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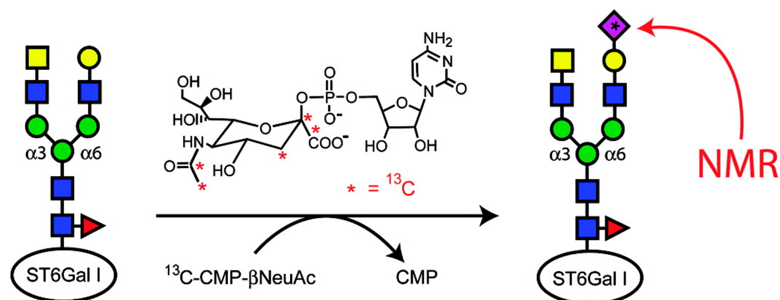

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^{13}C-Sialic Acid Labeling of Glycans on Glycoproteins Using ST6Gal-I

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Glycans that are either N-linked to asparagine or O-linked to serine or threonine are the hallmark of glycoproteins, a class of protein that dominates the mammalian proteome. These glycans ensure quality of protein folding, dictate transport to various regions of the cell, mediate interactions of the cell with its environment, regulate clearance of spent proteins from the system, and in some cases are required for proper protein function.[1] Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for studying glycan structure and interactions, particularly in a form that exploits heteronuclei such as ^{13}C. It has been used effectively to determine the structure of oligosaccharides, polysaccharides, and protein-bound oligosaccharides,[2] but studies of glycans attached to their native proteins by NMR are rare.[3] Here an approach to label glycans is presented that uses α-2,6-sialyltransferase (ST6Gal-I, EC 2.4.99.1) to add ^{13}C-*N*-acetylneuraminic acid (NeuAc or sialic acid) to glycoproteins after their preparation using nonbacterial hosts. ST6Gal-I is itself a glycoprotein, and in this initial application labeling and observation of its own glycans by NMR are illustrated.

ST6Gal-I is a member of the glycosyltransferase family 29 of the CAZY database (www.cazy.org).[4] Members of this family transfer NeuAc from the donor substrate, cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-β-NeuAc), to terminal galactose (Gal) or *N*-acetylgalactosamine (GalNAc) residues of glycans to create α-2,6, α-2,3, and α-2,8 linkages. ST6Gal-I from most species has two *N*-glycosylation sites, which are necessary for normal function.[5] For this reason, recombinant ST6Gal-I has been expressed primarily in cells that produce glycosylated forms.[5−7]

The isotopic labeling approach used here is illustrated in Figure 1. An NH_2-terminal His-tagged form of the catalytic domain from rat ST6Gal-I (residues 97−403, GenBank P13721)[8] was expressed in mammalian HEK293 cells as described previously, but without amino acid specific isotopic labeling.[6] Native NeuAc residues were then removed from the glycans of ST6Gal-I with a general neuraminidase (New England Biolabs, Inc.) capable of catalyzing the hydrolysis of α-2,3, α-2,6, and α-2,8 linked NeuAc residues (38 μg ST6Gal-I, 50 units neuraminidase, 24 h, 0.21 mL, 37 °C). Neuramindase was separated from the ST6Gal-I using a Concanavalin A sepharose affinity column (GE Healthcare, 1 mL), which has weak affinity for biantennary and truncated glycans like those of ST6Gal-I.[9] The column was washed with 3 mL of buffer (20 mM bis-tris, pH 6.5, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$) to remove the neuraminidase.[10] Desialylated ST6Gal-I was then eluted from the column with α-D-methylmannoside (Sigma-Aldrich, 15 mL, 0.2 M, buffer) and stored with 400 μM *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (Sigma-Aldrich), a neuraminidase inhibitor. Before the addition of ^{13}C-NeuAc, the desialylated ST6Gal-I was washed into NMR buffer (10 mM potassium phosphate, 200 mM NaCl, pH 6.5, 400 μM inhibitor, D_2O). ^{13}C-CMP-NeuAc (27 μg) was added to 176 μL of a 45 μM solution of the desialylated ST6Gal-I, and its addition to ST6Gal-I was monitored with ^{1}H NMR.

