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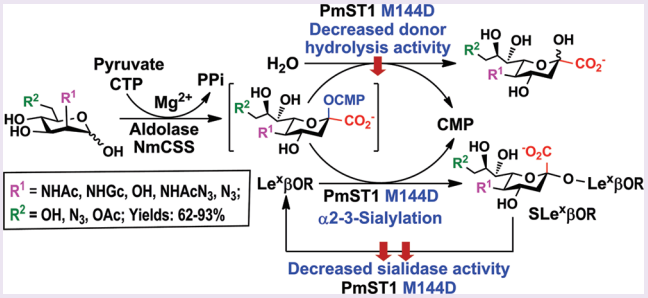
A Sialyltransferase Mutant with Decreased Donor Hydrolysis and Reduced Sialidase Activities for Directly Sialylating Lewis^x

Go Sugiarto,[†,§] Kam Lau,[†,§] Jingyao Qu,[†] Yanhong Li,[†] Sunghyuk Lim,[†] Shengmao Mu,[†] James B. Ames,[†] Andrew J. Fisher,*[,†,‡] and Xi Chen*[,†]

[†]Department of Chemistry and [‡]Department of Molecular and Cellular Biology, University of California-Davis, One Shields Avenue, Davis, California 95616, United States

Supporting Information

ABSTRACT: Glycosyltransferases are important catalysts for enzymatic and chemoenzymatic synthesis of complex carbohydrates and glycoconjugates. The glycosylation efficiencies of wild-type glycosyltransferases vary considerably when different acceptor substrates are used. Using a multifunctional *Pasteurella multocida* sialyltransferase 1 (PmST1) as an example, we show here that the sugar nucleotide donor hydrolysis activity of glycosyltransferases contributes significantly to the low yield of glycosylation when a poor acceptor substrate is used. With a protein crystal structure-based rational design, we generated a single mutant (PmST1 M144D) with decreased donor hydrolysis activity without significantly affecting its α2−3-sialylation activity when a poor fucose-containing acceptor substrate was used. The single mutant also has a drastically decreased α2−3-sialidase activity. X-ray and NMR structural studies revealed that unlike the wild-type PmST1, which changes to a closed conformation once a donor binds, the M144D mutant structure adopts an open conformation even in the presence of the donor substrate. The PmST1 M144D mutant with decreased donor hydrolysis and reduced sialidase activity has been used as a powerful catalyst for efficient chemoenzymatic synthesis of complex sialyl Lewis^x antigens containing different sialic acid forms. This work sheds new light on the effect of donor hydrolysis activity of glycosyltransferases on glycosyltransferase-catalyzed reactions and provides a novel strategy to improve glycosyltransferase substrate promiscuity by decreasing its donor hydrolysis activity.



Glycosyltransferase-catalyzed reactions have gained increasing attention and application for the synthesis of complex carbohydrates and glycoconjugates.[1−3] Most mammalian glycosyltransferases suffer from no or low expression in *E. coli* systems and more restricted substrate specificity.[4] In comparison, bacterial glycosyltransferases are generally easier to access using *E. coli* expression systems[5] and have more promiscuous substrate flexibility.[6,7] Nevertheless, despite the discovery of many bacterial glycosyltransferases that have promiscuities for both donor and acceptor substrates,[6,7] the application of glycosyltransferases in the synthesis of carbohydrate-containing structures is limited by the availability and the substrate specificity of wild-type enzymes.[8−10]

For example, sialyltransferases, the key enzymes that catalyze the transfer of a sialic acid residue from cytidine 5′-monophosphate-sialic acid (CMP-sialic acid) to an acceptor, have been commonly used for the synthesis of sialic acid-containing structures.[11−16] Sialyl Lewis^x [SLe^x, Siaα2−3Galβ1−4(Fucα1−3)GlcNAcβOR] is an important carbohydrate epitope involved in inflammation as well as adhesion and metastasis of cancer cells.[17,18] It is a well-known tumor-associated carbohydrate antigen[19−23] and has been used as a candidate for cancer vaccine.[21,24] The biosynthesis of SLe^x involves the formation of Siaα2−3Galβ1−4GlcNAcβOR catalyzed by an α2−3-sialyltransferase followed by an α1−3-fucosyltransferase-catalyzed fucosylation (Supplementary Scheme S1). This biosynthetic sequence usually cannot be altered as common α2−3-sialyltransferases do not use fucose-containing Lewis^x [Le^x, Galβ1−4(Fucα1−3)GlcNAcβOR] as a substrate.[25]

As common terminal monosaccharides, sialic acids constitute a family of great structural diversity. So far, more than 50 structurally distinct sialic acid forms have been identified in nature.[26] To obtain SLe^x with different sialic acid forms to elucidate the biological significance of naturally occurring sialic acid modifications, an efficient enzymatic approach is to use Le^x [Galβ1−4(Fucα1−3)GlcNAcβOR] as a fucose-containing acceptor to add different sialic acid forms by a suitable α2−3-sialyltransferase.[25] This process of introducing different forms of sialic acid onto the common fucosylated acceptor Le^x in the last step has significant advantages compared to the normal SLe^x biosynthetic pathway in which fucosylation is the last glycosylation process (Supplementary Scheme S1). It not only simplifies the synthetic scheme as a less number of reactions are

Received: December 25, 2011
Accepted: April 26, 2012
Published: May 14, 2012

needed but also makes the purification process much easier as negatively charged SLe^x product is separated from neutral Le^x oligosaccharide instead of separating both negatively charged oligosaccharides SLe^x and non-fucosylated sialosides if fucosylation occurs in the last step.

