Engineering Bifidobacterium longum Endo--N-acetylgalactosaminidase for Neu5Ac2-3Ga11-3Ga1NAc reactivity on Fetuin

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Engineering *Bifidobacterium longum* Endo-α-N-acetyl-β-mannosaminidase for Neu5Acx2-3Galβ1-3GalNAc reactivity on Fetuin

Dennis K. Hansen a,1, Anders Lønstrup Hansen a,1, Johanna M. Koivistö a, Bashar Shuoker b, c, Maher Abou Hachem b,1, Jakob R. Winther b, Martin Willemoës b,1

**A R T I C L E   I N F O**

Keywords: Endo-α-N-acetyl-β-mannosaminidase from *Bifidobacterium longum* (EngBF) belongs to the glycoside hydrolase family GH101 and has a strict preference towards the mucin type glycan, Galβ1-3GalNAc, which is O-linked to serine or threonine residues on glycopeptides and -proteins. While other enzymes of the GH101 family exhibit a wider substrate spectrum, no GH101 member has until recently been reported to process the synthetic substrates [1–3] as have other members of the CAZy GH101 family [4]. However, to a varying extent some enzyme family members do release alternative glycans including GalNAc, Galβ1-3(NeulNAcβ1-6)GalNAc, GlcNAcβ1-3GalNAc from para-nitrophenyl in synthetic substrates [1–3]. The main species of O-glycosylation on bovine fetuin is the sialylated glycan, Neu5Acox2-3Galβ1-3GalNAc (Fig. 1B), linked to serine or threonine residues [5]. Until recently, no GH101 family member has been shown to release glycans from fetuin without prior sialidase treatment to convert these to Galβ1-3GalNAc [1–3]. However, during preparation for the present manuscript, activity towards the extended glycan, Neu5Acox2-3Galβ1-3GalNAc, was demonstrated for four endo-α-N-acetylgalactosaminidases: EngSP (Streptococcus pneumoniae), EngCP (Clostridium perfringens), EngSN (Streptomyces natalensis) and EngCS (Collinsella stercoris) utilizing a

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Endo-α-N-acetyl-β-mannosaminidase from *Bifidobacterium longum* (EngBF) belongs to the glycoside hydrolase family GH101 and has a strict preference towards the mucin type glycan, Galβ1-3GalNAc, which is O-linked to serine or threonine residues on glycopeptides and -proteins. While other enzymes of the GH101 family exhibit a wider substrate spectrum, no GH101 member has until recently been reported to process the synthetic substrates [1–3] as have other members of the CAZy GH101 family [4]. However, to a varying extent some enzyme family members do release alternative glycans including GalNAc, Galβ1-3(NeulNAcβ1-6)GalNAc, GlcNAcβ1-3GalNAc from para-nitrophenol in synthetic substrates [1–3]. The main species of O-glycosylation on bovine fetuin is the sialylated glycan, Neu5Acox2-3Galβ1-3GalNAc (Fig. 1B), linked to serine or threonine residues [5]. Until recently, no GH101 family member has been shown to release glycans from fetuin without prior sialidase treatment to convert these to Galβ1-3GalNAc [1–3]. However, during preparation for the present manuscript, activity towards the extended glycan, Neu5Acox2-3Galβ1-3GalNAc, was demonstrated for four endo-α-N-acetylgalactosaminidases: EngSP (Streptococcus pneumoniae), EngCP (Clostridium perfringens), EngSN (Streptomyces natalensis) and EngCS (Collinsella stercoris) utilizing a

1. Introduction

Endo-α-N-acetyl-β-mannosaminidase from *Bifidobacterium longum* (EngBF), has been shown to be quite specific for the O-linked Galβ1-3GalNAc glycan [1–3] (Fig. 1A) as have other members of the CAZy GH101 [4] family. However, to a varying extent some enzyme family members do release alternative glycans including GalNAc, Galβ1-3(GlcNAcβ1-6)GalNAc, GlcNAcβ1-3GalNAc from para-nitrophenol in synthetic substrates [1–3]. The main species of O-glycosylation on bovine fetuin is the sialylated glycan, Neu5Acox2-3Galβ1-3GalNAc (Fig. 1B), linked to serine or threonine residues [5]. Until recently, no GH101 family member has been shown to release glycans from fetuin without prior sialidase treatment to convert these to Galβ1-3GalNAc [1–3]. However, during preparation for the present manuscript, activity towards the extended glycan, Neu5Acox2-3Galβ1-3GalNAc, was demonstrated for four endo-α-N-acetylgalactosaminidases: EngSP (Streptococcus pneumoniae), EngCP (Clostridium perfringens), EngSN (Streptomyces natalensis) and EngCS (Collinsella stercoris) utilizing a

