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Binding Epitopes of Gangliosides to their Neuronal Receptor, Myelin-Associated Glycoprotein, from Saturation Transfer Difference NMR

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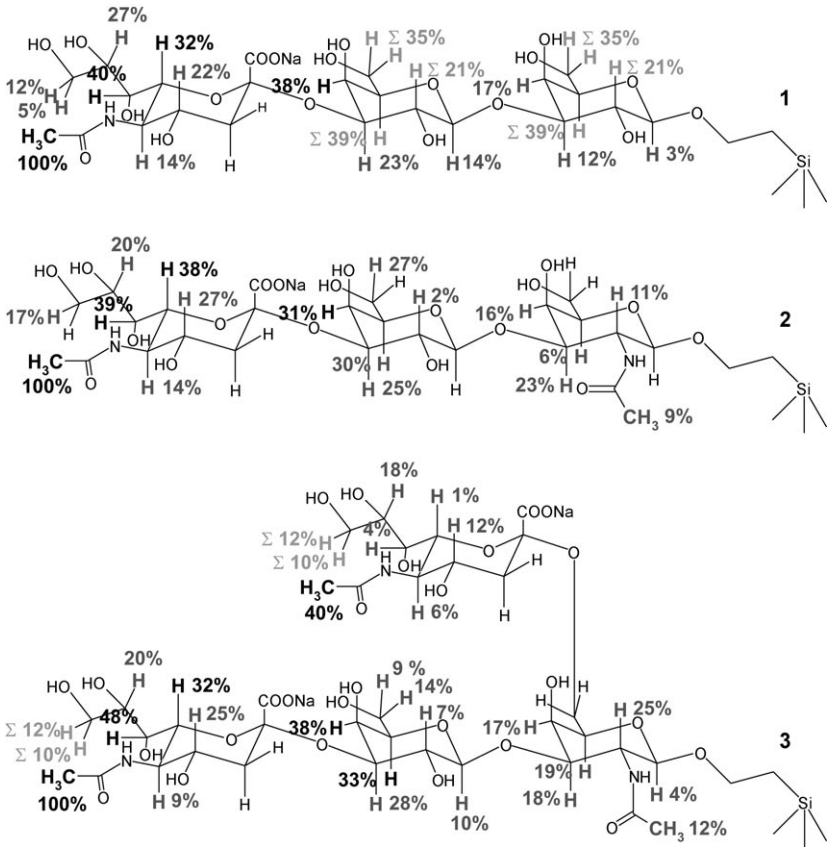
The myelin-associated glycoprotein (MAG) inhibits neurite outgrowth in the central nervous system.[1–11] Certain gangliosides can block this action of MAG and can thus stimulate the regrowth of severed neurons. In this study we analyzed the binding epitopes of fragments of the ligand with the highest binding affinity to MAG, that is, the ganglioside GQ1bα.[12] Specifically, we studied the binding of three trisaccharides and one tetrasaccharide to the MAG chimera $FcMAG_{d1\rightarrow3}$[8] using saturation transfer difference (STD) NMR.[13–15] Trisaccharide Siaα-(2→3)Galβ(1→3)Gal*N*Ac (**2**) and the tetrasaccharide, Siaα(2→3)Galβ(1→3)[Siaα(2→6)]Gal*N*Ac (**3**), are fragments of GQ1bα, whereas the trisaccharide Siaα(2→3)Galβ(1→3)Gal (**1**) is a close mimetic.[16] For reference purposes, the commercially available sialyllactose Siaα(2→3)Galβ(1→4)Glc was also included in the study to obtain its K_D value. Understanding the detailed binding mechanisms is essential for developing efficient inhibitors of this interaction.



Scheme 1. Binding epitopes of the three ligands obtained from the integration of signals in the STD HSQC. The strongest signal (*N*-acetyl group, left) was scaled to 100%, and all other signals were related to it. The signal intensity was divided in three categories: black for 31–100% and mid gray for 1→30%, while overlapping signals are coded in light gray and designated by Σ, meaning that analysis was only possible for the sum of the signals. The absolute STD values of the *N*-acetyl groups were 22% for **1**, 24% for **2** and 16% for **3**.

