugates.

receptors, thereby altering antibody effector function.[29−35] Therefore, methods for preparing specific Fc domain glycoforms have been the subject of considerable interest.[8,32,36−44]

Here, we demonstrate that the aldehyde tag can serve as a site for internal chemical glycosylation of the Fc domain. We expressed in Chinese hamster ovary (CHO) cells an Fc construct in which the *N*-glycosylation sequence was replaced with the FGE consensus motif. The protein underwent minimal Cys-to-fGly conversion during expression, but could be efficiently converted *in vitro* by reaction with recombinant FGE from *Mycobacterium tuberculosis* (*M. tb*). We conjugated aldehyde-tagged Fc to aminooxy *N*-acetylglucosamine (AO-GlcNAc), the protein-proximal residue in native *N*-glycans. The oxime-linked GlcNAc residue was then elaborated by enzymatic transfer of complex *N*-glycan moieties using an engineered bacterial endo-*β*-*N*-acetylglucosaminidase from *Streptococcus pyogenes* (EndoS-D233Q) (Figure 1B).[37] This ability to install a reactive aldehyde at the Fc glycosylation site offers a new route to make glycosylated antibodies and a platform to study glycan-mediated functions.

RESULTS AND DISCUSSION

Replacement of the Fc Glycosylation Site with an Aldehyde Tag. The natural Fc *N*-glycosylation site lies within the C′E-loop; we sought a motif that introduced negligible structural perturbations while also introducing the minimal recognition elements for FGE conversion of Cys-to-fGly. Human FGE can convert peptide substrates containing the minimal CxPxR motif; however, the CTPSR motif is more efficiently converted in peptide and model protein substrates.[45,46] Extending the FGE recognition motif to 13 residues (LCTPSRAALLTGR) can offer increased conversion efficiency in model systems as well.[45] Based on this information, we constructed the three aldehyde tag Fc sequences shown in Table 1: CxPxR (Fc1), CTPSR (Fc2), and CTPSRxxxLTGR (Fc3), where x denotes the native amino acids in the Fc sequence. In all cases, the Cys/fGly site replaced the Asn residue that is normally glycosylated. Notably, each construct possessed the same total number of residues as the wild type

Table 1. Aldehyde Tag Sequences Installed at Fc Glycosylation Site[a]

Fc construct	Sequence	Experimental Outcome
Wild Type Fc	Y-N-S-T-Y-R-V-V-S-V-L-T-V	N/A
Fc1 *CxPxR*	Y-C-S-P-Y-R-V-V-S-V-L-T-V	No fGly formation
Fc2 *CTPSR*	Y-C-T-P-S-R-V-V-S-V-L-T-V	*In vitro* fGly formation
Fc3 *CTPSRxxxLTGR*	Y-C-T-P-S-R-V-V-S-L-T-G-R	No expression

[a]N: Natural *N*-glycosylation site. N/A: Not Applicable.

sequence. Amino acid changes were made via point mutagenesis rather than, for example, sequence additions or insertions.

Genes encoding the Fc1, 2, and 3 constructs were introduced into the commercial pFuse vector (InvivoGen). As controls, analogous Fc constructs in which the Cys residue was mutated to an Ala residue were also generated. Following expression in CHO cells, the Fc proteins were purified using protein A/G agarose. The expression levels of Fc1 and Fc2 were similar to that of wild type glycosylated Fc (∼1 mg/L). The Fc3 construct showed little to no expression, perhaps because the longer aldehyde tag sequence disrupted its overall structure; this construct was therefore no longer pursued.

