Glycosyltransferase Activity Can Be Modulated by Small Conformational Changes of Acceptor Substrates†

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ABSTRACT: A range of *N*-acetyllactosamine derivatives (compounds **4**−**7**) that have restricted mobilities around their glycosidic linkages have been employed to determine how small changes in conformational properties of an oligosaccharide acceptor affect catalytic efficiencies of glycosylations by α-2,6- and α-2,3-sialyltransferases and α-1,3-fucosyltransferases IV and VI. Restriction of conformational mobility was achieved by introducing tethers of different length and chemical composition between the C-6 and C-2′ hydroxyl of LacNAc. Compound **4** is a 2′,6-anhydro derivative which is highly constrained and can adopt only two unusual conformations at the LacNAc glycosidic linkage. Compound **5** is modified by a methylene acetal tether and can exist in a larger range of conformations; however, the Φ dihedral angle is restricted to values smaller than 30°, which are not entirely similar to minimum energy conformations of LacNAc. The ethylene-tethered **6** can attain conformations in the relatively large energy plateau of LacNAc that include syn conformations A and B, whereas compound **7**, which is modified by a methylamide tether, can only reside in the B-conformer. 2′,6-Dimethoxy derivative **2** was employed to determine the effect of alkylation of the C-6 and C-2′ hydroxyls of **5** and **6** whereas **3** was used to reveal the effects of the C-6 amide and C-2′ alkylation of **7**. The apparent kinetic parameters of transfer to the conformationally constrained **4**−**7** and reference compounds **1**−**3** catalyzed by α-2,6- and α-2,3-sialyltransferases and α-1,3-fucosyltransferases IV and VI were determined, and the results correlated with their conformational properties. The data for **4**−**6** showed that each enzyme recognizes *N*-acetyllactosamine in a low minimum energy conformation. A small change in conformational properties such as in compound **5** resulted in a significant loss of catalytic activity. Larger conformational changes such as in compound **4** abolished all activity of the sialyltransferases whereas the fucosyltransferases showed some activity, albeit very low. The kinetic data for compounds **4** and **5** demonstrate clearly that different glycosyltransferases respond differently to conformational changes, and the fucosyltransferases lost less activity than the sialyltransferases. Correlating apparent kinetic parameters of conformationally constrained **6** and **7** and their reference compounds **2** and **3** further supports the fact that different enzymes respond differently and indicates that sialyltransferases and fucosyltransferases recognize *N*-acetyllactosamine in a different conformation. Collectively, the data presented here indicate that small conformational changes of an oligosaccharide acceptor induced by, for example, the protein structure can be employed to modulate the patterns of protein glycosylation.

Protein glycosylation is more abundant and structurally diverse than all other types of posttranslational modifications combined (*1*−*4*). Protein-bound saccharides range from dynamic monosaccharides on nuclear and cytoplasmic proteins to enormously complex structures on extracellular N- or O-linked glycoproteins and proteoglycans. At the cellular level, *N*- and *O*-glycans have been shown to contribute to a myriad of functions, including cell adhesion events during immune surveillance, inflammatory reactions, hormone action, arthritis, and viral and bacterial infections (*5*−*9*). The cell- and tissue-specific changes in cell surface oligosaccharides during development have indicated that these structures may be involved in cell adhesion and migration events during embryogenesis. Alterations in the branching and extension of *N*-glycans have also been found on the surfaces of cells that have undergone oncogenic transformation, and these changes correlate with the alterations in cell adhesion, invasiveness, and metastasis of transformed cells (*10*−*12*).

Glycan chains are assembled by a myriad of membrane-bound glycosyltransferases, which catalyze the transfer of monosaccharide residues from nucleoside mono- or diphosphate sugars to growing oligosaccharide chains, resulting in remarkably complex structures. Glycosyltransferases are highly specific for their donor and acceptor substrates, and with a few exceptions each type of glycosidic linkage requires a unique glycosyltransferase. It has been estimated that the mammalian cells require well over 100 different glycosyltransferases to biosynthesize all known oligosaccharide structures (*13*−*15*).

According to the current consensus, glycosyltransferases are sequentially distributed within the Golgi compartments

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in approximately the observed order of glycosylation (*16*, *17*). Glycosyltransferases that act early in protein-linked oligosaccharide biosynthesis are located in the medial and trans-Golgi (e.g., GNT-I)[1] whereas the late glycosyltransferases that are involved in the terminal decoration of oligosaccharide motifs (e.g., α-2,3/6-sialyltransferases and α-3/4-fucosyltransferases) are localized later within the trans-Golgi and trans-Golgi network. Thus, an important determinant of the pattern of oligosaccharide biosynthesis is the level of expression of particular glycosyltransferases and their localization in the subcompartments of the secretory pathway.

