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Complete switch from α₂,3- to α₂,6-regioselectivity in *Pasteurella dagmatis* β-D-galactoside sialyltransferase by active-site redesign

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Structure-guided active-site redesign of a family GT-80 β-o-galactoside sialyltransferase (from *Pasteurella dagmatis*) to change enzyme regioselectivity from α₂,3 in wild type to α₂,6 in a P7H-M117A double mutant is reported. Biochemical data for sialylation of lactose together with protein crystal structures demonstrate highly precise enzyme engineering.

α₂,3- and α₂,6-sialic acid capped oligosaccharides are highly important in human glycobiology.¹ Sialylated human milk oligosaccharides (HMOs) are of interest due to their roles in the development and health protection of newborn infants.² Nearly 20% of the total HMOs comprise sialic acid,² and sialyllactose is one of their main components,³ with both regiosomers being biologically active.⁴ Sialylated HMOs contain N-acetylmuramidic acid (Neu5Ac) attached to α-galactosyl (Scheme 1) or N-acetyl-D-glucosaminyl residues through α₂,3- or α₂,6-linkage.²,⁵ Since natural availability of sialylated HMOs is limited, synthetic sialyloligosaccharides (e.g. sialyllactose) are demanded as infant formula,²,⁵ and nutraceutical ingredients.⁴

Stereo- and regiocontrol are problems requiring special attention when installing a sialyl group on a nascent oligosaccharide.⁶ Selective sialylation bivalent sialylation avoids use of protecting group chemistry and therefore presents a highly attractive route for sialylated HMO synthesis.⁷ Sialyltransferases (STs; EC 2.4.99) catalyze transfer of a Neu5Ac residue from CMP-Neu5Ac to an acceptor oligosaccharide. Complementarily regioselective STs from bacterial,⁸ mammalian,²,⁹ and viral¹⁰ sources, were successfully applied for enzymatic α₂,3- and α₂,6-sialylation of various acceptor substrates. Sialyllactose and sialyl-(poly)-N-acetyllactosamine derivatives were produced in gram quantities.⁸,¹⁰ Engineered STs exhibiting tailored selectivities could be of interest for flexible sialoside synthesis.

Hitherto unprecedented switch in ST regioselectivity from α₂,3 in wild type to α₂,6 in a designed enzyme variant is reported. High-resolution protein structures show the active-site remodeling of the parent ST to have been precise at the atomic level. Results provide deepened insights into determinants of ST selectivity. They are also relevant for practical synthesis: a pair of "ST twins" is created that differ in regioselectivity, but otherwise have uniform synthesis conditions and substrate preferences. We show their application to alternative 3'- or 6'-sialylation of lactose and N-acetyllactosamine.

Glycosyltransferase family GT-80 comprises α₂,3-, α₂,6- and also mixed α₂,3/α₂,6-selective STs.⁶,¹⁰,¹¹,¹² These STs are furthermore characterized by high specific activity and broad acceptor substrate scope, which typically includes lactose.⁸,¹² Redesign of the naturally α₂,3-selective β-D-galactoside ST from *Pasteurella multocida* (PmST1)¹³ was developed from two family GT-80 protein structures that delineate distinct lactose binding modes in the α₂,3/α₂,6-selective ST from *Pasteurella multocida* (PmST1)¹³ and the α₂,6-selective ST from *Photobacterium sp.* JT-ISH-224¹⁴ (Fig. 1). PmST is 70% identical in amino acid sequence to PmST1, and residues of their acceptor binding sites (Fig. 1a) are completely identical. Different orientations of the lactose's β-D-galactosyl
moiety relative to the proposed catalytic base of the enzyme (Asp$^{141}$, Asp$^{235}$) that either the 3-OH (Fig. 1a) or the 6-OH (Fig. 1b) is brought into a reactive position, appeared to have been evoked by a two amino acid residue substitution where Pro$^{117}$ and Met$^{146}$ in PmST1 are exchanged to, respectively, His$^{233}$ and Ala$^{235}$ in Photobacterium ST. Family-wide sequence comparison of GT-80 STs revealed clear sub-categorization of β-galactosides α2,3- and α2,6-STs according to a conserved Pro/Met or His/Ala(Ser) sequence pattern (see Table S1 in ESI†) that was therefore hypothesized to be decisive for α2,3 compared to α2,6 ST regioselectivity. Note, however, that a group of α2,3-selective STs from within family GT-80 that are flexible in using α/β-galactosides for sialylation also possess an Ala instead of a Met (Table S1 in ESI†). To graft α2,6-selective ST activity on PdST, the relevant residues Pro$^{117}$ and Met$^{146}$ were replaced to generate P7H and P7H-M117A variants of the naturally α2,3-selective wild-type enzyme.