To probe for the conversion of Cys-to-fGly, we incubated Fc1 and 2 with AlexaFluor 488 (AF488) hydroxylamine (Invitrogen) in sodium acetate buffer (pH 4) and analyzed the products by SDS-PAGE with fluorescence scanning. Neither Fc1 nor Fc2 exhibited detectable fluorophore labeling, indicating that the CHO cells' endogenous FGE did not convert Cys-to-fGly at a level detectable by in-gel fluorescence. These unconverted proteins were thus ideal substrates to test *M. tb* FGE's ability to convert an internal sequence *in vitro*. The Fc constructs were incubated with *M. tb* FGE (0.1 equiv) in Tris buffer (pH 9) with 0.5 mM dithiothreitol (DTT) at 30 °C. An appealing feature of this FGE ortholog is its tolerance for a variety of CxPxR sequences.[47] After *in vitro* treatment with FGE, the Fc1 and 2 proteins were again reacted with AF488 hydroxylamine. Fc2 showed labeling by in-gel fluorescence analysis, indicating that fGly conversion had occurred. The control Fc2 Cys-to-Ala mutant showed no detectable fluorescence (Figure 2A), confirming that fGly was present exclusively at the desired glycosylation site. As further verification of enzymatic conversion, we treated Fc2 with heat killed FGE and saw no reactivity with AF488 hydroxylamine (Figure 2B). In contrast to Fc2, Fc1 exhibited no detectable labeling after incubation with active FGE. This observation suggests that despite its promiscuity among CxPxR sequences in short peptides, *M. tb* FGE requires the more native CTPSR substrate sequence in folded proteins.

We next sought to optimize the efficiency of *in vitro* Cys-to-fGly conversion by *M. tb* FGE. The reaction was relatively insensitive to different buffer salts but showed a strong preference for alkaline pH (optimum conversion was obtained at pH 9) (Figure S1). We observed a pronounced effect of reaction temperature on conversion efficiency as assessed qualitatively by in-gel fluorescence intensity. We performed identical reactions (Fc2 with 0.4 equiv *M. tb* FGE in Tris buffer (pH 9) with 0.5 mM DTT) at temperatures ranging from 25 to 45 °C. fGly-Fc2 was then labeled with AF488 hydroxylamine and analyzed by SDS-PAGE (Figure 3A). The intensity of Fc2's fluorescence increased with reaction temperature, indicating

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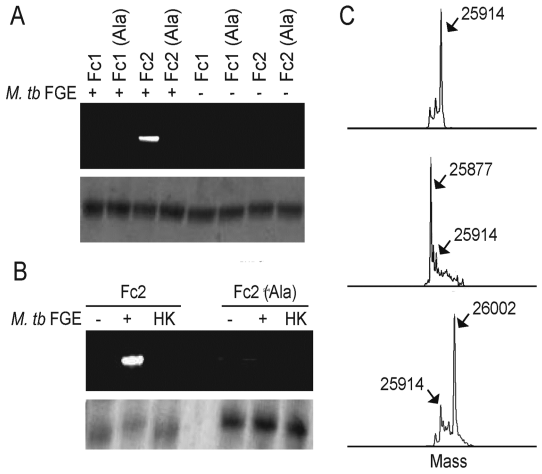

Figure 2. Incorporation of aldehyde tags at the glycosylation site of Fc. (A) SDS-PAGE of fGly formation in Fc monomer. Purified Fc was treated with (+) or without (-) *M. tb* FGE. Following FGE incubation, tagged Fcs were reacted with AF488 hydroxylamine. AF488 fluorescence (Top); colloidal blue stain (Bottom). (B) SDS-PAGE of fGly formation of aldehyde-tagged Fc dimer as a result of *M. tb* FGE activity. Fcs were incubated with either no (-), active (+), or heat killed (HK) FGE followed by reaction with AF488 hydroxylamine. AF488 fluorescence (Top); colloidal blue stain (Bottom). (C) Deconvoluted mass spectra of Fc2 treated with 1 equiv *M. tb* FGE at pH 9 for 20 h at 42 °C followed by reaction with *O*-benzylhydroxylamine at pH 4 for 20 h at 30 °C. (Top) untreated Fc2. (Middle) Fc2 treated with *M. tb* FGE. (Bottom) Fc2 treated with *M. tb* FGE and reacted with *O*-benzylhydroxylamine. Expected masses (Da): Fc2, 25914; fGly-Fc2, 25896; fGly-Fc2(M-H_2O), 25878; Oxime-benzyl Fc2, 26001.