Genetic and localization control cannot explain all features of protein glycosylation. For example, many glycoproteins display different oligosaccharide chains at different glycosylation sites of the same glycoprotein. These differences in chain compositions on the same glycoprotein cannot arise from indirect genetic control by modulation of glycosyltransferase activity. An obvious explanation would be that certain glycosylation sites are less accessible to processing enzymes by virtue of steric constraints imposed by the three-dimensional structure of the protein (*18*). Several studies, however, have indicated that this is an incomplete picture and that other parameters contribute to site-selective glycosylation (*19*, *20*). For example, there are some indications that not only primary structures but also conformational properties of oligosaccharides are important for glycosyl acceptor specificities (*21*−*24*). In particular, flanking saccharide residues or attachments to proteins and lipids may modulate conformational properties of glycosidic linkages, which may affect acceptor activity. The generality of this concept is, however, difficult to prove due to complicating factors such as the difficulties of obtaining well-defined complex oligosaccharides, the difficulties of determining their conformational properties, difficulties of crystallizing oligosaccharides, and the fact that very few X-ray crystal structures of acceptor−glycosyltransferase complexes have been reported.

We report here a novel method to study how conformational properties of oligosaccharides can affect the biosynthesis of glycoconjugates. This approach employs a series of preorganized *N*-acetyllactosamine (LacNAc) derivatives with different conformational properties as substrates for several glycosyltransferases. Correlating the conformational properties of these compounds with their enzyme activities established that small changes in conformational properties could have a dramatic effect on oligosaccharide biosynthesis. Furthermore, different enzymes responded differently to conformational changes, providing a way to tune selectively glycosyl acceptor specificities.

EXPERIMENTAL PROCEDURES

Compounds **1**−**7** were synthesized as reported previously (*25*, *26*). The rat liver α-2,6-sialyltransferase was purchased from Sigma, and α-2,3-sialyltransferase was obtained from Calbiochem. CMP-[^{14}C]Neu5Ac and GDP-[^{14}C]fucose were obtained from Amersham Corp. Human recombinant fucosyltransferases VI, CTP, CMP-Neu5Ac, and calf alkaline phosphatase were purchased from Calbiochem. Stable transfected CHO cells with fucosyltransferase IV were a generous gift from Dr. Theodora de Vries. Protein assay reagents were obtained from Sigma. Fetal calf serum was purchased from HyClone. GDP-fucose was synthesized following a reported procedure (*27*). ACS liquid scintillation cocktail was obtained from Fisher Scientific. All other chemicals of the highest purity available were obtained from commercial sources.

Cell Extract Preparation. Stable transfected CHO cells with FucT IV were cultured in nutrient mixture F-12 HAM, supplemented with 2 mM L-glutamine, 100 units/mL penicillin/100 μg/mL streptomycin (Sigma P0781), and 10% fetal calf serum (FCS). The cells were maintained in a humid 5% CO_2 atmosphere at 37 °C.

The CHO-FucT IV cells from confluent monolayers in seven T75 flasks were washed with PBS, trypsinized with trypsin−EDTA solution (1×), and collected in PBS. After centrifugation for 5 min at 1400 rpm, the cell pellet was mixed with 1.75 mL of 1% Triton X-100 in PBS containing a protease inhibitor cocktail (final concentration of inhibitors: AEBSF, 0.1 mM; EDTA, 50 μM; bestatin, 6.5 μM; E-64, 0.7 μM; leupeptin, 50 nM; and aprotinin, 15 nM). The solution was kept on ice for 30 min and then sonicated five times for 10 s. Next the cell lysate was centrifuged at 14000 rpm for 10 min at 4 °C. The clear supernatant was aliquoted as 100 μL/vial and stored at −80 °C before use.

The protein concentration was determined using the bicinchonic acid protein assay (Sigma P 0914 and C2284) with bovine serum albumin (BSA) as standard. The protein concentration was found to be 6.2 μg/μL.

Sialyltransferase Assays. Reported methods (*28*−*31*) were employed for assaying sialyltransferase activity. For studies of the relative rates, incubation mixtures contained CMP-[^{14}C]Neu5Ac (9 nmol, 6180 cpm/nmol), and substrate (120 nmol), bovine serum albumin (1 mg/mL), 57 microunits of α-2,6-sialyltransferase, and 370 microunits of α-2,3-sialyltransferase in sodium cacodylate (50 mM, pH 6.5) containing 0.1% Triton X-100 in a total volume of 60 μL were incubated at 37 °C for a period of 30 min. The radiolabeled product was isolated using a procedure modified by Horenstein et al. (*31*) based on Paulson's ion-exchange chromatography on a Dowex 1-X8−200 (PO_4^{2-}, 100−200 mesh) Pasteur pipet column (*28*). Columns (5 cm high) were eluted twice with 1 mM PO_4^{2-} (4 mL) buffer to ensure that no radiolabeled product was left on the column.