**Abbreviations:** PmST1, *Pasteurella multocida* strain P-1059 Sialyltransferase; NanL, NanL sialidase from *Clostridium perfringens*; Asialo-fetuin, fetuin treated with NanL; DMAB, para-dimethylaminobenzaldehyde; EngBF, EngSP; EngCP and EngSN and EngCS, endo-α-N-acetylgalactosaminidase from *Bifidobacterium longum*; Streptococcus pneumoniae, *Clostridium perfringens*; Streptomyces natalensis and Collinsella stercoris, respectively; Gal, galactose; GalNAc, N-acetylgalactosamine; Neu5Ac, N-acetylenuraminic acid; GlcNAc, N-acetylglucosamine; TLC, thin-layer chromatography; HPEAC-PAD, High-performance Anion Exchange Chromatography coupled to Pulsed Amperometric Detection.

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synthetic substrate [6]. In addition, EngSN and EngSP wild-type enzyme with the EngSP variants showing up to four-fold increased release of the substrate that were previously inferred from docking experiments reported. The EngSP(TIGR4) structure [9] confirmed interactions with the substrate that were previously described [15].

Fig. 1.

Crystal structures of EngBF [7], as well as the enzyme EngSP from two Streptococcus pneumoniae strains, R6 and TIGR4 [9], have been reported. The EngSP(TIGR4) structure [9] confirmed interactions with the substrate that were previously inferred from docking experiments [7,8,10]. In addition, detailed substrate interactions of EngSP were resolved from complexes with both substrate and product that also confirmed the role of a dual tryptophan lid closing over the active site, as proposed previously [7].

N-glycans are removed by a single enzyme, peptide:N-glycosidase or PNGase, which is responsible for releasing the branched and matured glycan [11,12]. In an analogy for O-glycans and their release from glycoproteins and -peptides, endo-α-N-acetylgalactosaminidase is sometimes referred to as O-glycanase despite that the activity of this enzyme is the last to follow several enzymatic steps in mucin like O-glycan removal [13]. Not only for analytical purposes could an enzyme that can catalyze the release of full-size O-glycans seem attractive, but also if such an enzyme could facilitate transglycosylation by these O-glycans on to e.g. peptides or peptide synthesis precursors.

The present work describes the successful mutational analysis of EngBF to generate an enzyme releasing trisaccharide Neu5Ac2-3Galβ1-3GalNAc glycan, compared to wild-type enzyme [6].

2. Experimental procedures

Materials. GalNAc for standard solutions and DMAB for colorimetric detection of GalNAc and for use in the enzyme assay were purchased from Carl Roth (Germany). Fetuin from fetal bovine serum and all other chemicals were from Sigma Aldrich (United States). Standards used for peak identification in product characterization by anion chromatography, were all diluted to 50 μM in 10 mM NaOH.

Expression plasmids, protein synthesis and purification. Expression plasmids for synthesis of the mutant variants of EngBF were prepared by the two-reaction site directed mutagenesis procedure [14] as described previously [15] using the following oligo-deoxyribonucleotides: EngBF E1294K Rv: GGATGCCGGTTGTCCTAAGGCTTCCGG.

A codon optimized reading frame encoding PmST1 sialytransferase was used with plasmids encoding wild-type EngBF or variants and subsequent protein purification was performed as previously described [15].

Molecular ligand docking. Ligand dockings were performed with Glide [17–20] accessed from in the Maestro interface [21]. Prior to Glide docking, the EngSP(TIGR4) [9] E796Q variant crystal structure (PDB ID: 5A59) containing the Galβ1-3GalNAc ligand was prepared for docking by optimizing hydrogen bond networks and subsequent energy minimization with charges calculated for pH 6. A Glide grid was calculated from the prepared structure [20].