We and others have demonstrated that a myxoma virus α2−3-sialyltransferase can use Le^x as an acceptor substrate for synthesizing SLe^x.[25,27,28] Nevertheless, the low expression level of the enzyme in *E. coli* (<0.1 mg L^{-1} culture) limits its application in preparative and large-scale synthesis of SLe^x.[25]

We have previously shown that a multifunctional α2−3-sialyltransferase from *Pasteurella multocida* (PmST1)[11,29,30] has a good expression level in *E. coli* (100 mg L^{-1} culture). It can use Le^x as an acceptor for the synthesis of SLe^x, but the yields are poor (<20%) despite different conditions tested.[25] Our previous protein crystal structure-based mutagenesis studies led to the design of a PmST1 double mutant E271F/R313Y with a significantly (6333-fold) decreased sialidase activity when a nonfucose lactoside was used as an acceptor substrate.[31] Nevertheless, the PmST1 E271F/R313Y does not efficiently sialylate fucose-containing oligosaccharides such as Le^x. In this study, we show that the donor hydrolysis activity of PmST1 contributes significantly for the low yield sialylation of Le^x. Based on protein X-ray crystal structure studies, a PmST1 mutant (PmST1 M144D) is successfully generated to decrease the donor hydrolysis activity without affecting its efficiency in sialylating Le^x. The resulting PmST1 mutant also shows drastically decreased α2−3-sialidase activity and has been successfully applied in efficient sialylation of a poor fucose-containing Le^x acceptor for the synthesis of complex and biologically important SLe^x oligosaccharides containing different sialic acid forms. Therefore, using PmST1 as the model system, we demonstrate that decreasing donor hydrolysis by site-directed mutagenesis may be a general approach to improve glycosyltransferase-catalyzed reactions when a poor glycosylation acceptor is used.

RESULTS AND DISCUSSION

Donor Hydrolysis by PmST1 Causes Low Yield Sialylation of Le^x. In order to understand why PmST1-catalyzed sialylation of Le^x resulted in low yields, time course studies were carried out using a fluorescently labeled Le^x acceptor (4-methylumbelliferyl β-Le^x or Le^{x}βMU) in a high performance liquid chromatography (HPLC) assay.[25] As shown in Figure 1, PmST1-catalyzed sialylation of Le^{x}βMU (1 mM) using 1 equiv of donor CMP-Neu5Ac reached a low yield (1.1−1.3%) plateau quickly within 2 min. Every additional dose of donor substrate CMP-Neu5Ac increased the product formation, which always reached a plateau quickly. Monitoring the CMP-Neu5Ac consumption (% consumption numbers are shown in parentheses in Figure 1) in the reaction mixture by capillary electrophoresis studies confirmed a quick consumption of CMP-Neu5Ac. These indicated that donor (CMP-Neu5Ac) hydrolysis activity of PmST1, where water molecules compete with the poor Le^x acceptor for the consumption of sugar nucleotide (CMP-Neu5Ac) donor of the sialyltransferase (Supplementary Scheme S2), contributed significantly to the low yield of PmST1-catalyzed sialylation. In fact, donor hydrolysis has been observed in other glycosyltransferase-catalyzed reactions that lead to lower synthetic yields.[32] The donor hydrolyses were observed frequently in co-crystallization of glycosyltransferases with a corresponding sugar nucleotide donor where its sugar component was usually cleaved off and only the hydrolyzed nucleotide was observed in the substrate-binding pocket of the enzyme.[29,33] Therefore, inert donor derivatives of glycosyltransferases have been commonly applied in the X-ray crystal structure studies of glycosyltransferases.[30,33,34] Two recent papers discussed the donor hydrolysis activities of human blood group A and B glycosyltransferases (GTA and GTB) that are Mn^{2+}-dependent[35] and the increased UDP-Gal hydrolysis activity of GTB in the presence of an acceptor substrate analogue.[36] Nevertheless, the effect of donor hydrolysis of glycosyltransferases on glycosylation processes has not been investigated in detail. In addition, no strategy has been reported for improving the yields of glycosyltransferase-catalyzed reactions by decreasing donor hydrolysis activity.

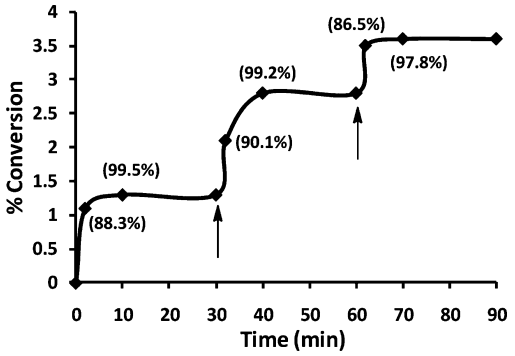


Figure 1. HPLC-based time course studies of PmST1-catalyzed α2−3-sialylation of Lewis^x trisaccharide (1 mM) with periodical addition of sialyltransferase donor CMP-Neu5Ac (indicated by arrows). As shown, the initial 1 equiv of donor CMP-Neu5Ac resulted in a low yield (1.1%) product formation in 2 min. Every additional dose of donor substrate CMP-Neu5Ac (shown by arrows) increased the product formation, which always reached a plateau quickly, indicating the quick consumption of the donor substrate. This was confirmed by capillary electrophoresis (CE) assays (numbers in parentheses represent the % consumption of CMP-Neu5Ac). When a poor acceptor substrate such as Lewis^x was used, quick hydrolysis of the donor substrate contributes significantly to the low yield formation of SLe^x in PmST1-catalyzed reaction.

Mechanism of PmST1-Catalyzed CMP-Neu5Ac Donor Hydrolysis. ^{1}H NMR-based time course studies of the donor (CMP-Neu5Ac, 25 mM) hydrolysis activity of wild-type (WT) PmST1 (10.8 μg) (Figure 2) in either sodium tartrate buffer (10 mM, pH 5.5) or sodium phosphate buffer (20 mM, pH 8.0) indicate that both α-Neu5Ac and β-Neu5Ac were formed in CMP-Neu5Ac hydrolysis reactions. This conclusion is based on the comparison of Figure 2 with results of previously reported ^{1}H NMR-based time course studies of the sialidase activity (Neu5Acα*p*NP, 20 mM, was used as the substrate) of WT PmST1 (65.0 μg) in the same sodium tartrate buffer (10 mM, pH 5.5) as Figure 2A.[31] As shown previously, α-Neu5Ac was the initial product formed from α-linked sialoside (Neu5Acα*p*NP) catalyzed by the α2−3-sialidase activity of PmST1 in sodium tartrate buffer (10 mM, pH 5.5). The mutarotation of the α-Neu5Ac formed in the α2−3-sialidase reaction with sodium tartrate buffer (10 mM, pH 5.5) to β-Neu5Ac took more than 20 min, and α-Neu5Ac was the predominant form in reaction mixtures with at least up to 2 h of reaction time.[31] This slow mutarotation process is most likely due to the spontaneous mutarotation of α-Neu5Ac to β-Neu5Ac, which is a predominant form (>90%)[37,38] at equilibrium and not due to the activity of a mutarotase.[39] In