Kinetic Studies. Apparent kinetic parameters of the α-2,6-ST and α-2,3-ST for synthetic acceptors were determined under the above standard conditions using a saturating concentration of CMP-[^{14}C]Neu5Ac (*29*). Assays were performed in duplicate using the appropriate amount of each of enzyme. The concentration of the oligosaccharide acceptor was varied around the K_m value (see Supporting Information), whereas the concentration of CMP-[^{14}C]Neu5Ac was kept constant at 200 μM (1655 cpm/nmol). The time of incubation at 37 °C was limited to 15 min to limit the CMP-[^{14}C]Neu5Ac consumption to 10−15% to ensure initial rate conditions. The kinetic parameters V_{max} and K_m were

[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; Asn, asparagine; CHO, Chinese hamster ovary; CMP, cytidine monophosphate; Conf_, conformation; EDTA, ethylenediaminetetraacetic acid; FucT, fucosyltransferases; Gal, galactose; GDP, guanosine diphosphate; GlNAc, *N*-acetylglucosamine; GNT-I, glucosaminyltransferase I; IgG, immunoglobulin G; LacNAc, *N*-acetyllactosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; NMR, nuclear magnetic resonance.

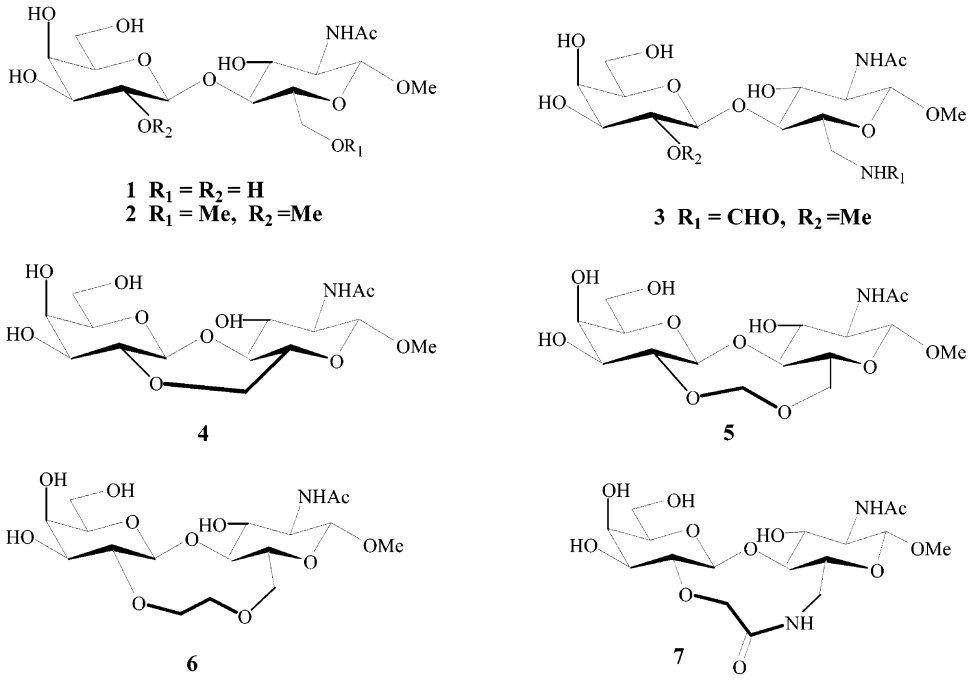

FIGURE 1: Disaccharide derivatives **1**−**7**.

determined using the GraphPad computer program obtained from Prism.

Fucosyltransferase Assays. Reported methods (*29*, *32*) were employed for assaying fucosyltransferase activity. For studies of the relative rates, incubation mixtures contained GDP-[^{14}C]fucose (2.3 nmol, 6532 cpm/nmol), and substrate (100 nmol) and an amount of enzyme corresponding to the initial velocity for each fucosyltransferase [56 microunits of FucT VI and 4 μL of FucT IV (6 μg of protein/μL)] assayed in sodium cacodylate (25 mM, pH 6.5) containing $MnCl_2$ (8 mM), ATP (1.6 mM), and NaN_3 (1.6 mM) in a total volume of 50 μL were incubated at 37 °C for a period of 60 min. The radiolabeled product was isolated using ion-exchange chromatography on a Dowex 1-X8−200 (Cl^-, 100−200 mesh) Pasteur pipet column (*32*). Columns (2.5 cm high) were eluted twice with ice-cold water (1.5 mL) to ensure that no radiolabeled product was left on the column.