Purified preparations of wild type and variant PdST were obtained from Escherichia coli overexpression culture producing target protein equipped with a C-terminal His$_x$-tag for purification by metal chelate chromatography. Specific activity for sialyltransfer to lactose (1 mM) from CMP-Neu5Ac (1 mM) was determined at pH 8.0, measuring the consumption of CMP-Neu5Ac and the release of CMP by HPLC, and the formation of sialyllactose by HPAEC-PAD (high-performance anion exchange chromatography with pulsed amperometric detection). The specific activity of P7H mutant was identical (5.8 U mg$^{-1}$) to that of the wild-type enzyme. The specific activity of the P7H-M117A double mutant was lowered somewhat in comparison (2.2 U mg$^{-1}$). Sialyllactose regiosomers formed in the different enzymatic reactions were identified from their elution in HPAEC-PAD referenced against authentic standards of 3'- and 6'-sialyllactose, as shown in Fig. 2. The wild-type enzyme produced 3'-sialyllactose exclusively. Single mutation of Pro$^7$ to His resulted in drastic change of enzyme regioselectivity, so that 6'-sialylation of lactose was now strongly favored by the enzyme. However, 3'-sialyllactose was still present to ~4% of total transfer product (Fig. 2), showing that α2,3-ST activity had not been completely abolished in the P7H mutant. The double mutant P7H-M117A, by contrast, featured complete α2,3 to α2,6 switch in ST regioselectivity. No 3'-sialyllactose was detectable (~1%) next to 6'-sialyllactose as product of the enzymatic sialyltransfer (Fig. 2). Therefore, these results suggested distinct and divergingly important roles for His and Ala in conferring α2,6-regioselectivity to STs of family GT-80. While the His was clearly essential, the Ala seemed to fulfil an auxiliary function in fine-tuning of enzyme selectivity. Consistent with this notion, the M117A mutant of PdST did not exhibit significant change in regioselectivity as compared to the wild-type enzyme (Table 1). Evidence from enzyme kinetic characterization (Table 1) supports the idea that in terms of catalytic efficiency ($k_{cat}/K_m$) and also turnover frequency ($k_{cat}$), the replacement Pro$^7$→His is tolerated much better by the enzyme than the Met$^117$→Ala replacement. Interestingly, Pro$^7$→His replacement in M117A variant resulted in clear recovery of activity parameters that had been decreased in the single-site variant as compared to wild-type PdST.

### Table 1 Activity and selectivity parameters of wild-type PdST and site-directed variants thereof.

<table>
<thead>
<tr>
<th>Regioselectivity</th>
<th>WT</th>
<th>M117A</th>
<th>P7H</th>
<th>P7H-M117A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity [U mg$^{-1}$]</td>
<td>α2,3</td>
<td>α2,3$^*$</td>
<td>α2,6</td>
<td>α2,6$^*$</td>
</tr>
<tr>
<td>$K_m$ [mM]</td>
<td>1.5</td>
<td>15</td>
<td>3.8</td>
<td>6.4</td>
</tr>
<tr>
<td>$k_{cat}$ [s$^{-1}$]</td>
<td>24</td>
<td>11</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ [s$^{-1}$ mM$^{-1}$]</td>
<td>16</td>
<td>0.74$^*$</td>
<td>4.3</td>
<td>2.8</td>
</tr>
</tbody>
</table>

$^*$ 4% of 6'-sialyllactose and 4% of 3'-sialyllactose were detected.