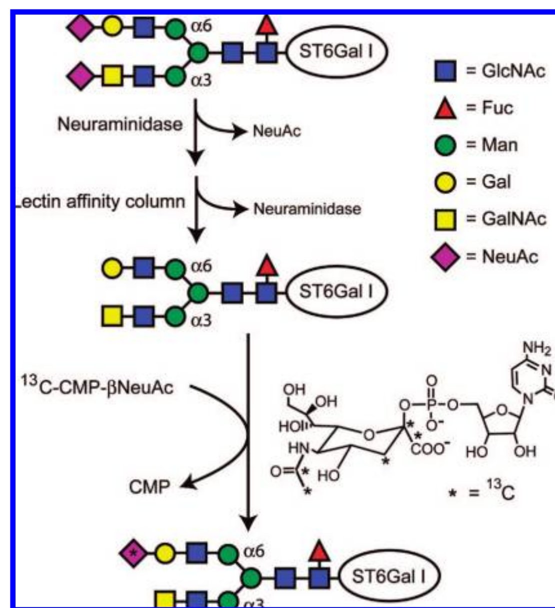

Figure 1. Isotopic labeling of ST6Gal-I glycans.

^{13}C-CMP-NeuAc was made in a four-step synthesis process following published procedures.[11] Briefly, *N*-hydroxysuccinimide and ^{13}C-acetyl chloride were combined to produce *N*-^{13}C-[1,2]-acetoxy-succinimide, which was further reacted with D-mannosamine to make *N*-^{13}C-[1,2]-acetyl-mannosamine (^{13}C-ManNAc). The enzyme, NeuAc aldolase, was used to react ^{13}C-ManNAc with ^{13}C-pyruvate to produce ^{13}C-[1,2,3,10,11]-NeuAc. ^{13}C-[1,2,3,10,11]-CMP-β-NeuAc was then synthesized using the enzyme CMP-NeuAc synthetase and cytidine-5′-triphosphophate to produce the final product. Details are provided in the Supporting Information.

The original glycans of ST6Gal-I as expressed in HEK293 cells were analyzed using PNGaseF digestion, permethylation, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), and exoglycosidase digestion. Two major glycoforms were identified as NeuAc-α-2-3/6GalNAc-β-1-4GlcNAc-β-1-2Man-α-1-3(NeuAc-α-2-3/6Gal-β-1-4GlcNAc-β-1-2Man-α-1-6)Man-β-1-4GlcNAc-β-1-4(Fuc-α-1-6)GlcNAc and a similar structure with a Gal instead of a GalNAc residue and without the fucose residue. The NeuAc-α-2-6GalNAc linkage of the major glycans is unusual but not unprecedented.[12] β-*N*-acetylhexosaminidase and α-1-2,3 mannosidase digestions, composition analysis, and MALDI-MS were used to confirm the GalNAc-β-1-4GlcNAc structure on the α3 arm of the glycan (details provided in the Supporting Information). After neuraminidase treatment, the glycan structures of ST6Gal-I should have multiple sites for NeuAc addition.

The addition of ^{13}C-NeuAc to desialylated ST6Gal-I was monitored with a standard 2D ^{1}H−^{13}C heteronuclear single quantum coherence (HSQC) experiment (gChsqc, Varian Biopack; see

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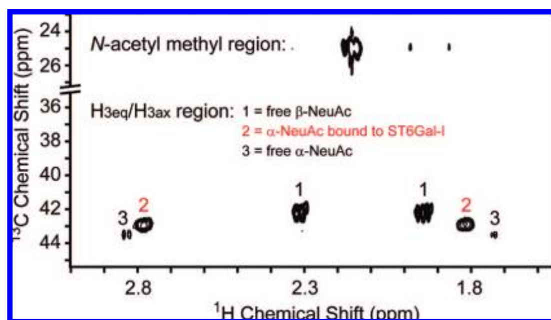

Figure 2. 2D $^1H-^{13}C$ decoupled HSQC of ST6Gal-I reaction mixture after the addition of CMP-^{13}C-β-NeuAc. A chemical shift table is included in the Supporting Information.