Kinetic Studies. Apparent kinetic parameters of the human recombinant FucT IV and FucT VI for synthetic acceptors were determined under the above standard conditions using a saturating concentration of GDP-fucose (*32*). Assays were performed in duplicate using the appropriate amount of each enzyme. The concentration of oligosaccharide acceptor was varied around the K_m value (see Supporting Information), whereas the concentration of GDP-fucose was kept constant at 45 μM. The time of incubation at 37 °C was limited to 15 min to limit the GDP-[^{14}C]fucose consumption to 10−15% to ensure initial rate conditions. The kinetic parameters V_{max} and K_m were determined using the GraphPad 3.0 program obtained from Prism.

RESULTS

The apparent kinetic parameters of sialylation and fucosylation of acceptors **1**−**7** by α-2,6- and α-2,3-sialyltransferases and α-1,3-fucosyltransferases IV and VI were determined, and the results correlated with the conformational properties of these derivatives. Compounds **4**−**7** (Figure 1) are derived from LacNAc, and due to the presence of tethers between the C-6 and C-2′ hydroxyls, they have restricted mobilities around their glycosidic linkages. These hydroxyls, which in a low-energy conformation are at the same face of the molecule, are not critical for sialyltransferase (*30*) or fucoyltransferase activity (*32*). However, it is to be expected that these modifications will have some effect on the apparent kinetic parameters, and therefore **2** and **3** were employed as reference compounds. Compound **2** will determine the effect of alkylation of the C-6 and C-2′ hydroxyls of **5** and **6**. Furthermore, data for **3** will reveal the effects of the C-6 amide and C-2′ alkylation of **7**.

Previously, we determined the conformational properties of LacNAc (**1**) and compounds **4**−**7** by molecular mechanics simulations and NMR spectroscopy (*25*, *26*). It was shown that LacNAc is a highly flexible disaccharide with a global minimum energy conformation around $\Phi = 50°$ ($\Phi = H_1'-C_1'-O_1'-C_4$) and $\Psi = 0°$ ($\Psi = C_1'-O_1'-C_4-H_4$) (Conf_A) and a second low minimum around $\Phi = 30°$ and $\Psi = -60°$ (Conf_B). Regions C and D correspond to remote parts of the main low-energy region and should be considered as secondary minima (Figure 2). Compound **4** is a 2′,6-anhydro derivative that is highly constrained and can adopt only two conformations, both with similar but unusual conformations at the LacNAc glycosidic linkage. Compound **5** can exist in a larger range of conformations; however, the Φ dihedral angle is restricted to values smaller than 30°, which are not entirely similar to minimum energy conformations of LacNAc. Compound **6** can attain conformations in the relatively large energy plateau of LacNAc that include syn conformations A and B. However, the anti (Conf_C) and gauche−gauche conformers (Conf_D) cannot be adopted. Compound **7** also adopts a low-energy conformation on the LacNAc energy surface, but it is significantly more rigid than **6** and resides in the B-conformer.

Acceptor Specificities of Rat Liver α-2,6- and α-2,3-Sialyltransferases. The apparent kinetic parameters of transfer

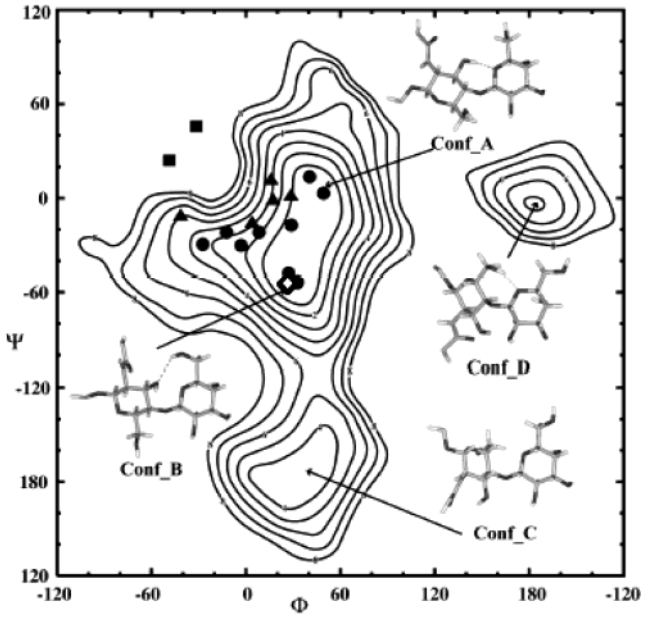

FIGURE 2: Energy map of the LacNAc glycosidic linkage calculated with MM3 (*60*). Conformations accessible to compounds **4**−**7** have been superimposed as □ = **4**, ▲ = **5**, ● = **6**, and ◇ = **7** (*25*, *26*).

Table 1: Apparent Kinetic Parameters for the Transfer of *N*-Acetylneuraminic Acid to Various Acceptors by α-2,6-Sialyltransferase[a]

	α-2,6-sialyltransferase		
acceptor	K_m (mM)	rel V_{max}	rel V_{max}/K_m (mM^{-1})
1	1.7 ± 0.2	1.0	0.6
2	11.2 ± 0.8	0.5	0.04
3	3.3 ± 0.8	1.0	0.3
4	na[b]	na	na
5	11.6 ± 1.9	1.0	0.09
6	1.1 ± 0.1	1.3	1.2
7	1.0 ± 0.1	1.1	1.1

[a] See ref *25*. [b] na = not active up to 4.0 mM.