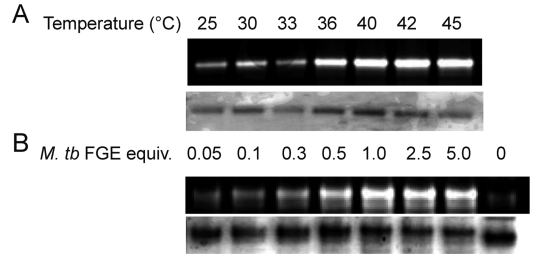

Figure 3. Optimization of the *in vitro* Cys-to-fGly conversion efficiency by *M. tb* FGE. (A) Temperature optimization. Fc2 was treated with 0.4 equiv *M. tb* FGE at 25−45 °C for 20 h before labeling with AF488 hydroxylamine. Reactions were reduced and resolved by SDS-PAGE. fGly formation was assessed by AF488 fluorescence (Top) and protein loading by colloidal blue stain (Bottom). (B) FGE dosage. Fc2 was treated with 0.5−5 equiv *M. tb* FGE at 42 °C for 20 h before conjugation to AF488 hydroxylamine. Reactions were resolved by SDS-PAGE. fGly formation was assessed by AF488 fluorescence (Top) and protein loading by colloidal blue stain (Bottom).

more efficient fGly formation. This observation may reflect temperature-dependent conformational fluctuations that give FGE better access to its internal substrate sequence. Since maximum conversion occurred at 42 °C, all subsequent FGE reactions were performed at this temperature.

Next, we focused on optimizing the stoichiometry of FGE to Fc2. Reactions containing Fc were incubated with various amounts of FGE ranging from 0.05 to 5 equiv. After the enzyme reaction, fGly-Fc2 constructs were probed with AF488 hydroxylamine and analyzed by SDS-PAGE (Figure 3B). The in-gel fluorescence reached a maximum at 1 equiv of FGE, suggesting that the enzyme is being consumed in the reaction rather than functioning catalytically. In the proposed mechanism of human FGE catalysis,[22,23] completion of the catalytic cycle requires consumption of a reducing equivalent from the medium. DTT was proposed to fulfill this function in the context of *in vitro* reactions.[22] *M. tb* FGE does not seem to follow this paradigm; even in the presence of excess DTT, the enzyme appears to function stoichiometrically. Further optimization with *M. tb* FGE might focus on identifying a reducing agent that can complete its catalytic cycle *in vitro*.

Having optimized the *in vitro* FGE reaction by qualitative assessments, we next sought to quantitate both the Cys-to-fGly conversion process as well as the yield of oxime formation. We confirmed fGly formation by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) analysis of Fc monomers generated by DTT reduction. Following FGE treatment, we observed ions corresponding to aldehyde-tagged (fGly-Fc2) and unconverted (Cys-Fc2) Fc2 monomers by MS (Figure 2C). Based on their relative mass spectral abundances, we calculated the Cys-to-fGly conversion to be 76% under optimized conditions. To assess the efficiency of oxime formation, we incubated fGly-Fc2 with *O*-benzylhydroxylamine. Ions corresponding to oxime-conjugated (oxime-benzyl Fc2) and unconverted Cys-Fc2 were observed (Figure 2C). Unconjugated fGly-Fc2 was below the limit of detection, indicating that quantitative oxime conjugation had occurred. Control reactions using the Cys-to-Ala Fc2 mutant, which does not contain fGly, showed no FGE conversion or reaction with *O*-benzylhydroxylamine (Figure S2). LC-ESI-MS analysis was also performed on the Fc dimer to verify that both Fc subunits contained the aldehyde modification (Figure S3).