dx.doi.org/10.1021/cb300125k | *ACS Chem. Biol.* 2012, 7, 1232−1240

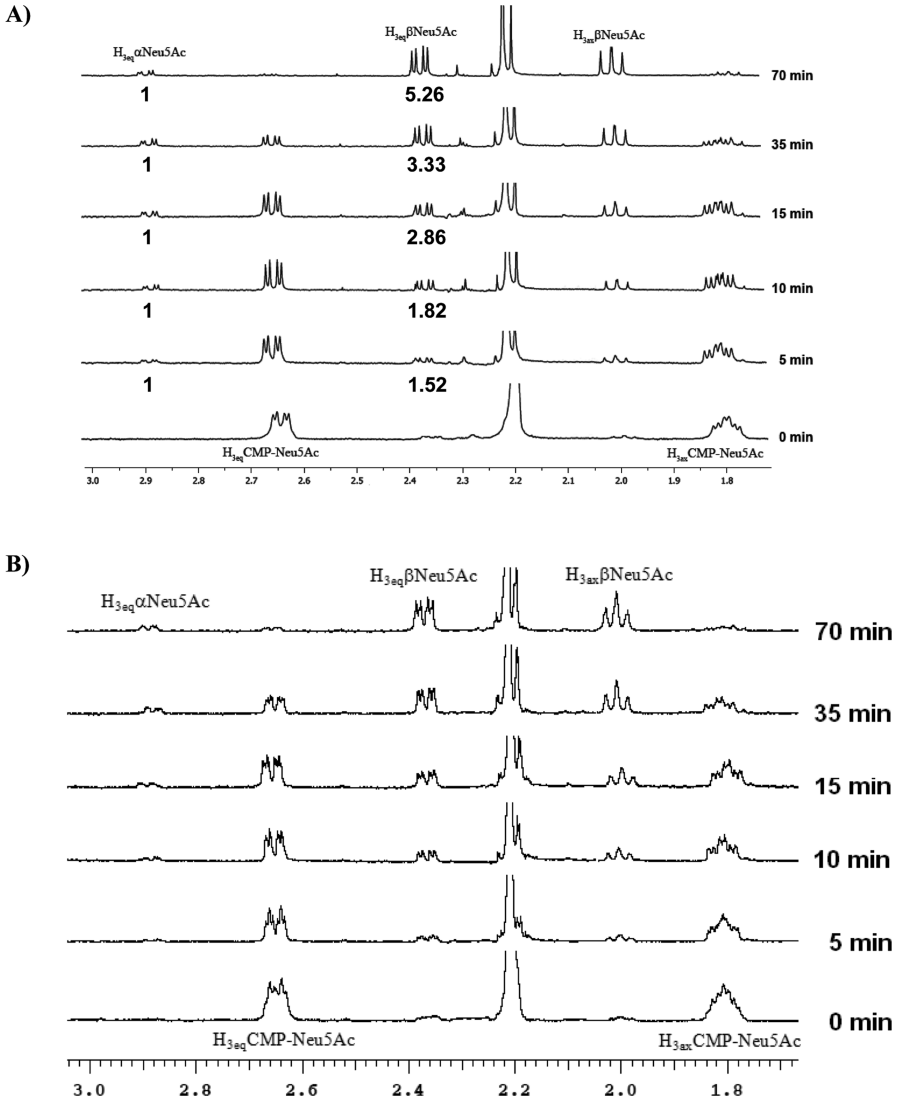


Figure 2. Time course studies of PmST1 donor (CMP-Neu5Ac) hydrolysis by ^{1}H NMR (600 MHz) analysis in (A) sodium tartrate buffer (10 mM, pH 5.5) or (B) sodium phosphate buffer (20 mM, pH 8.0) containing CMP-Neu5Ac (25 mM) and PmST1 (10.8 μg). The initial observation of both α-Neu5Ac and β-Neu5Ac indicates a combination of inverting and retaining mechanisms for PmST1-catalyzed CMP-Neu5Ac hydrolysis reaction. Ratios of peak integration of H_{3eq}αNeu5Ac peaks (~2.90 ppm) and H_{3eq}βNeu5Ac peaks (~2.38 ppm) for the PmST1-catalyzed CMP-Neu5Ac hydrolysis reaction in sodium tartrate buffer (10 mM, pH 5.5) are shown below the NMR spectra in panel A.

contrast, both α-Neu5Ac and β-Neu5Ac were observed in 5 min in the PmST1-catalyzed CMP-Neu5Ac hydrolysis reaction. Due to the same buffer (sodium tartrate buffer, 10 mM, pH 5.5) condition and a less amount of PmST1 (10.8 μg) used in the CMP-Neu5Ac hydrolysis reaction (comparing to 65.0 μg of PmST1 used in the sialidase reaction), the mutarotation between α-Neu5Ac and β-Neu5Ac will not be faster than that in the sialidase reaction, and the only explanation is that both α-Neu5Ac and β-Neu5Ac are formed in the PmST1-catalyzed donor hydrolysis reaction. Comparison of the integration of H_{3eq}αNeu5Ac peaks around 2.90 ppm and H_{3eq}βNeu5Ac peaks around 2.38 ppm provides ratios of 1:1.52, 1:1.82, 1:2.86, 1:3.33, and 1:5.26 for reaction mixtures at 5, 10, 15, 35, and 70 min, respectively. The percentage increase of the β-Neu5Ac product with the increase of reaction time may be contributed at least partially by spontaneous mutarotation of α-Neu5Ac to β-Neu5Ac.

The observation of the formation of both α-Neu5Ac and β-Neu5Ac in the PmST1-catalyzed CMP-Neu5Ac hydrolysis reaction was a surprise as it indicates that the donor hydrolysis can undergo two different paths. One is the formation of α-Neu5Ac from β-linked Neu5Ac in CMP-Neu5Ac, which goes through an inverting process similar to the inverting mechanism of the α2−3-sialyltransferase reaction catalyzed by the same enzyme in which an α2−3-sialyl linkage is formed from β-linked Neu5Ac in the CMP-Neu5Ac donor. In this process, a water molecule competes with, most likely by replacement of, an acceptor molecule for the same donor substrate in PmST1-catalyzed reaction. The other is the formation of β-Neu5Ac from β-linked Neu5Ac in CMP-Neu5Ac, which undergoes a retaining process and is opposite to the inverting mechanism of α2−3-sialyltransferase reaction. In this process, the water and the acceptor may occupy different binding pockets, and therefore the amino acid residues in the substrate binding pocket may play different roles in donor hydrolysis and α2−3-sialylation reactions.