Table 2: Apparent Kinetic Parameters for the Transfer of *N*-Acetylneuraminic Acid to Various Acceptors by α-2,3-Sialyltransferase

	α-2,3-sialyltransferase		
acceptor	K_m (mM)	rel V_{max}	rel V_{max}/K_m (mM^{-1})
1	5.5 ± 0.8	1	0.18
2	9.1 ± 1.0	0.6	0.07
3	>4.8	<1	0.10
4	na[a]	na	na
5	>20.0	<1	0.01
6	1.6 ± 0.4	0.5	0.31
7	0.6 ± 0.1	0.3	0.50

[a] na = not active up to 4.0 mM.

Table 3: Apparent Kinetic Parameters for the Transfer of Fucose to Various Acceptors by α-1,3-Fucosyltransferase IV

	α-1,3-fucosyltransferase IV		
acceptor	K_m (mM)	rel V_{max}	rel V_{max}/K_m (mM^{-1})
1	1.2 ± 0.2	1.0	0.8
2	2.7 ± 0.5	1.6	0.6
3	1.3 ± 0.1	1.2	0.9
4	>25	<0.4	0.02
5	11 ± 3	0.5	0.05
6	5 ± 1	1.2	0.24
7	16 ± 3	1.05	0.06

Table 4: Apparent Kinetic Parameters for the Transfer of Fucose to Various Acceptors by α-1,3-Fucosyltransferase VI

	α-1,3-fucosyltransferase VI		
acceptor	K_m (mM)	rel V_{max}	rel V_{max}/K_m (mM^{-1})
1	0.35 ± 0.05	1	2.8
2	0.12 ± 0.03	0.8	6.7
3	0.075 ± 0.07	0.7	9.3
4	>3.0	<0.3	0.04
5	1.10 ± 0.10	0.2	0.2
6	0.40 ± 0.05	0.6	1.5
7	0.61 ± 0.10	0.5	0.8

of CMP-[^{14}C]Neu5Ac to acceptors **1**−**7** catalyzed by α-2,3- and α-2,6-sialyltransferase were determined using a reported assay (*30*). In each case, the K_m for **1** was in close agreement with previous data, and the V_{max} values were set at 1. The apparent kinetic parameters of the sialylation of compound **2** by rat liver α-2,6-sialyltransferase show that the methyl groups at C-6 and C-2′ hydroxyls significantly lower the catalytic efficiency (V_{max}/K_m) of the transfer due to a notable increased K_m and a somewhat smaller V_{max} (Table 1). The formamide-containing compound **3** displayed only a slightly decreased K_m, indicating that its C-6 functionality does not impact the catalytic efficiency. Thus, these findings indicate that the enzyme prefers polar substituents at the periphery of its binding site. The anhydro derivative **4** is not a substrate, whereas the methylene-bridged compound **5** has kinetic parameters similar to those of **2** but is a much worse substrate than LacNAc (**1**). The ethylene and the methylene amide tethered derivatives **6** and **7**, respectively, have slightly higher catalytic efficiencies than LacNAc (**1**), indicating that they are preorganized in a conformation favorable for the sialyltransferase. The low-energy conformations of **6** are centered around the A- and B-conformers of LacNAc, whereas **7** is constrained in the B-conformer. Thus, our findings indicate that rat liver α-2,6-sialyltransferase recognizes LacNAc in a low-energy conformation, with Conf_B being the most probable one. Correlating the properties of compounds **5** and **6** reveals that small differences in conformational properties may result in significant differences in apparent kinetic parameters. In the case of **5**, a small shift outside the main minimum resulted in a much larger K_m, but importantly, the compound can still act as an acceptor. When larger conformational changes are induced, such as in compound **4**, glycosyl-accepting properties are lost. The similarity of apparent kinetic parameters for compounds **2** and **4** was surprising, and probably, the unfavorable conformation of **4** is compensated by less reduction in flexibility upon binding. The K_m of conformationally constrained **6** is approximately 10 times smaller than that of the dimethoxy analogue **2**. A similar trend was observed when the kinetic parameters of reference compound **3** were compared with those of **7**. Each set of compounds essentially has similar hydrogen-bonding potential and hydrophobic and hydrophilic surface but differs in conformational properties. Therefore, the higher catalytic efficiency of **6** and **7** probably results from a more favorable enzyme−substrate association due to preorganization of the acceptor in a conformation that is recognized by the enzyme.