Chemoenzymatic Glycosylation of fGly-Fc2. After the successful introduction of a reactive aldehyde at the glycosylation site of Fc, we sought to introduce defined glycans by oxime formation and enzymatic elaboration. Aminooxy (AO) GlcNAc was synthesized as previously described[48] and conjugated to fGly-Fc2 in sodium acetate buffer (pH 4) (Figure 4A). The formation of the corresponding glycoconjugate was confirmed by lectin blot probing with wheat germ agglutinin conjugated to AlexaFluor 647 (WGA-AF647). WGA binding was observed only for the product of fGly-Fc2. Control reactions using either non-FGE treated Fc2 or the Cys-to-Ala Fc2 mutant did not give products that bound to WGA (Figure 4B). LC-ESI-MS analysis verified oxime-GlcNAc formation on the Fc dimer (Figure 4C). To ensure that the Fc domain retained its overall structure after FGE conversion and oxime formation, we confirmed that the glycosylated Fc2 dimer could still bind to protein A/G resin (Figure S4).

With oxime-GlcNAc Fc2 in hand, we next sought to elaborate the glycans analogously to native glycosylated Fc. Previous work has shown that IgG glycans can be remodeled using endo-β-*N*-acetylglucosaminidases, which can be engineered to transfer oxazoline glycans onto single core GlcNAc-Asn residues at natural glycosylation sites.[36−39] Moreover, the endoglycosidase EndoA has promiscuous transglycosylation activity on GlcNAc moieties attached to peptides or proteins via non-natural linkages (i.e., triazole, thioether, and disulfide-linked GlcNAc).[11,49] However, this enzyme is limited with regard to the complexity of its glycan substrates. A mutant form of EndoS (EndoS-D233Q), by contrast, can transfer complex glycan oxazolines to GlcNAc-modified IgG and Fc, recapitulating the native structures,[37,39] and is thus ideally suited for Fc glycoengineering. However, EndoS is highly specific for IgG Fc *N*-linked GlcNAc residues and will not elaborate Asn-GlcNAc

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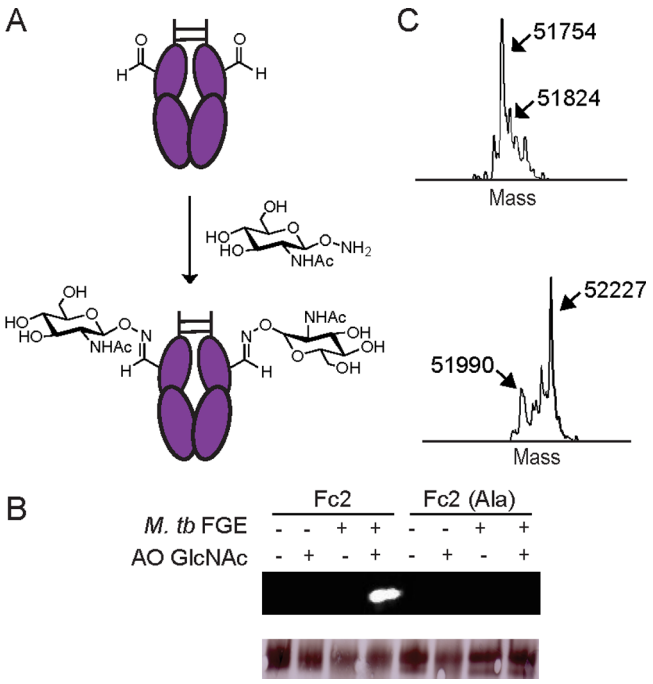

Figure 4. AO GlcNAc conjugation to aldehyde-tagged Fc2 dimer. Fc constructs were treated with *M. tb* FGE at pH 9 for 20 h at 42 °C followed by conjugation to AO GlcNAc at pH 4 for 20 h at 30 °C. (A) Schematic of AO GlcNAc conjugation to fGly-Fc2. (B) Lectin blot of Fc construcs following conjugation to AO GlcNAc. Conjugation of AO GlcNAc was assessed using WGA-AF647 (Top) and protein loading by India ink (Bottom). (C) Deconvoluted mass spectrum of fGly-Fc2 (Top) and oxime-GlcNAc Fc2 (Bottom). Expected masses (Da): Cys-Fc2, 51824; fGly-Fc2 (M-2H_2O), 51754; FGly-Fc2 + 1GlcNAc (M-H_2O), 51990; fGly-Fc2 +2GlcNAc, 52226.

sites on other proteins or on denatured IgG,[50,51] suggesting sensitivity to the protein structure surrounding the glycosylation site. Thus, the ability of EndoS to elaborate oxime-GlcNAc within the aldehyde tag motif on the Fc was an open question.