PmST1 Crystal Structural Analysis. Examining the co-crystal structures of PmST1 with a donor analogue CMP-

dx.doi.org/10.1021/cb300125k | ACS Chem. Biol. 2012, 7, 1232−1240

3F(*axial*)-Neu5Ac in the presence (PDB ID 2IHZ) and absence (PDB ID 2IHJ) of an acceptor (lactose) indicated that Asp141 and His311 are in close proximity to the donor analogue.[29,30] Previous structural and kinetic studies[30] indicated that Asp141 serves as a general base to activate the C3′-hydroxyl group in the lactose acceptor for PmST1-catalyzed α2−3-sialylation with a direct displacement mechanism involving an oxocarbenium ion-like transition state that leads to the overall inversion of the configuration of the anomeric center of the sialic acid. His311 was believed to act as a general acid that helps to stabilize the CMP leaving group. The potential roles were supported by previously reported mutagenesis studies that showed that H311A mutation resulted in 28-fold decrease in the efficiency of α2−3-sialyltransferase activity, whereas D141A mutation decreased the α2−3-sialyltransferase activity by more than 20,000-fold.[30]

Asp141 and His311 Influence PmST1 Donor Hydrolysis Activity. The important roles of Asp141 and His311 in the CMP-Neu5Ac donor hydrolysis activity of PmST1 were demonstrated by the kinetic studies of previously generated D141A and H311A mutants.[30] As shown in Table 1, D141A mutation decreased the efficiency of CMP-Neu5Ac hydrolysis activity of PmST1 by 1,000-fold mainly due to the decrease in the turnover number. H311A mutation also decreased the CMP-Neu5Ac hydrolysis activity by 16-fold, mainly contributed by a decreased turnover number without affecting the binding affinity significantly. The data indicate that Asp141 plays a more important function than His311 in the donor hydrolysis activity of PmST1.

Table 1. Apparent Kinetics of the CMP-Neu5Ac Hydrolysis Activity of WT PmST1 and Mutants

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
WT	1.5 ± 0.2	27 ± 1	18
D141A[a]	1.4 ± 0.2	(2.5 ± 0.1) × 10^{-2}	1.8 × 10^{-2}
H311A[a]	1.8 ± 0.2	2.1 ± 0.1	1.1
M144D	7.3 ± 0.5	6.5 ± 0.1	0.89
M144H	13 ± 3	71 ± 6	5.5

[a]PmST1 D141A and H311A mutants were generated previously.[30]

PmST1 Mutants with Decreased CMP-Neu5Ac Hydrolysis Activity. To decrease the CMP-Neu5Ac hydrolysis activity of PmST1 and to improve its application in α2−3-sialylation of fucose-containing Le^x, the two amino acid residues Met144 and Ala35 that are in close proximity to Asp141 shown in the PmST1 ternary crystal structure with sialyltransferase donor analogue and acceptor[30] were chosen for site-directed mutagenesis studies. The hypothesis was that changing a hydrophobic residue to a charged Asp or His residue near Asp141, an important amino acid residue for both CMP-Neu5Ac hydrolysis and α2−3-sialyltransferase activities, may alter the PmST1 activities by shifting the pK_a or the position of Asp141. Initial tests on their α2−3-sialyltransferase activity using Le^x as an acceptor indicated that both A35D and A35H PmST1 mutants were inactive. In comparison, both M144D and M144H were active while M144D mutant provided a better yield than M144H mutant for the formation of SLe^x.

As shown in Table 1, both M144D and M144H mutations decreased the efficiency of donor hydrolysis with varied degrees. M144D mutation decreased the efficiency of donor hydrolysis by 20-fold due to a 4.9-fold increase of the K_m value and a 4.2-fold decrease of the k_{cat} value. M144H mutation caused a less significant 3.3-fold decrease in the efficiency of donor hydrolysis due to a significant 8.7-fold increase in the K_m value which is offset by a 2.6-fold increase in the k_{cat} value.

α2−3-Sialyltransferase Activities of PmST1 Mutants. M144D and M144H mutations also have different effects on the α2−3-sialyltransferase activity of the enzyme. As shown in Table 2, when a good sialyltransferase acceptor 4-methylumbelliferyl β-lactoside (Lacβ**MU**) was used, the M144D mutation decreased the α2−3-sialyltransferase activity by 18-fold due to a 9-fold increase of K_m value and a 2-fold decrease of k_{cat} value. Interestingly, when a poor sialyltransferase acceptor Le^xβ**MU** was used, the M144D mutation did not change the efficiency of the α2−3-sialyltransferase activity of PmST1 significantly. In comparison, M144H mutation only decreased the α2−3-sialyltransferase activity weakly (1.3-fold) when Lacβ**MU** was used as an acceptor and increased the efficiency of α2−3-sialyltransferase activity by 2.6-fold when Le^xβ**MU** was used as an acceptor. These discrepancies may be due to the stronger acidity of the amino acid side chain in the aspartic acid compared with that in the histidine.

PmST1 M144D Mutant Has a Drastically Decreased α2−3-Sialidase Activity. To our delight, M144D and M144H mutations also decreased the α2−3-sialidase activity of PmST1 drastically by 5588- and 594-fold respectively when Neu5Acα2−3Lacβ**MU** was used as the sialidase substrate (Table 3). Interestingly, while the PmST1 M144D mutant showed no sialidase activity when Neu5Acα2−3Le^xβ**MU** was used as the substrate, PmST1 M144H has increased sialidase activity compared to the WT PmST1 using the SLe^x substrate. For example, the PmST1 M144H mutant cleaved 10.0%,

Table 3. Apparent Kinetics of the α2−3-Sialidase Activity of WT PmST1, M144D, and M144H Mutants Using Neu5Acα2−3LacβMU** as the Sialidase Substrate**

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
WT[a]	24[a]	2.3 × 10^{2}[a]	9.5[a]
M144D	20 ± 2	(3.5 ± 0.1) × 10^{-2}	1.7 × 10^{-3}
M144H	1.7 ± 0.3	(2.7 ± 0.2) × 10^{-2}	1.6 × 10^{-2}

[a]Data are from ref 11.