The ligand Neu5Acα2-3Galβ1-3GalNAc was built using the carbohydrate builder at Glycome.org [22] and prepared for docking in the Maestro LigPrep wizard with charges calculated for pH 6. Ligand docking used the Glide SP algorithm [19,20] with flexible ligand sampling, in which hydrogen bond formation is rewarded. The initial output was set to a maximum of 10,000 poses, which were subsequently energy minimized in a full force-field to optimize bond length, angles and torsional angles before scoring of the poses using the modified Glide Energy score [18,19].

The RMSD values between the co-crystallized ligand, Galβ1-3GalNAc, and this moiety of the docked trisaccharide Neu5Acα2-3Galβ1-3GalNAc in the poses were calculated and plotted against the Glide Energy Score. The poses were evaluated for lowest values of RMSD for the docked ligand compared to the co-crystallized disaccharide and the Glide Energy Score.

Surface electrostatic potentials were calculated using the Adaptive Poisson-Boltzmann Solver package [23] in PyMol (Schrödinger Inc.) and displayed using the electrostatic potential from ±5 K/˚e.

Analysis of enzymatically released saccharides. The identity of the glycan released from fetuin by the EngBF variants was analyzed and established in two ways. The first method using HPEC-PAD was performed as follows, 1 mL of 10 mg/mL fetuin was incubated with 1.5 μM of the EngBF variant at 37 °C for 72 h in 50 mM Acetic acid, 100 mM NaCl, pH 6.0. After incubation, the released saccharides were isolated from enzyme and fetuin, by filtering through an Amicon Ultra-0.5 mL 3 K MWCO centrifugal filter (Merck, Germany). The pH of 250 μL of the filtrate was adjusted to 5.0 by addition of HCl, and subsequently added 2 μL of 200 mM CMP and 36 μg of purified PmST1 sialytransferase and incubated for 2 h at 37 °C. PmST1 was then removed by filtering as above. The filtrate of fetuin reacted with E1294K and the sample additionally treated with PmST1 as well as the standard markers to be used for anion chromatography, were all diluted to 50 μM in 10 mM NaOH.
were then analyzed on an ICS-3000 (Dionex) chromatography system which was also used for visualization.

Based on the concentration determined by the Morgan Elson colorimetric assay [24,25] for the glycans (see above) or the known stock concentrations for small-molecule markers. The samples and standards containing asialo-fetuin with E1294K was performed as follows: Fetuin (10 mg/mL) was incubated with E1294K for 24 h at 37 °C, after which no further increase in Galβ1-3GalNAc was observed, indicating complete hydrolysis.

**Table 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat} \ (s^{-1})$</th>
<th>$K_a \ (\mu M)$</th>
<th>$k_{cat}/K_a \ (\text{mM}^{-1} \ s^{-1})$</th>
<th>Substrate range (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>111 ± 5</td>
<td>163 ± 21</td>
<td>679 ± 64</td>
<td>800–35</td>
</tr>
<tr>
<td>E1294K</td>
<td>108 ± 6</td>
<td>128 ± 23</td>
<td>846 ± 109</td>
<td>700–30</td>
</tr>
<tr>
<td>E1294A</td>
<td>118 ± 3</td>
<td>133 ± 11</td>
<td>883 ± 52</td>
<td>970–35</td>
</tr>
<tr>
<td>E1294 M</td>
<td>151 ± 7</td>
<td>218 ± 28</td>
<td>693 ± 63</td>
<td>870–35</td>
</tr>
<tr>
<td>E1294H</td>
<td>178 ± 14</td>
<td>119 ± 3</td>
<td>669 ± 36</td>
<td>910–35</td>
</tr>
<tr>
<td>E1294KQ894A</td>
<td>477 ± 79</td>
<td>119 ± 3</td>
<td>250 ± 26</td>
<td>1400–30</td>
</tr>
<tr>
<td>E1294K1199A</td>
<td>201 ± 16</td>
<td>109 ± 3</td>
<td>539 ± 32</td>
<td>1130–30</td>
</tr>
</tbody>
</table>

Experiments were performed as described in Experimental procedures.

The concentration range of the substrate calculated as released GalNAc residues determined with the Morgan-Elson colorimetric assay and obtained from end points fitted to the progress curves of substrate turnover (see Supplementary Materials).