We incubated oxime-GlcNAc Fc2 with EndoS-D233Q and two oxazoline glycans previously shown to exhibit good substrate activity: a sialylated complex-type glycan (S2) and its unsialylated congener (G2) (Figure 5A). Reactions were performed in Tris buffer (pH 7.5) at 30 °C for 1.5 h. Product formation was monitored by LC-ESI-MS. Ions corresponding to S2-glycosylated Fc2 (S2-Fc2), oxime-GlcNAc Fc2, and fGly-Fc2 were observed, the latter presumably derived from hydrolysis of either oxime conjugate. The relative abundances of oxime-GlcNAc Fc2 and S2-Fc2 indicated a transfer yield[52] of 51% (Figure 5C). The analogous reaction with G2 oxazoline proceeded similarly, with a glycan transfer yield[52] of 61% (Figure 5D). Further, we analyzed the enzymatic reaction products by lectin blot probing with the sialic acid-binding *Sambucus nigra* agglutinin conjugated to fluorescein isothiocyanate (SNA-FITC) (Figure 5E) or the terminal *N*-acetyllactosamine-binding *Erythrina crista-galli* agglutinin conjugated to FITC (ECA-FITC) (Figure 5F). Significant lectin binding was observed only for the transglycosylation products. These results demonstrate that oxime-linked GlcNAc at the glycosylation site of Fc2 is a suitable substrate for EndoS-D233Q. This is the first report of transglycosylation activity with this non-natural GlcNAc linkage, as well as the first to show transfer of complex-type glycans onto a non-natural linkage on a fully folded protein.

■ CONCLUSIONS

In summary, an internal aldehyde tag sequence can replace native *N*-glycosylation sites in the IgG Fc domain without perturbing protein structure. Despite their location within a folded protein, these sites can be converted by FGE *in vitro*, generating a reactive aldehyde for subsequent chemical glycosylation. EndoS, previously thought to be highly specific for the Fc *N*-glycan sequon, can elaborate oxime-linked GlcNAc residues conjugated to the aldehyde tagged Fc. We used these tools to generate specific Fc glycoforms that emulate native structures. While this work focused on Fc glycoengineering, it can serve as a model for tailored glycosylation of other proteins by a combination of genetic, chemical, and enzymatic methods.

■ EXPERIMENTAL SECTION

Expression and Purification of Aldehyde Tagged Fc Constructs. The plasmids encoding Fc constructs were transiently transfected into CHO cells using TransIT-LT1 (MirusBio) following the manufacturer's protocol. CHO cells were cultured in F-12 media (HyClone) containing 5% ultralow IgG fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2 at 37 °C. Two to four days after transfection, the media was collected and centrifuged at 3700*g* for 15 min. The media was then concentrated using a 10-kDa spin concentrator (Millipore). The concentrate was diluted (1:1) with phosphate buffered saline (PBS) and purified using Protein A/G agarose (Pierce).

Expression and Purification of *M. tb* FGE. Recombinant *M. tb* FGE was expressed in *E. coli* as previously reported.[53] Briefly, BL21 (DE3) *E. coli* harboring a pBAD plasmid containing a His_6-tagged *M. tb* FGE were grown in LB media supplemented with 75 μg/mL ampicillin at 37 °C. When OD_{600} reached 0.5, 100 mM isopropyl-1-thio-β-D-galactopyranoside was added, and the temperature was lowered to 18 °C. After 18 h, the cells were harvested and lysed. The His_6-tagged protein was purified using Ni-NTA-agarose beads (Qiagen) following the manufacturer's protocol. FGE was eluted with 50 mM Tris, 500 mM NaCl, 250 mM imidazole, 10% glycerol, and 1 mM DTT, pH 7.5. The eluted protein was concentrated to <2 mL and further purified on a Superdex 75 column (GE Healthcare) using elution buffer without imidazole. Purified FGE was concentrated to 7 mg/mL and stored at −80 °C.