Table 2. Apparent Kinetics of the α2−3-Sialyltransferase Activity of WT PmST1 and Mutants

	K_m (mM)			k_{cat} (s^{-1})			k_{cat}/K_m (s^{-1} mM^{-1})		
	WT	M144D	M144H	WT	M144D	M144H	WT	M144D	M144H
Lacβ**MU**	1.4[a]	12 ± 1	0.79 ± 0.04	47[a]	22 ± 1	21 ± 1	34[a]	1.9	27
CMP-Neu5Ac[b]	0.44[a]	0.30 ± 0.05	0.81 ± 0.06	32[a]	1.9 ± 0.1	21 ± 1	73[a]	6.1	27
Le^xβ**MU**	17 ± 2	13 ± 2	8.1 ± 0.9	6.7 ± 0.3	4.0 ± 0.2	8.4 ± 0.3	0.38	0.32	1.0
CMP-Neu5Ac[c]	0.39 ± 0.03	2.1 ± 0.1	0.4 ± 0.05	0.55 ± 0.01	0.59 ± 0.01	0.93 ± 0.02	1.4	0.28	2.2

[a]Data are from ref 11. [b]With Lacβ**MU**. [c]With Le^xβ**MU**.

dx.doi.org/10.1021/cb300125k | *ACS Chem. Biol.* 2012, 7, 1232−1240

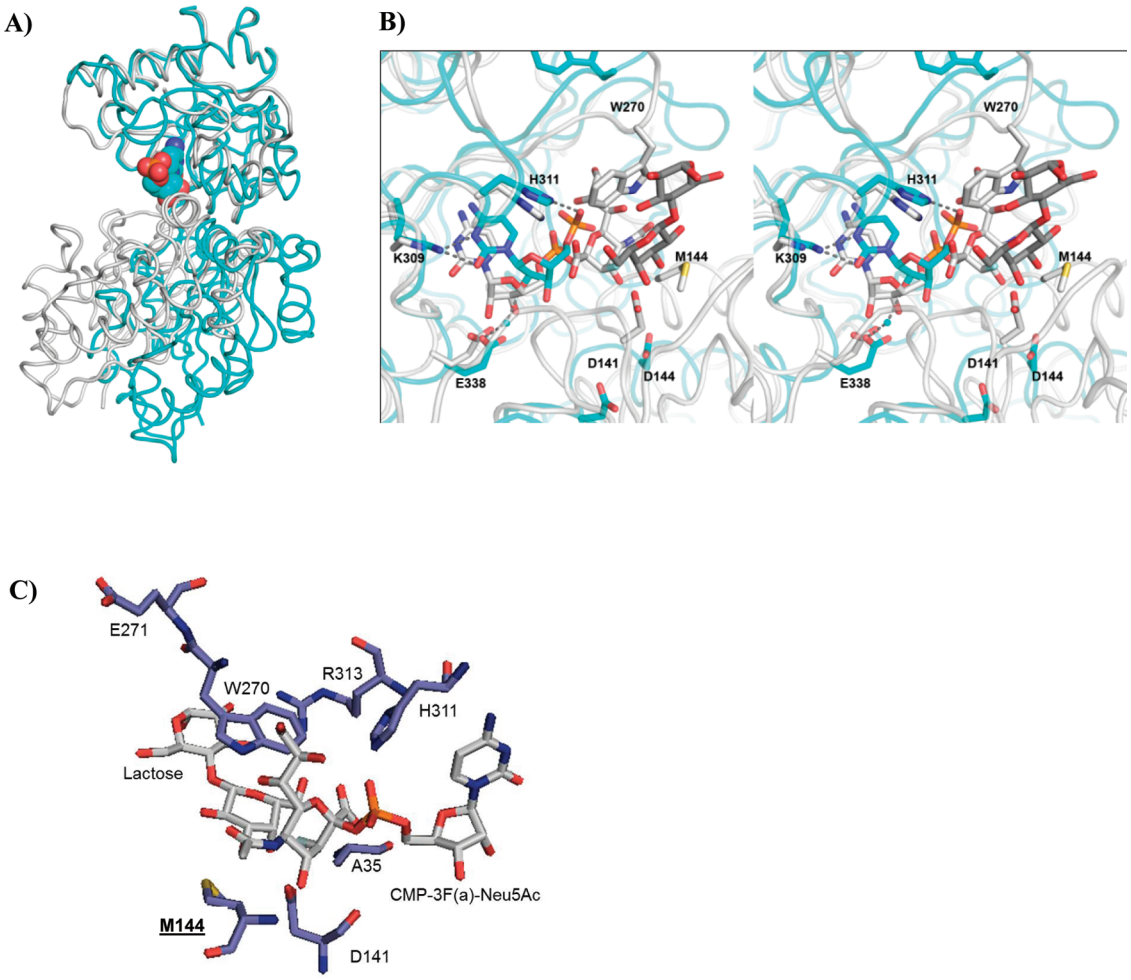


Figure 3. Structural comparison between WT PmST1 and M144D mutant with bound CMP donor. (A) Overall structure of WT PmST1 with CMP bound (white), aligned with the C-terminal domain of the M144D mutant (cyan) also with CMP bound (space filled atoms). (B) Stereoview of the superposition near the active site. WT PmST1 is shown in white with bound CMP-3F(*axial*)-Neu5Ac (sticks with white carbon bonds) and lactose acceptor (sticks with gray carbon bonds). The M144D mutant in shown in cyan with CMP bound (sticks with cyan carbon bonds). (C) Active site of the ternary crystal structure of PmST1 (PDB ID: 2IHZ) with bound CMP-3F(*axial*)-Neu5Ac and lactose. The mutation site M144 is underlined.

24.5%, and 34.0% of Neu5Ac from Neu5Acα2−3Le$^x\beta$MU in 1 h, 6 h, and 20 h, respectively. In comparison, WT PmST1 removed 2.0%, 7.0%, and 7.5% of Neu5Ac from Neu5Acα2−3Le$^x\beta$MU under the same reaction conditions. The drastically decreased α2−3-sialidase activity by M144D mutation allows the potential application of the PmST1 M144D mutant in sialylation of glycoconjugates containing terminal galactoside or Le^x where the decreased α2−3-sialidase activity has the most advantages as these reactions are challenging for prompt monitoring.[31]

PmST1 M144D Mutant Has a Similar Expression Level as the WT PmST1. The PmST1 M144D mutation did not change the enzyme expression level in *E. coli*. About 98 mg of C-His$_6$-tagged PmST1 M144D protein can be routinely purified from one liter of *E. coli* cell culture using Ni^{2+}-affinity column (Supplementary Figure S1). This expression level is very similar to that (100 mg) of the WT PmST1[11] and allows the application of the mutant in preparative and large-scale synthesis of SLe^x antigens.