In the case α-2,3-sialyltransferase, modification of the C-6 and C-2′ hydroxyls (compounds **2** and **3**) resulted only in relatively small loss of catalytic efficiency (Table 2),

indicating that these functionalities make no significant interactions with the periphery of its binding site. Compound **4** was not a substrate whereas the methylene derivative **5** was poorly accepted. The apparent kinetic parameters for compounds **6** and **7** were intriguing, and in this case they displayed 3.5 and 9 times lower K_m values, respectively, than LacNAc (**1**). The V_{max} values of these derivatives were, however, somewhat lower, resulting in a modest increase in catalytic efficiency compared to LacNAc. The combined data indicate that α-2,6- and α-2,3-sialyltransferases respond similarly to conformational changes of LacNAc; however, it appears that the α-2,3 enzyme gains significantly more affinity when it is locked in a conformation required by the enzyme. Furthermore, it appears that the periphery of the binding site of the two enzymes has different architectures since they respond differently to modifications at C-6 and C-2′.

Acceptor Specificities of Human Recombinant Fucosyltransferases IV and VI. In the case of α-1,3-fucosyltransferase IV, compound **2**, which has a methoxy group at C-6 and at C-2′, had a 2-fold higher K_m and V_{max}, which resulted in a catalytic efficiency similar to that observed for LacNAc (**1**). Also, the presence of the formamide at the C-6 of compound **3** did not affect the catalytic efficiencies. Thus, these findings indicate that these functionalities are not interacting with the protein surface, and therefore it is likely that they are outside the binding pocket of the enzyme. Intriguingly, the anhydro derivative **4**, which adopts an unusual conformation on the LacNAc energy surface, was accepted by the transferase although the K_m and V_{max} values were dramatically decreased. The methylene derivative **5** had a 10-fold higher K_m and a 2-fold lower V_{max} than **1**, making it a poor substrate. The methylene amide derivative **7** has a 15 times higher K_m, while the more flexible ethylene compound **6** had a 4-fold higher K_m and similar V_{max} values. Thus, these data illustrate that the α-1,3-fucosyltransferases IV responds differently to imposed conformational constraints and chemical modifications at C-6 and at C-2′ than the two sialyltransferases. On one hand, this enzyme tolerates larger conformational changes since **4** is still accepted as a substrate. On the other hand, imposing restrictions in mobility did not result in compounds that displayed improved catalytic efficiencies. This observation may indicate that, upon binding to the enzyme, LacNAc loses less conformational flexibility than when interacting with the sialyltransferases. Compound **6** displayed the most favorable kinetic parameters of the conformationally constrained molecules, indicating that this enzyme prefers the A-conformation.

The apparent kinetic data for α-1,3-fucosyltransferase VI show that this enzyme can make favorable interactions with substituents at C-6 and at C-2′, and this observation indicates that its binding pocket for the acceptor substrate differs from that of α-1,3-fucosyltransferase IV. The dimethoxy derivative **4** displayed an increased catalytic efficiency due to a 3-fold lower K_m than **1**. Compound **3**, which has a methoxy group at C-2′ and a formamide functionality at C-6, was a remarkable good substrate and had the lowest K_m of all the compounds tested. The anhydro derivative **4** was also accepted as a substrate, albeit with very poor efficiency. The methylene-bridged compound **5** also displayed rather poor kinetic data when compared to **1** or reference derivative **2** but was markedly better than **4**. The ethylene-tethered derivative **6** and methylene amide compound **7** have similar K_m values and a slightly reduced V_{max} than **1**.