**Table 2**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>$k_{cat}/K_a \ (\text{mM}^{-1} \ s^{-1})$</th>
<th>Substrate range (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>4.5</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>E1294K</td>
<td>–</td>
<td>0.095 ± 0.001</td>
<td>1120–160</td>
</tr>
<tr>
<td>E1294A</td>
<td>–</td>
<td>0.030 ± 0.001</td>
<td>1350–160</td>
</tr>
<tr>
<td>E1294 M</td>
<td>–</td>
<td>0.0176 ± 0.0008</td>
<td>850–110</td>
</tr>
<tr>
<td>E1294 H</td>
<td>–</td>
<td>0.26 ± 0.01</td>
<td>710–150</td>
</tr>
<tr>
<td>E1294KQ894A</td>
<td>–</td>
<td>0.14 ± 0.005</td>
<td>1240–140</td>
</tr>
<tr>
<td>E1294K1199A</td>
<td>–</td>
<td>0.031 ± 0.001</td>
<td>1200–200</td>
</tr>
<tr>
<td>Wild-type</td>
<td>6.0</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>E1294K</td>
<td>–</td>
<td>1.14 ± 0.03</td>
<td>490–10</td>
</tr>
<tr>
<td>E1294 A</td>
<td>–</td>
<td>1.47 ± 0.06</td>
<td>570–20</td>
</tr>
<tr>
<td>E1294 M</td>
<td>–</td>
<td>0.78 ± 0.06</td>
<td>470–60</td>
</tr>
<tr>
<td>E1294 H</td>
<td>–</td>
<td>1.26 ± 0.09</td>
<td>370–20</td>
</tr>
<tr>
<td>E1294KQ894A</td>
<td>–</td>
<td>4.4 ± 0.13</td>
<td>350–30</td>
</tr>
<tr>
<td>E1294K1199A</td>
<td>–</td>
<td>0.76 ± 0.04</td>
<td>350–20</td>
</tr>
</tbody>
</table>

Experiments were performed as described in Experimental procedures.

NA, no detectable activity.

The concentration range of the substrate calculated as released GalNAc residues determined with the Morgan-Elson colorimetric assay and obtained from end points fitted to the progress curves of substrate turnover (see Supplementary Materials).

$k_{cat}/K_a$ was derived as the slope from a linear fit of catalytic rate versus substrate concentration.

**3. Results and discussion**

Molecular ligand docking in the endo-α-N-acetylgalactosaminidase active site. To probe the accommodation of the Neu5AcO2-3Galβ1-3GalNAc glycan in the endo-α-N-acetylgalactosaminidase active site, molecular dockings with this ligand were performed using the software Glide as described in Experimental procedures. The high-resolution structure of the EngBF homolog, EngSP, in complex with Galβ1-3GalNAc (PDB ID: 5A59) was used as a template for the docking. For each docking experiment the RMSD obtained from comparing the position of the docked Galβ1-3GalNAc moiety of the extended glycan to that of the co-crystallized disaccharide Galβ1-3GalNAc in the EngSP structure were plotted against the Glide energy score (Fig. 2A). Intriguingly, the extended glycan could readily be docked into the active site to position itself close to the subsites of the Galβ1-3GalNAc glycan. Analyzing the best scoring poses for the extended glycan, demonstrated a marked favorable decrease in the Glide energy score concomitant with lowered RMSD values. In the docked complexes, the galactose C6-hydroxyl in the extended glycan was either within hydrogen bonding.
distance of the Asp-658 carboxylate, common to the crystal structure of the EngSP Galβ1-3GalNAc complex, or rotated towards the Asp-1254 carboxylate (Fig. 2B). Inspection of the best poses, judged by lowest values of Glide energy score and RMSD, showed that the Neu5Ac C1 carboxylate was either pointing towards the enzyme surface (Fig. 2C) or the solvent (Fig. 2D). Not surprisingly, the docking experiments with the Neu5Acα2-3Galβ1-3GalNAc ligand in the EngSP active site show that binding of this glycan is restricted by accommodation of the Neu5Ac moiety. And perhaps more important, the docking experiments indicate that the Neu5Ac residue exerts restrictions on correctly locating the galactose residue to its subsite as defined in the binding of the Galβ1-3GalNAc glycan. Thus, a simple metric for a rational substitution of side chains in the active site of the enzyme that facilitate docking of Neu5Acα2-3Galβ1-3GalNAc, seemed to be the aim for a further concomitant decrease in the Glide energy score and RMSD.