X-ray Crystal Structure of PmST1 M144D Mutant. The structure of the PmST1 M144D mutant with CMP-3F(*axial*)-Neu5Ac was determined to 1.45 Å resolution with R_{factor} and R_{free} values of 18.7% and 21.5% respectively (Supplementary Table S1). To our surprise, the structure resides in the open conformation similar to the wild-type structure with no substrate (rmsd of 0.50 Å for 385 equivalent α-carbons).[29] However, the M144D structure contains well-ordered electron density in the active site that clearly defines the CMP nucleotide. The sialic acid moiety is disordered, likely due to dynamics and/or multiple conformations in the open state of the enzyme. In the M144D structure, the CMP moiety does not bind as deeply into the pocket of the active site as the WT PmST1. The base and ribose are situated about 1.5 and 2.0 Å, respectively, farther out of the active site compared to the WT PmST1 (Figure 3). In the wild-type structure, Glu338 forms bidentate hydrogen bond interactions with both the 2′ and 3′ OH of the CMP ribose. In the M144D structure, an ordered water molecule mediates the interaction between the ribose and Glu338 (Figure 3). The more shallow binding of the donor nucleotide in the M144D structure does not pull down the β-strand and the ensuing loop that contains Trp270. In comparison, in the wild-type enzyme, donor-nucleotide binding pulls down a β-strand causing Trp270 to pop out of the C-terminal domain, where it helps define the acceptor binding site in the sialyltransferase reaction.[30]

dx.doi.org/10.1021/cb300125k | *ACS Chem. Biol.* 2012, 7, 1232−1240

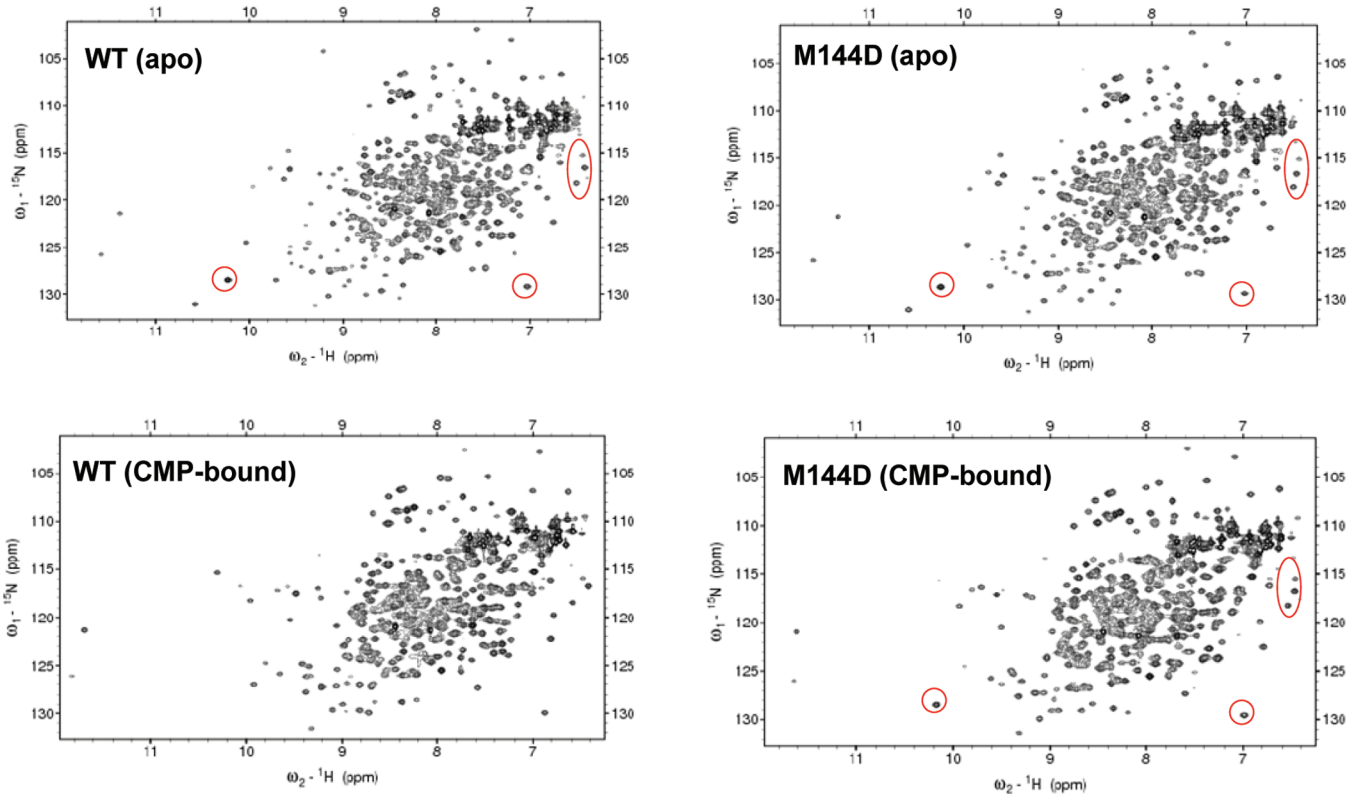
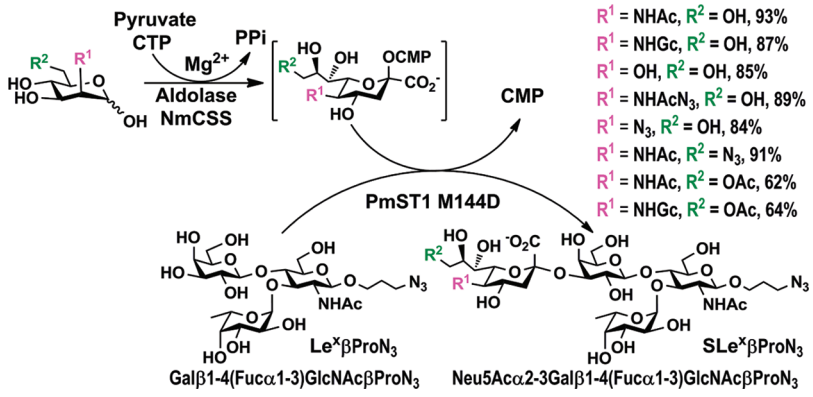


Figure 4. $^{15}N-^{1}H$ HSQC NMR spectra of ^{15}N-labeled PmST1 (WT *versus* M144D and apo *versus* CMP-bound).