DISCUSSION

Secretory *N*-glycoproteins synthesized by a given cell must pass the same enzymes of the oligosaccharide processing machinery. A remarkable observation is, therefore, that different proteins or even glycosylation sites may acquire different oligosaccharide structure, and it appears that the protein itself largely determines final oligosaccharide structures on a glycoprotein (*18*−*20*, *33*). For example, when expressed in BHK-21 or Chinese hamster ovary cells, human erythropoietin is decorated with predominantly tetraantennary oligosaccharides with 1−3 *N*-acetyllactosamine repeating units, and human antithrombin III is modified by a mixture of di- and tetraantennary glycans and some tetraantennary chains whereas human b-TP contains only highly sialylated diantennary *N*-lactosamine-type structures. Selective protein glycosylation may result from inaccessibility of particular Golgi membrane-anchored glycosyltransferases to a glycosylation site (*33*−*38*). Several studies, however, have indicated that this is an incomplete picture and that other parameters contribute to site-selective glycosylation. Carver and co-workers (*39*) proposed that conformational properties of oligosaccharides can be modulated by the protein structure it is part of, and the resulting differences in conformational properties may affect the activity of glycosyltransferases. For example, it was shown (*40*, *41*) that a human myeloma IgG antibody contains an oligosaccharide at Asn107 of the light chain, as were all of the Neu5Ac(α2,6) terminated bisected biantennary complex type, whereas those at Asn297 of the heavy chain were found to comprise primarily unbisected complex types terminated in Gal or Neu5Ac. GlcNAc transferase III (*42*), the enzyme responsible for incorporation of the bisecting GlcNAc moiety, acts before galactosylation by β-1,4-galactosyltransferase, and therefore inaccessibility of the oligosaccharide chain is an unlikely reason for the absence of the bisecting structure. A possible explanation came by comparing the conformations of the Man(α1,6)Man glycoside at the two glycosylation sites. It was found that this moiety of the Asn297 glycosylation site adopts an unusual conformation in which $\omega = -60°$. In solution and at Asn107, this glycoside adopts a very different conformation ($\omega = 180°$). It is highly likely that GlcNAc transferase III requires the mannoside of the 1−6 arm for recognition, and thus, the unusual conformation of the 1−6 arm of Asn297 accounts for the absence of the bisecting GlcNAc moiety. Although several other studies have indicated that the local environment can modulate conformational properties of glycosidic linkages (*43*−*45*), it is largely unknown whether glycosyltransferase activity can be modulated by subtle differences in conformational properties of an oligosaccharide substrate. This important issue has been complicated by the fact that only very few X-ray structures of glycosyltransferases have been reported (*46*). Although unproven, it may well be possible that different transferases respond differently to changes in the conformational properties of a common substrate, and thus site-specific glycosylation of proteins may arise from modulation of oligosaccharide conformation by the macromolecular structure it is part of.

In this study, we have probed in which way catalytic efficiencies of several sialyl- and α-1,3-fucosyltransferases are affected by small changes in conformational properties of an oligosaccharide substrate. This objective was achieved by employing a series of modified LacNAc derivatives (**4**−**7**) that contain tethers between its two monosaccharide units to modify conformational properties. An important design feature of these compounds was the selection of hydroxyls that allow introduction of tethers without loss of activity. We were guided by previous studies that have identified substrate requirements of several glycosyltransferases (*30*, *32*, *47*). For example, it has been shown that α-2,6-sialyltransferase recognizes type II (Gal*β*1−4GlcNAc) but not type I (Gal*β*1−3GlcNAc) or type III (Gal*β*1−3GalNAc) acceptors. Furthermore, the C-6 hydroxyl of Gal and the acetamido moiety of GlcNAc are essential for recognition (*30*) whereas the C-3 or C-4 hydroxyls of Gal can be deoxygenated or substituted with fluorine with only partial loss of catalytic efficiency. Furthermore, deoxygenation of C-2′ and C-6 hydroxyls was also permitted, albeit with some loss of catalytic activity. The substrate specificity of rat liver α-2,3-sialyltransferase (ST3Gal III) has been mapped in detail, and in this case the C-3, C-4, and C-6 hydroxyls of Gal are essential for recognition (*30*). A 4″-*O*-methyl derivative of the Gal*β*1−4GlcNAc*β*1−2Manα-*O*oct was also shown to be an acceptor, and a variety of modifications of the acetamido function of either type I or type II acceptors were tolerated (*48*, *49*).

Five distinct human α-1,3-fucosyltransferases have been cloned and overexpressed, and studies with synthetic substrates have shown that these enzymes have overlapping but somewhat different acceptor specificities (*50*). FucT IV is a myeloid-type enzyme that only transfers to type II acceptors (*50a*−*c*), and in this case sialylation results in poor recognition (*51*). On the other hand, FucT VI can employ both Gal*β*1−4GlcNAc and NeuAcα2−3Gal*β*1−4GlcNAc to give Le^x or sialyl $Lewis^x$, respectively. Identical key polar groups have been identified for FucT III, IV, and V, and all three enzymes display an absolute requirement for the C-6 hydroxyl of galactose in addition to the C-3 and C-4 hydroxyls of GlcNAc (*32*, *52*). FucT VI allows replacement or modification of the acetamido group of the GlcNAc moiety (*53*−*55*), and for example, compounds that contain a glucal or cyclohexanediol moiety instead of GlcNAc are appropriate acceptors (*56*). FucT III and FucT VI have been used to construct libraries of sialyl Le^a or sialyl Le^x derivatives using both unnatural donors and unnatural acceptors (*54*, *57*, *58*).