To provide some guidance for the engineering of the EngBF active site, we tested both wild-type enzyme and different EngBF variants with alanine substituted amino acid residues for the ability to cleave the Neu5Acα2-3Galβ1-3GalNAc glycan of fetuin. Most variants are previously described [7,15] and form part of the active site lid, W750A, or comprise the binding site for the galactose moiety, Q894A, K1199A and D1295A (Fig. 3A). To complement these, we also constructed the E1294A variant, as Glu-1294 is also potentially blocking an extended binding site for Neu5Acα2-3Galβ1-3GalNAc (Fig. 3A). Despite recent findings with endo-α-N-acetylgalactosaminidases from other sources [6], no activity on fetuin was detected for the wild-type EngBF or the variants W750A, Q894A, K1199A, D1295A under the reaction conditions and assay method used in the present work. Nevertheless, the side-chain substitutions at the positions corresponding to Gln-894 and Lys-1199 in EngBF were recently shown to improve the activity of EngSP.
Fig. 4. Molecular docking of Neu5Acα2-3Galβ1-3GalNAc in EngSP(TIGR4) (PDB ID: 5A59) and variants substituted at the position of Glu-1253 (Glu-1294 in EngBF). A) The Glide energy score plotted as a function of the RMSD calculated for the superimposition of the Galβ1-3GalNAc moiety of the docked glycan, Neu5Acα2-3Galβ1-3GalNAc, and that of Galβ1-3GalNAc co-crystallized with the EngSP enzyme. The inset shows all the data using an extended x-axis. The data points in the square of the main plot represents the poses with lowest RMSD and Glide energy score for the model EngSP E1253K variant (homologous to the E1294K variant of EngBF). B) Superimposition of the Galβ1-3GalNAc co-crystallized with the EngSP enzyme (dark grey) and the best poses (green) from dockings of Neu5Acα2-3Galβ1-3GalNAc into the model EngSP E1253K structure. Side chains interacting with the Neu5Ac moiety are displayed as sticks (dark grey). The side-chain numbering is according to EngSP and numbering for the corresponding residues in EngBF is shown in parentheses. The figure was created using PyMol (Shrödinger Inc.). C) The Glide energy score plotted as a function of the RMSD calculated for the superimposition of the Galβ1-3GalNAc moiety of the docked glycan, Neu5Acα2-3Galβ1-3GalNAc, and that of Galβ1-3GalNAc co-crystallized with the EngSP enzyme. The inset shows all the data using an extended x-axis. The main plot shows the poses with lowest RMSD and Glide energy score for the model EngSP E1253K variant and the double variants, E1253K:Q868A and E1253K:K1156A (homologous to the E1294K, E1294K:Q894A and E1294K:K1199 variants of EngBF). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
was found to release the Neu5Ac reaction conditions that we have chosen. However, the E1294A variant was sensitive enough to detect activity at the level reported for EngSP under the on fetuin up to four-fold [6]. EngBF is almost identical to EngSP in the region of the active site (Fig. 3B) suggesting that our assay is not sensitive enough to detect activity at the level reported for EngSP under the reaction conditions that we have chosen. However, the E1294A variant was found to release the Neu5Ac glycan when fetuin was used as a substrate. This observation was followed up by analysis of several in silico side-chain substitutions at the homologous position, Glu-1253, in the EngSP structure used for the docking experiments. Here, the E1253K variant was found to produce docking poses with the Neu5Ac glycan having a markedly lower RMSD and Glide energy score compared to wild type enzyme and that of other side-chain substitutions at this position (Fig. 4A). Dissection of the best poses from docking of the extended glycan in the E1253K variant (Fig. 4B) shows the hydroxyl groups of C5 and C6 of the galactose moiety to be within hydrogen bond distance of the Asp-658 and Asp-1254 carboxylates. Thus, the interactions of the wild-type enzyme and the Galβ1-3GalNAc ligand found in the crystal structure of the EngSP complex and those of this moiety of the Neu5Ac glycan in dockings performed on the E1253K variant, are in better agreement than what is found with the latter glycan in the dockings with the wild-type EngSP enzyme. In addition, the positioning of the Neu5Ac moiety is identical for all the dockings in this group of best poses for the E1253K variant with a solvent exposed positioning of the Neu5Ac side-chain substitutions at this position (Fig. 4A). Dissection of the best poses from docking of the extended glycan in the E1253K variant with a solvent exposed positioning of the Neu5Ac moiety is identical for all the dockings in this group of best poses for the E1253K variant, which are in better agreement than what is found with the latter glycan in the dockings with the wild-type EngSP enzyme. In addition, the positioning of the Neu5Ac moiety is identical for all the dockings in this group of best poses for the E1253K variant with a solvent exposed positioning of the Neu5Ac carbohydrate (Fig. 4B). Products from fetuin incubation with E1294K analyzed by HPEAC-PAD and TLC. A) HPEAC-PAD glycan analysis of E1294K treated fetuin (black line) before and (red line) after treatment with PmST1 sialyltransferase. Labeled peaks were identified from chromatography of standards. B) TLC of samples containing 1) asialo-fetuin, 2) and 3) fetuin and 4) Galβ1-3GalNAc α1-paranitrophenol. The chromatogram shows glycan release after incubation in the (−) absence or (+) presence of E1294K. In 3), the E1294K treated sample from 2) was subsequently incubated with Nant sialidase to release Neu5Ac. The composition of the released glycans and carbohydrates are indicated above the chromatogram. Experiments were performed as described in Experimental procedures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. Products from fetuin incubation with E1294K analyzed by HPEAC-PAD and TLC. A) HPEAC-PAD glycan analysis of E1294K treated fetuin (black line) before and (red line) after treatment with PmST1 sialyltransferase. Labeled peaks were identified from chromatography of standards. B) TLC of samples containing 1) asialo-fetuin, 2) and 3) fetuin and 4) Galβ1-3GalNAc α1-paranitrophenol. The chromatogram shows glycan release after incubation in the (−) absence or (+) presence of E1294K. In 3), the E1294K treated sample from 2) was subsequently incubated with Nant sialidase to release Neu5Ac. The composition of the released glycans and carbohydrates are indicated above the chromatogram. Experiments were performed as described in Experimental procedures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. Progress-curves and end-point determination of glycan product formation from processing of fetuin and asialo-fetuin. Data points are the mean of three determinations and error bars are visible where they extend beyond the data point symbol. A) Progress-curves from fetuin (10 mg/mL) incubated up to 24 h with a final concentration of (filled squares) 3.4 μM EngBF and (filled circles) 3.9 μM EngBF E1294K. B) Progress-curves from asialo-fetuin (10 mg/mL) incubated 90 min with a final concentration of (filled squares) 6.7 nM EngBF and (filled circles) 6.7 nM EngBF E1294K.