Scheme 1. One-Pot Three-Enzyme Synthesis of Sialyl Le$^x\beta$ProN$_3$ (SLe$^x\beta$ProN$_3$) Containing Different Forms of Sialic Acids from Le$^x\beta$ProN$_3$[a]



[a]Aldolase, *Pasteurella multocida* sialic acid aldolase; NmCSS, *Neisseria meningitidis* CMP-sialic acid synthetase.

NMR Analysis of PmST1 M144D Mutant. The open conformation of the M144D mutant bound to donor nucleotide is not a crystallization artifact. Solution NMR studies corroborate the open conformation of the mutant with bound donor. $^{15}N-^{1}H$ HSQC NMR spectra of both WT and M144D mutant (apo and CMP-bound forms) all exhibited the expected number of dispersed peaks with uniform intensities (Figure 4), indicating that the protein adopts a stable three-dimensional fold, consistent with the X-ray crystal structures. The peaks in the HSQC spectra represent the main chain amide groups that serve as fingerprints of the overall main chain conformation. The HSQC spectra of apo-WT, apo-M144D and CMP-bound M144D all exhibit a similar pattern of peaks (Figure 4, see red circles), consistent with all three adopting an open conformation in solution like that observed in the crystal structures. By contrast, the HSQC spectrum of CMP-bound WT has a distinct pattern of peaks not observed in the other three spectra, suggesting that CMP-bound PmST1 adopts a distinct closed conformation in solution like that observed in the crystal structure.

It is unclear whether the open conformation of PmST1 M144D, which may be able to accommodate a bigger acceptor such as Le^x, is responsible for its decreased donor hydrolysis activity and similar activity in sialylation of Le^x. It is also possible that the closed conformation of PmST1 M144D mutant may still exist, but at a much lower abundance. The closed conformation may be responsible for sialylation and/or donor hydrolysis as the WT PmST1 has similar efficiency as the M144D mutant in sialylating Le^x. The M144D mutation may cause a weaker interaction of the active sites (Asp141 and/or

dx.doi.org/10.1021/cb300125k | *ACS Chem. Biol.* 2012, 7, 1232–1240

His311) with water and a better binding of the enzyme with Le^{x}βMU as supported by the K_m data (M144D, 13 ± 2 mM; WT PmST1, 17 ± 2 mM). We are trying to get co-crystal structures of WT PmST1 or the M144D mutant with CMP-3F(*axial*)Neu5Ac and Le^x to solve this puzzle.

PmST1 M144D Mutant Is More Efficient than M144H Mutant in Sialylating Le^x. Overall, the M144D mutation decreased the undesired CMP-Neu5Ac hydrolysis activity significantly (20-fold) without appreciably changing the efficiency of the α2−3-sialyltransferase activity when Le^x was used as an acceptor. As a result, M144D showed an overall improved activity in sialylation of Le^x for the formation of sialyl Le^x (SLe^x) structures. In comparison, M144H mutant which has a 3.3-fold decreased CMP-Neu5Ac hydrolysis activity and 2.6-fold increased α2−3-sialyltransferase activity using Le^x as an acceptor was less effective for directly sialylating Le^x.

Synthesis of SLe^x Containing Diverse Sialic Acid Forms Using PmST1 M144D Mutant. The application of the PmST1 M144D mutant obtained by protein structure-based rational design in the synthesis of SLe^x containing diverse naturally occurring and non-natural sialic acid forms was demonstrated using an efficient one-pot three-enzyme chemoenzymatic synthetic system (Scheme 1). The system contained PmST1 M144D mutant, an *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),[40] and a *Pasteurella multocida* sialic acid aldolase.[41] *N*-Acetylmannosamine (ManNAc), mannose, and their derivatives[11,15] were used for *in situ* synthesis of CMP-sialic acids and derivatives. Le^x trisaccharide used as the sialyltransferase acceptor was synthesized using a one-pot two-enzyme system containing a bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (FKP) cloned from *Bacteroides fragilis*[42] and a recombinant *Helicobacter pylori* α1−3-fucosyltransferase as shown previously.[25] As shown in Scheme 1, SLe^x tetrasaccharides containing natural sialic acid forms including *N*-acetylneuraminc acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn), as well as 9-*O*-acetylated Neu5Ac and Neu5Gc were obtained in excellent (85−93%) to good yields (62−64%). The relatively lower yields for the synthesis of SLe^x containing the 9-*O*-acetyl sialic acid forms were due to the de-*O*-acetylation process leading to the formation of non-*O*-acetylated SLe^x oligosaccharides. In addition, SLe^x containing non-natural sialic acid forms including those with an *N*-azidoacetyl group or an azido group at C-5 or a C-9 azido group were also successfully obtained in excellent yields (84−91%). We would like to point out that the synthesis of SLe^x containing 9-*O*-acetyl sialic acid forms was not possible previously by fucosyltransferase-catalyzed fucosylation of sialyl LacNAc due to easy loss of the 9-*O*-acetyl group in longer reaction time or purification process. The efficiency of the WT PmST1 for sialylation of Le^x was too low to achieve reasonable yields.

Conclusion. In conclusion, we show here that the donor hydrolysis activity contributes significantly to the low yield synthesis of sialyl Le^x catalyzed by a multifunctional *Pasteurella multocida* sialyltransferase (PmST1) when a poor acceptor Le^x is used. We also show that protein X-ray crystal structure-based site-directed mutagenesis can be used as an effective strategy to generate a sialyltransferase single mutant (M144D) with decreased donor hydrolysis activity without affecting the activity in sialylating Le^x. The PmST1 M144D mutant retains a high expressing level (98 mg L^{-1} culture) and the donor substrate promiscuity of the wild-type enzyme. It is a powerful catalyst for synthesizing SLe^x containing different sialic acid forms that are unable or difficult to obtain by any other synthetic or isolation strategies. The study presented here sheds new light on the effect of donor hydrolysis in glycosyltransferase-catalyzed reactions and supplies a new powerful catalyst for efficient chemoenzymatic synthesis of important SLe^x structures. The study also opens a new revenue by decreasing donor hydrolysis activity of glycosyltransferases for improving the efficiency of glycosyltransferase-catalyzed reactions, especially when poor acceptor substrates are used. Although a specific strategy is unclear at the moment, a general approach to achieve this is by mutating the amino acid residues that are located near the key catalytic base of glycosyltransferases. It will also be interesting to characterize the donor hydrolysis activities of other glycosyltransferases and their mutants[8−10] to see whether changes in donor hydrolysis contribute to broadening the substrate specificity by mutagenesis.