The above-described studies have shown that the C-6 and C-2′ hydroxyls of LacNAc are not essential for recognition by a range of fucosyl- and siayltransferases, and furthermore, in a low-energy conformation these functionalities are close in space. Thus, these groups were utilized to install tethers to modify conformational properties of LacNAc. Derivatives **4**, **5**, and **6** contain an alkyl tether between C-6 and C-2′ of differing length whereas compound **7** is modified by a methylene amide linker. The different linkers constrain these compounds in different conformations, and therefore, correlation of the conformational properties of these compounds with enzyme activities should establish in which way particular changes in conformational properties of an acceptor affect oligosaccharide biosynthesis. The apparent kinetic parameters of transfer to **4**, **5**, and **6** by α-1,3-fucosyltransferases IV and VI and α-2,6- and α-2,3-sialyltransferases show that, for each enzyme, compound **6** is markedly the best substrate. Each of these three compounds is modified by an alkyl tether between C-6 and C-2′; thus, the differences in catalytic activity should be mainly due to differences in conformational properties. Compound **6** is confined in a conformational space that corresponds to the relatively large low-energy plateau of LacNAc that includes syn conformations A and B. Thus, this implies that all four transferases recognize LacNAc in a low minimum energy conformation. A small change in conformational properties such as in compound **5** resulted in a significant loss of catalytic activity. Different enzymes responded, however, differently to this conformation change, and in particular the sialyltransferases endured a larger loss of catalytic activity. The fact that compound **4** was recognized by the two fucosyltransferases but not by the sialyltransferases further supports that the former enzymes allow larger conformational changes of the acceptor substrate. The implications of these findings are that only small changes in conformational properties, induced by, for example, protein or flanking saccharide residues, are required to affect dramatically the type of oligosaccharide that is biosynthesized.

The apparent kinetic parameters of conformationally constrained **6** and **7** provided further evidence that different enzymes respond differently to conformational changes in LacNAc. Compound **7** is contained in the B-conformer on the energy surface of LacNAc whereas the above-discussed **6** can adopt the A- and the B-conformer. When a transferase prefers the A-conformer, it is to be expected that only **6** gives favorable kinetic parameters. On the other hand, when the B-conformer is recognized, both compounds should display good activities. It is important to note that tethers of different chemical composition modify **6** and **7**, and this feature complicates the comparison of the two data sets. To address this problem, reference compound **2** was employed to account for alkylation of C-6 and C-2′ of **6**, and compound **3** was used to determine the effect of the C-6 amide and C-2′ methylation of **7**. In the case of the sialyltransferases, compounds **6** and **7** showed an improved catalytic efficiency when compared to the corresponding reference compounds, indicating that the B-conformer is preferred. The fucosyltransferases displayed a different profile, and in this case, only compound **6** was a markedly better substrate when **6** and **7** were compared to the reference compounds **2** and **3**, respectively. This finding indicates that the fucosyltransferases recognize LacNAc in the A-conformer. It is important to note, however, that a tether between C-6 and C-2′ changes the rotamer population of the C-6 hydroxyl. In the case of fucosyltransferase VI, functional groups at C-6 are able to make favorable interactions with the protein binding site. Thus, when for example the C-2′ methoxy and the C-6 formamide of **3** are linked to give the methylene amide tethered **7**, the orientation of the C-6 functional group changes, which may result in a reduction of favorable interactions leading to lowering of catalytic efficiency. Thus, differences in activity between **3** and **7** may in part be due to a combination of differences in conformational properties of the glycosidic linkages and the rotamer population of C-6.

Comparing the data of reference compounds **2** and **3** showed that different enzymes respond differently to chemi-

cal modifications at C-6 and C-2′. In the case of α-2,6-sialyltransferase, alkylation resulted in a 15-fold lower catalytic efficiency, and it appears that this effect is caused by loss of hydrogen-bonding potential. The latter is supported by the observation that compound **3**, which has a formamide at C-6 that has more possibilities for hydrogen bonding (*59*), is a significantly better substrate than **2**. Methylation had little impact on α-1,3-fucosyltransferase IV, whereas α-1,3-fucosyltransferase VI gained some activity. The incorporation of the C-6 formamide in **3** resulted in a significant gain in catalytic efficiency when α-1,3-fucosyltransferase VI was used as the transferase whereas this modification has little impact on α-1,3-fucosyltransferase IV. The C-6 and C-2′ hydroxyls are supposed to be on the periphery of the binding site of the transferases, and the observation that the four transferases respond very differently to chemical modifications indicates that their binding sites have different architectures.

Previous studies identified key polar functionalities of an oligosaccharide by selective methylation or deoxygenation of particular hydroxyls (*30*, *32*, *47*). These functionalities, which are important but not critical for recognition, are probably located at the periphery of the binding site and may form indirect interaction. Our results show that, by employing a wider range of functionalities, a more detailed picture of the architecture of a binding site may be obtained. Furthermore, our studies also reveal that chemical modification of these hydroxyls may lead to compounds that display exquisite selectivity for particular transferases and this feature may be important for the design of selective inhibitors for particular transferases.

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SUPPORTING INFORMATION AVAILABLE

Enzyme kinetic plots of the α-2,6-sialyltransferase, α-2,3-sialyltransferase, α-1,3-fucosyltransferase IV, and α-1,3-fucosyltransferase VI and nomenclature and detailed geometrical description of all optimized conformers of **1**, **4**, **5**, and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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