on fetuin up to four-fold [6]. EngBF is almost identical to EngSP in the region of the active site (Fig. 3B) suggesting that our assay is not sensitive enough to detect activity at the level reported for EngSP under the reaction conditions that we have chosen. However, the E1294A variant was found to release the Neu5Ac glycan when fetuin was used as a substrate. This observation was followed up by analysis of several in silico side-chain substitutions at the homologous position, Glu-1253, in the EngSP structure used for the docking experiments. Here, the E1253K variant was found to produce docking poses with the Neu5Ac α1-3Galβ1-3GalNAc glycan having a markedly lower RMSD and Glide energy score compared to wild type enzyme and that of other side-chain substitutions at this position (Fig. 4A). Dissection of the best poses from docking of the extended glycan in the E1253K variant (Fig. 4B) shows the hydroxyl groups of C5 and C6 of the galactose moiety to be within hydrogen bond distance of the Asp-658 and Asp-1254 carboxylates. Thus, the interactions of the wild-type enzyme and the Galβ1-3GalNAc ligand found in the crystal structure of the EngSP complex and those of this moiety of the Neu5Ac α1-3Galβ1-3GalNAc glycan in dockings performed on the E1253K variant, are in better agreement than what is found with the latter glycan in the dockings with the wild-type EngSP enzyme. In addition, the positioning of the Neu5Ac moiety is identical for all the dockings in this group of best poses for the E1253K variant with a solvent exposed positioning of the Neu5Ac carbohydrate (Fig. 4B).