Expression and Purification of EndoS-D233Q. The EndoS-D233Q plasmid was previously constructed[37] and was transformed into BL21 (DE3) *E. coli*. The transformants were cultured in 2YT broth at 37 °C. When OD_{600} reached 0.8, 1 mM isopropyl β-D-thiogalactoside was added, and the temperature was lowered to 25 °C. After overnight growth, cells were harvested and frozen at −80 °C prior to protein purification. Bacterial pellets were resuspended and lysed in B-Per Bacterial Protein Extraction Reagent (Pierce) supplemented with 10 μg/mL of DNase I. Cell lysate was centrifuged at 26000*g* for 20 min at 4 °C, and the supernatant was applied to Glutathione-Superflow resin (Clontech). Samples were incubated at 4 °C for 60 min with gentle agitation. The resin was first washed with PBS, then 125 mM Tris, 125 mM NaCl, pH 8.0 until no protein was detected. EndoS-D233Q was eluted with 125 mM Tris, 125 mM NaCl, pH 8 supplemented with 10 mM reduced

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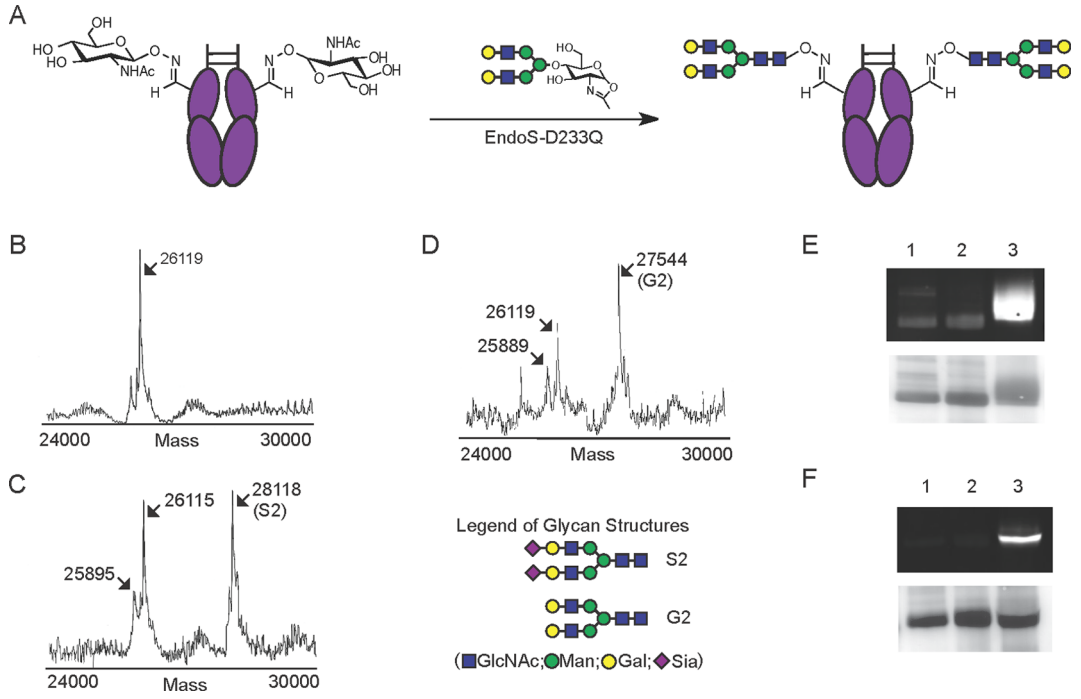