METHODS

Site-Directed Mutagenesis, Expression, and Purification of PmST1 Mutants. Site-directed mutagenesis was performed using the QuikChange multisite-directed mutagenesis kit from Stratagene according to the manufacturer's protocol. The primers used were 5′ AATCTTTATGACGATGGCTCAGATGAATATGTTGATTTAGAAAAAG 3′ for M144D; 5′ AATCTTTATGACGATGGCTCACATGAATATGTTGATTTAGAAAAAG 3′ for M144H; 5′ ATCACGCTGTATTTAGATCCTGATTCCTTACCGGCATTAAATCAG 3′ for A35D; and 5′ ATCACGCTGTATTTAGATCCTCATTCCTTACCGGCATTAAATCAG 3′ for A35H. The expression and purification of the mutants were performed as previously described for the WT PmST1.[11,29]

Kinetics of the Donor Hydrolysis Activity of PmST1 and Mutants by Capillary Electrophoresis Analysis. The reactions were carried out in duplicate in a total volume of 10 μL at 37 °C for 15 min (WT), 40 min (D141A), 20 min (H311A), or 15 min (M144D and M144H) in Tris-HCl buffer (200 mM, pH 8.5) containing CMP-Neu5Ac (1, 2, 5, 10, 20, and 40 mM) and an enzyme (WT, 4 μg mL^{-1}; D141A, 1500 μg mL^{-1}; H311A, 40 μg mL^{-1}; M144D, 39 μg mL^{-1}; M144H, 5 μg mL^{-1}). The reactions were stopped by adding 10 μL of prechilled ethanol. The mixtures were incubated on ice for 30 min and centrifuged at 13,000 rpm for 5 min. The supernatants were diluted with borate buffer (25 mM, pH 9.5), and aliquots of 5 μL each were injected to a Beckman Coulter P/ACE MDQ Capillary Electrophoresis system equipped with a capillary (60 cm × 75 μm i.d.) and monitored at 254 nm. The apparent kinetic parameters were obtained by fitting the experimental data (the average values of duplicate assay results) into the Michaelis−Menten equation using Grafit 5.0.

Kinetics of the α2−3-Sialyltransferase Activity of PmST1 Mutants by HPLC Analysis. With Lacβ MU as the acceptor substrate, the reactions were performed in duplicate at 37 °C for 10 min (M144D) or 4 min (M144H) in a reaction mixture (10 μL) containing Tris-HCl (100 mM, pH 8.5), an enzyme (5 μg mL^{-1}), and different concentrations (0.2, 0.5, 1.0, 2.0, and 5.0 mM) of Lacβ MU with a fixed concentration (1 mM) of CMP-Neu5Ac or different concentrations (0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mM) of CMP-Neu5Ac with a fixed concentration (1 mM) of Lacβ MU. With Le^{x}β MU as the acceptor substrate, the reactions were carried out in duplicate at 37 °C for 9 min (M144D) or 10 min (M144H) in a reaction mixture (10 μL) containing CAPSO (100 mM, pH 9.5), an enzyme (M144D, 39 μg mL^{-1} or M144H, 5 μg mL^{-1}), and various concentrations of Le^{x}β MU (1.0, 5.0, 10.0, 15.0, 25.0, and 35.0 mM) with a fixed concentration (1 mM) of CMP-Neu5Ac or various concentrations (0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, and 40.0 mM) of CMP-Neu5Ac with a fixed concentration (1 mM) of Le^{x}β MU. Reactions were stopped by adding 10 μL of prechilled ethanol. The mixtures were incubated on ice for 30 min and centrifuged at 13,000 rpm for 5 min. The supernatants were diluted with 25% acetonitrile and kept on ice until aliquots of 8 μL were injected and analyzed by the Shimadzu LC-6AD system equipped with

a membrane online degasser, a temperature control unit, and a fluorescence detector (Shimadzu RF-10AXL). A reverse-phase Premier C18 column (250 mm × 4.6 mm i.d., 5 μm particle size, Shimadzu) protected with a C18 guard column cartridge was used. The mobile phase was 25% acetonitrile. The fluorophore (MU)-labeled compounds were detected by excitation at 325 nm and emission at 372 nm.[11,43] The apparent kinetic parameters were obtained by fitting the experimental data (the average values of duplicate assay results) into the Michaelis–Menten equation using Grafit 5.0.

Kinetics of the α2–3-Sialidase Activity of PmST1 Mutants. The reactions were performed in duplicate in a total volume of 10 μL at 37 °C for 60 min (M144D) or 15 min (M144H) in MES buffer (100 mM, pH 5.5) containing Neu5Acα2–3Lacβ*MU (0.4, 1, 2, 4, 10, 20, 40, and 60 mM) and an enzyme (M144H, 1.36 mg mL^{-1} or M144D, 1.05 mg mL^{-1}). Sample treatment after the reaction and analysis were carried out by HPLC similar to that described above for the α2–3-sialyltransferase assays.

α2–3-Sialidase Activity Assays of PmST1 and Mutants. The reactions were carried out in duplicate in a total volume of 10 μL at 37 °C for 20 h in MES buffer (100 mM, pH 5.5) containing Neu5Acα2–3Le^xβ*MU (1 mM) and an enzyme (4 mg mL^{-1}). Aliquots of 1 μL were withdrawn at 1, 6, and 20 h, and analyzed by HPLC as described above for the α2–3-sialyltransferase assays.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

Accession Codes

The structure of PmST1 M144D mutant in complex with CMP-3F(*axial*)-Neu5Ac was deposited with a PDB ID code 3S44.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: chen@chem.ucdavis.edu; fisher@chem.ucdavis.edu.

Author Contributions

[§]These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by NIH grants R01GM076360 and R01HD065122 (to X.C.) and R01EY012347 (to J.B.A.). X.C. is an Alfred P. Sloan Research Fellow, a Camille Dreyfus Teacher-Scholar, and a UC-Davis Chancellor's Fellow. Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource (SSRL), a Directorate of SLAC National Accelerator Laboratory and an Office of Science User Facility operated for the U.S. Department of Energy Office of Science by Stanford University. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program (P41RR001209), and the National Institute of General Medical Sciences.

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