In docking experiments, we also tested the influence of substituting the side chains of Gln-868 and Lys-1156 in EngSP, corresponding to Gln-894 and Lys-1199 in EngBF, when combined with the E1253K substitution (E1294K in EngBF). The E1253K:Q868A variant showed poses of docking with Neu5Ac α1-3Galβ1-3GalNAc C with a decreased Glide energy score and reduced RMSD as also found for the E1253K variant above (Fig. 4C).

**Product analysis.** The finding that the EngBF variants substituted at Glu-1294 were able to produce a detectable product from incubation with fetuin, in contrast to wild-type enzyme, gave a strong indication that these variants were able to process the extended glycan, Neu5Ac α1-3Galβ1-3GalNAc. To substantiate this we set out to identify the chemical composition of the glycan released by the EngBF variants, exemplified by E1294K.

The product released by E1294K upon incubation with fetuin was analyzed by HPEAC-PAD as described in Experimental procedures, before and after n2-3-specific Neu5Ac removal by PmST1 sialyltransferase in the presence of CMP [16]. Although the Galβ1-3GalNAc entity was fully hydrolyzed to galactose and GalNAc during the HPEAC-PAD analysis, as this is performed in 100 mM NaOH, distinct peaks that are likely to represent the full trisaccharide Neu5-Acr2-3Galβ1-3GalNAc or the partial hydrolysate Neu5Acα2-3Gal eluted at a position later than the Neu5Ac monomer (Fig. 5A). Supporting this attribution of the late eluting peaks, was the disappearance of these peaks after treatment of the released glycan with PmST1 sialyltransferase and instead the chromatogram showed increased amounts of Galβ1-3GalNAc hydrolysate (Gal + GalNAc) as well as free Neu5Ac (Fig. 5A).
We also performed a TLC analysis (Fig. 5B) of product from incubation of fetuin with E1294K. The application of standards and the treatment of the product with Nanl sialidase indicated that the released glycan is Neu5AcOc2-3Galj1-3GalNAc.

Progress curves of fetuin processed by E1294K showed a maximal release of two glycans per fetuin molecule (Fig. 6A), compared to 2-2.5 glycans obtained with wild-type EngBF or E1294K upon incubation with asialo-fetuin (Fig. 6B). This stoichiometry of Neu5AcOc2-3Galj1-3GalNAc glycans, or Galj1-3GalNAc after Nanl sialidase treatment, per fetuin molecule is in agreement with an average of 2-2.5 O-glycosylations per fetuin molecule with the majority being the Neu5AcOc2-3Galj1-3GalNAc-Ser/Thr as previously determined [5].

Steady-state kinetic analysis of wild-type EngBF and variants using asialo-fetuin or fetuin as a substrate. The EngBF variants altered at position Glu-1294 were subjected to a steady-state kinetic analysis on asialo-fetuin to evaluate the impact of the substituted side chains with respect to the native activity of EngBF of processing Galj1-3GalNAc O-glycosylations (Table 1). The steady-state kinetic parameters for all variants were close to those of wild-type EngBF (Table 1) and Glu-1294 appears not in any way critical for activity. In addition, this position seems functionally very tolerant to side-chain substitutions, both in terms of size and charge. With fetuin as a substrate, all the EngBF variants altered at Glu-1294 were active, but showed a linear increase in activity with increasing fetuin concentrations and no sign of substrate saturation at about 5 K bile. Both figures were created using PyMol (Shrödinger Inc.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 7. Surface electrostatic potential of the substrate bound EngSP(TIGR4) active site and Neu5AcOc2-3Galj1-3GalNAc calculated as described in Experimental procedures. A) Surface electrostatic potential of EngSP(TIGR4) (PDB ID: 5A59) in complex with Galj1-3GalNAc shown as sticks (green) and displayed using the electrostatic potential from ±5 K bile. B) Surface electrostatic potential of the extended glycan, Neu5AcOc2-3Galj1-3GalNAc, found on fetuin and shown using the electrostatic potential from ±5 K bile. Both figures were created using PyMol (Shrödinger Inc.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Additional figures and supplementary details are provided as additional files.
References