Figure 5. Chemoenzymatic glycan remodeling of oxime-GlcNAc Fc2. (A) Representative schematic of the transglycosylation reaction. LC-ESI-MS analysis of (B) oxime-GlcNAc Fc2, (C) transglycosylation product from the EndoS-D233Q catalyzed reaction between oxime-GlcNAc Fc2 and S2 oxazoline or (D) G2 oxazoline. Expected masses (Da): fGly-Fc2 (with disulfides), 25897; fGly-Fc2 (reduced disulfides), 25901; Oxime-GlcNAc Fc2 (with disulfides), 26115; Oxime-GlcNAc Fc2 (with reduced disulfides) 26119; S2-Fc2, 28117; G2-Fc2, 27539. Lectin blot of Fc2 dimer constructs following glycosylation remodeling. (E) Glycan elaboration to oxime-S2 was assessed using SNA-FITC (Top) and protein loading by india ink (Bottom). Lanes: 1, Fc2; 2, Oxime-GlcNAc Fc2; 3, S2-Fc. (F) Glycan elaboration to oxime-G2 was assessed using ECA-FITC (Top) and protein loading by India ink (Bottom). Lanes: 1, Fc2; 2, Oxime-GlcNAc Fc2; 3, G2-Fc2.

glutathione. Eluted protein was concentrated to 3 mg/mL and stored at −20 °C.

Verification of fGly via Coupling AF488 Hydroxylamine to Aldehyde Tagged Fc. To probe for the presence of aldehyde, Fc (5 μg) protein was reacted with 100 μM AF488 hydroxylamine in labeling buffer (100 mM sodium acetate, 150 mM NaCl, pH 4, 50 μL) at 30 °C overnight. Reaction mixtures were resolved by SDS-PAGE and fluorescence was detected using a Typhoon 9410 fluorescence scanner (GE Healthcare).

Initial *In Vitro* Formylglycine Conversion Using *M. tb* FGE. Fc constructs (5 μg) were incubated in 75 mM Tris, 100 mM NaCl, 0.5 mM DTT (pH 9, 95 μL) with 0.1 equiv of FGE for 18 h at 30 °C. Following FGE treatment, Fcs were buffered exchanged into labeling buffer and were reacted with AF488 hydroxylamine as stated above. Fc protein (2 μg) was separated by SDS-PAGE, and fluorescence was detected using a fluorescence scanner.

Temperature Optimization for *M. tb* FGE *In Vitro* Reaction. Fc2 (4 μg) was treated with 0.4 equiv of FGE in 75 mM Tris, 100 mM NaCl, 0.5 mM DTT (pH 9, 30 μL) for 18 h at 25, 30, 33, 36, 40, 42, or 45 °C. After FGE treatment, Fc was reacted with AF488 hydroxylamine as stated above. Fc2 (1 μg) from each reaction was separated by SDS-PAGE, and fluorescence was detected using a fluorescence scanner. Relative fluorescence was measured using Image J imaging software.

FGE Dosage Optimization for *M. tb* FGE *In Vitro* Reaction. Fc2 (4 μg) was incubated with 0.05, 0.15, 0.3, 0.5 1, 2, or 5 mol equiv of FGE in 75 mM Tris, 100 mM NaCl, 0.5 mM DTT (pH 9, 30 μL) overnight at 42 °C. After the FGE reaction, Fc2 was reacted with AF488 hydroxylamine as stated above. Fc2 (1 μg) from each reaction was separated by SDS-PAGE, and fluorescence was detected using a fluorescence scanner. Relative fluorescence was measured using Image J imaging software.

Optimized *In Vitro* Formylglycine Conversion Using *M. tb* FGE. Purified Fc (5 μg) was incubated in 75 mM Tris, 100 mM NaCl, 0.5 mM DTT (pH 9, 95 μL) with 1 mol equiv of *M. tb* FGE overnight at 42 °C.

Verification fGly Conversion Resulting from Active *M. tb* FGE Treatment. Heat-killed FGE was boiled at 100 °C for 10 min in 8 M urea. Fc2 (4 μg) was incubated with no FGE, FGE, or heat-killed FGE using the optimized protocol previously described. Following FGE treatment, Fc was reacted with AF488 hydroxylamine following the above protocol. Fc (1 μg) was separated by SDS-PAGE, and fluorescence was detected using a fluorescence scanner.