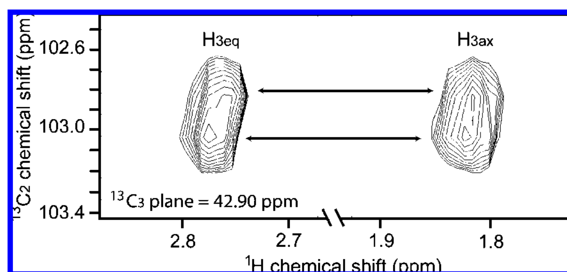

Figure 3. $^1H-^{13}C_2$ plane from a 3D $^1H-^{13}C_3-^{13}C_2$ correlation experiment. J_{C1C2} and J_{C2C3} were refocused during t_1 evolution with selective π pulses.

Figure 2). The $H_{3ax}-C_3$ and $H_{3eq}-C_3$ cross peaks of the NeuAc bound to ST6Gal-I (labeled 2 in the figure) are distinct due to the chemical shift change on production of an α linkage as well as the increase in line width. Peaks from free NeuAc in α and β anomeric configurations, produced by the action of some residual neuraminidase, are labeled 3 and 1, respectively. The bound cross peak volumes measured from Figure 2 were used to determine a minimum level of addition of ^{13}C-NeuAc to ST6Gal-I. The value determined (3 ^{13}C-NeuAc residues per molecule of ST6Gal-I) is reasonable, in view of the two glycosylation sites and the dominant glycan structures on ST6Gal-I, particularly if GalNAc terminated glycans can be substrates for ST6Gal-I.

By exploiting chemical shift differences at the multiple ^{13}C sites introduced in the NeuAc labels, it is possible to demonstrate the presence of chemically or structurally distinct glycan sites in ST6Gal-I. ^{13}C-NeuAc is ^{13}C-labeled at the methyl and carbonyl carbons of the *N*-acetyl group and the C_1, C_2, and C_3 carbons within the sugar ring. An 800 MHz Varian INOVA spectrometer was used to acquire several types of 2D and 3D $^1H-^{13}C$ correlation experiments. A $^1H-^{13}C$ HSQC experiment correlating the acetyl $^1H_{methyl}$ to the acetyl $^{13}C_{methyl}$ and 2D acetyl $^1H(^{13}C_{methyl})^{13}CO$ experiments did not reveal detectable chemical shift dispersion. Chemical shift dispersion due to the various ^{13}C-NeuAc adducts on ST6Gal-I was, however, observed in a 3D experiment correlating $^1H-^{13}C_3-^{13}C_2$ atoms of the sugar ring (shown in the $^1H-^{13}C_2$ plane of Figure 3). The C_3 chemical shift has no detectable difference, but the C_2 chemical shift is sensitive. At least two different ^{13}C-NeuAc environments are resolved in the 2D $^1H-^{13}C_2$ plane as indicated by the arrows connecting the H_{3ax} and H_{3eq} correlation peaks. C_2 is the anomeric linkage site for the α-2,6 glycosidic bond. The chemical shifts observed for C_2 are expected to be sensitive to structural and chemical differences in the linked residue and the surrounding environment. In particular, they may be sensitive to linkage to Gal vs GalNAc residues as occurs in the two branches of the major glycoform depicted in Figure 1. Assignment of the peaks is not straightforward, but it may be possible using differential isotopic labeling combined with mass spectrometry, as illustrated in our recent work on ^{13}C-methyl-lysine labeling.[13]

Additional chemical shift dispersion would be desirable. Neither the chemical shift of the $^{13}C_1$ of the carboxylate carbon nor the potential of 4D experiments that include this carbon has been exploited in the data presented. pK_a's of the carboxylate are expected to be sensitive to local environments, and the $^{13}C_1$ shifts would in turn be sensitive to differential protonation. Modern methods that allow efficient collection of multidimentional NMR including ^{13}C data may provide sufficient resolution to undertake site specific assignments and monitor site specific interactions.[14]

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Supporting Information Available: Complete ref 7a, details of the synthesis of ^{13}C-[1,2,3,10,11]-NeuAc, the analysis of glycans on ST6Gal-I, and chemical shifts of NeuAc compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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