Apparent kinetic parameters for lactose as sialyltransferase acceptor substrate. Kinetic parameters have S.D. of ≤10%, except 20%. Measurements were done using CMP-Neu5Ac (10 mM) and a varied concentration of lactose.
In a consequent next step, we applied the two regio-complementary STs (wild type; P7H-M117A) and also the P7H mutant to the synthesis of 2',3' and 6'-sialyllactose from CMP-Neu5Ac and lactose (each 1 mM) (Table 2). Full reaction time courses were determined (see Fig. S2 in ESI†), and the reported sample composition at each point was carefully verified by a closed mass balance. Using wild-type PdST (see Fig. S2a in ESI†), 3'-sialylation product was obtained in about 75% yield (0.75 mM). The enzyme's inherent hydrolytic activity caused partly non-productive utilization of the CMP-Neu5Ac donor substrate, thus restricting the 3'-sialyllactose yield under the conditions used. Hydrolytic competition was however strongly reduced at elevated lactose concentration (10 mM) where ≥ 95% of the initial CMP-Neu5Ac (1 mM) was utilized for sialyltransfer to acceptor substrate (see Fig. S3 in ESI†). Using the P7H-M117A double mutant, 6'-sialyllactose was synthesized in about 72% yield (0.72 mM) whereas 3'-sialyllactose was present at ≤ 0.003 mM (see Fig. S2b in ESI†). At the equivalent protein concentration used (0.1 µM), the space-time yield of sialyllactose product was 2.5-fold lower for the reaction of the double mutant as compared to the reaction of wild-type PdST, explained by the different specific ST activities of the two enzymes.

Table 2 Synthesis of sialyllactose and sialyl-N-acetyllactosamine using wild-type PdST and mutants thereof.

<table>
<thead>
<tr>
<th>Lactose* Yield SL [%]</th>
<th>WT</th>
<th>P7H</th>
<th>P7H-M117A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac</td>
<td>≤ 0.006 18 &gt; 240</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>N-acetyllactosamine* Yield SL [%]</th>
<th>WT</th>
<th>P7H</th>
<th>P7H-M117A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac</td>
<td>≤ 0.006 8 &gt; 180</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 1 mM of acceptor substrate was used. ° Ratio of 6' to 3'-sialylside, whereby product identity was verified by NMR. SL, sialyllactose; SLN, sialyl-N-acetyllactosamine.

To obtain molecular interpretation of the regioselectivity switch achieved in PdST (Tables 1 and 2), we determined the structures of wild-type enzyme, P7H and P7H-M117A in their respective apo-form (X-ray data collection and refinement statistics is shown in the ESI†, Table S2). For a detailed description of crystallization and structure determination see the ESI†. All structures show an open conformation with the same positions of mutated (Pro→His, Met→Ala) and catalytic (Asp284, His114) residues (Fig. 3). The two PdST variants were also co-crystallized in the presence of CMP-Neu5Ac and lactose or 2-nitrophenyl-β-D-galactopyranoside. Additionally, the crystals were soaked with lactose or 2-nitrophenyl-β-D-galactopyranoside.

Unfortunately, only CMP could be clearly placed in the electron density (X-ray data collection and refinement statistics see the ESI†, Table S2, Fig. S14). Note that co-crystallization of Photobacterium sp. α2,6-ST (PDB code 2Z4T) with CMP and lactose resulted in a ternary complex. However, overlay of the apo structure of P7H-M117A double mutant (open conformation) with the N- and C-terminal domains of Photobacterium sp. α2,6-ST (PDB code 2Z4T, closed conformation) shows almost perfect superimposition of important active site residues for catalytic function (Asp284, Asp235, His114, His115, and mutation sites (Pro284, Pro→His; Met117, Met→Ala) are drawn in sticks. This structural observations, taken together with the biochemical characterization, therefore clearly support a highly successful mutational strategy.

Alternative enzymatic α2,3- and α2,6-sialylation of various acceptor substrates, including lactose and sialyl-(poly)-N-acetyllactosamine derivatives, was demonstrated before. What is new here is the use of a designed pair of regio-complementary STs...
instead of two individual enzymes. The approach of ST engineering might offer a convenient exchange of enzymatic regioselectivity for synthesis of sialylactose and sialyl-N-acetyllactosamine (Table 2).

In conclusion, sequence motifs determining α2,3- and α2,6-regioselectivity in β-D-galactoside STs of family GT-80 were identified and exploited through protein engineering of PdST to create a unique pair of regio-complementary STs. Relationships between PdST atomic structure and enzyme selectivity were established. The two STs were applied for α2,3/α2,6-sialylation of lactose and LacNac from CMP-Neu5Ac.

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† Electronic Supplementary Information (ESI) available: Experimental procedures, sequence alignment, HPACE-PAD analysis, time-courses of synthesis experiments, H and HSQC NMR spectra, crystallization and structure determination. See DOI: 10.1039/c000000x/