Formylglycine Conversion Determined Using Mass Spectrometry. Mass spectrometry was used to confirm the presence of fGly after treatment with exogenous FGE. After fGly conversion, Fc2 (30 μg) was reacted with 13 mM *O*-benzylhydroxylamine hydrochloride (Sigma-Aldrich) in labeling buffer before being reduced in 5 mM DTT at 37 °C for 30 min. High-resolution LC-ESI-MS measurements were obtained using an Agilent 1200 LC that was equipped with a C8 column (100 mm × 1.0 mm, Restek) and connected in-line with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The instrumentation is located in the QB3/Chemistry Mass Spectrometry Facility at the University of California, Berkeley. Raw mass spectra were viewed using Xcalibur software (version 2.0.7 SP1, Thermo) and mass spectral deconvolution was performed using ProMass software (version 2.5 SR-1, Novatia). Percent conversion was

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determined by comparing mass spectral abundances of non-FGE treated protein and FGE-treated protein assuming the two components have the same ionization efficiencies.

Chemical Glycosylation of fGly-Fc2 with Aminooxy *N*-Acetylglucosamine. AO GlcNAc was synthesized as described.[48] Fc2 (5 μg) or the alanine mutant was treated with FGE as stated above. Following fGly formation, Fc constructs were reacted with 0.5 mM AO GlcNAc in labeling buffer at 30 °C overnight. Reaction mixtures were resolved by SDS-PAGE and analyzed by lectin blot with WGA-AF647. Oxime-GlcNAc Fc2 (30 μg) was subjected to LC-ESI-MS analysis in the QB3/Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Complex Type and Sialylated Complex Type Oxazoline Synthesis. Semisynthesis of the asialo complex type (G2) oxazoline and sialylated complex type (S2) oxazoline was performed as reported.[54,55] Briefly, S2 free glycan was obtained from wild-type EndoS cleaved sialylglycopeptide from chicken egg yolk. S2 was further purified with ion-exchange chromatography on a DEAE-sepharose column to give pure S2. A portion of the S2 was treated with neuraminidase (New England Biolabs) to give G2 glycan. Free S2 and G2 were converted to oxazolines by adding 25 mol equiv of 2-chloro-1,3-dimethylimidazolinium chloride and 45 mol equiv of triethylamine. The G2 and S2 oxaolines were then purified using gel filtration and lyophilized to give G2 oxazoline and S2 oxazoline.

Transglycosylation of Oxime-GlcNAc Fc2 with Complex-Type Glycan Oxazoline. Oxime-GlcNAc Fc2 (100 μg) and S2 or G2 glycan oxazolines (300 μg) dissolved in Tris buffer (pH 7.4, 30 μL) were incubated with EndoS-D233Q (6 μg) at 30 °C for 1.5 h. The reactions were monitored by LC-ESI-MS and lectin blot.

LC-ESI-MS Analysis of EndoS-Mediated Transglycosylation. LC-ESI-MS was performed on a LXQ system (Thermo Scientific) with an Agilent Poroshell 300SB-C8 column (5 μm, 75 × 1 mm). Fc samples were reduced in 40 mM tris (2-carboxyethyl) phosphine at 37 °C for 20 min before LC-ESI-MS measurement. LC separation of the resulting monomers was performed at 40 °C eluting with a linear gradient of 20–40% acetonitrile containing 0.1% formic acid within 10 min at a flow rate of 0.25 mL/min. Percent glycan transfer was determined by comparing mass spectral abundances of oxime-GlcNAc Fc2 and oxime-(S2/G2) Fc2.

■ ASSOCIATED CONTENT

Supporting Information

Plasmid construction, SDS-PAGE of differing pH and buffers for *M. tb* FGE reaction, LC-ESI-MS of Fc2 (Ala) and Fc2 dimer, and lectin blot of oxime-GlcNAc Fc2 binding to Protein A/G resin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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