We determined the binding epitopes with (^{13}C,^{1}H) HSQC STD NMR spectra and 1D STD spectra whenever the HSQC data were ambiguous (see the Supporting Information). The strongest STD effect was on the *N*-acetyl group at position 5 of the 2→3-linked sialic acid for trisaccharides **1** and **2** (Scheme 1). Therefore, the STD effect of this group was set to 100% in each spectrum. This can be explained by a hydrophobic interaction with the protein. Also, the glycerol side chain of the 2→3-linked sialic acid showed high STD effects, thus indicating that the region of H-6 to H-8 is involved in binding. The patch from H-4 to H-6 of the central galactosyl residue also shows strong STD contacts probably originating from hydrophobic interactions. The carboxy function of the α(2→3)-linked sialic acid is believed to interact with Arg118, and the proximity of this part of the oligosaccharide to the carboxy group makes an interaction likely. The low STD values for the protons of the first galactose or galactosamine residues indicate that no significant contribution to binding originates from these residues.

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The acetyl group of the galactosamine in **2** was obtained from the 1D STD spectrum and showed a drastically smaller STD effect of 9% (Supporting Information). Compounds **1** and **2** show an almost identical binding epitope; this suggests that the *N*-acetyl group in the 2 position of the galactose residue does not significantly contribute to binding. Due to the almost identical STD effects of **1** and **2**, it can be concluded that both trisaccharides bind in the same mode and orientation.

The tetrasaccharide **3** carries an additional sialic acid at position 6 of the GalNAc residue of **2** and represents the nonreducing terminus of GQ1bα. This tetrasaccharide shows a considerably stronger binding affinity than trisaccharides **1** and **2** (see below). Therefore, we wanted to understand the effect of the extra sialic acid. The largest STD response is still observed at the *N*-acetyl function of the α(2→3)-linked sialic acid. The binding epitope of the tetrasaccharide is similar to that of the trisaccharides. Differences are found for the α(2→3)-linked sialic acid, in which the STD value of H-5 is decreased and that of H-7 is increased compared to in the trisaccharides. This indicates that a slight rotation of the sialic acid is moving the glycerol side chain closer to the protein. This assumption is supported by the changes of the STD values in the H-6 protons of the galactose. H-6a does not show any STD response in the trisaccharide **2** and carries a STD value of 14% in **3**. In contrast, H-6b has an STD of 27% in **2** that is reduced in **3** to 9%.

The reducing galactosamine residue of **3** has only low STD effects, thus indicating that no close contacts to the protein are present. Compared to **2** it shows increased STD values for H-2, H-5 and the *N*-acetyl group; this also supports the notion that the additional sialic acid in position 6 stabilizes and slightly rotates the oligosaccharide relative to the protein.

The additional sialic acid shows only one sizable STD effect of 40% on its *N*-acetyl group. A direct interaction of the carboxy group with the protein is likely but cannot directly be detected by STD spectroscopy because of the lack of protons. Mutational analysis in the lab of S.K. has shown that Lys67 of MAG is probably also important for the binding of the tetrasaccharide, which in turn supports the hypothesis of a second ionic contact between the protein and the oligosaccharide. In summary, it seems likely that the tetrasaccharide is oriented in a similar way to the trisaccharides with a slight rotation of the trisaccharide backbone towards the protein.

The K_D values were determined by titration of a ligand solution to a protein sample and repeated acquisition of STD experiments (see the Supporting Information). The K_D values of trisaccharides **1** and **2** were ~400 and ~800 μM, respectively (see Figure 1). Thus, the *N*-acetyl group in position 2 of the reducing galactose reduces the binding affinity by a factor of two. The additional sialic acid in position 6 of the Gal*N*Ac residue enhances the binding affinity fourfold resulting in a K_D of 180 μM. Sialyllactose was used as a reference, and its K_D value was found to be better than the dissociation constant of the "native" trisaccharide with the correct linkage and the *N*-acetyl group. Its K_D of 650 μM was between those of the two other trisaccharides (Figure 2).

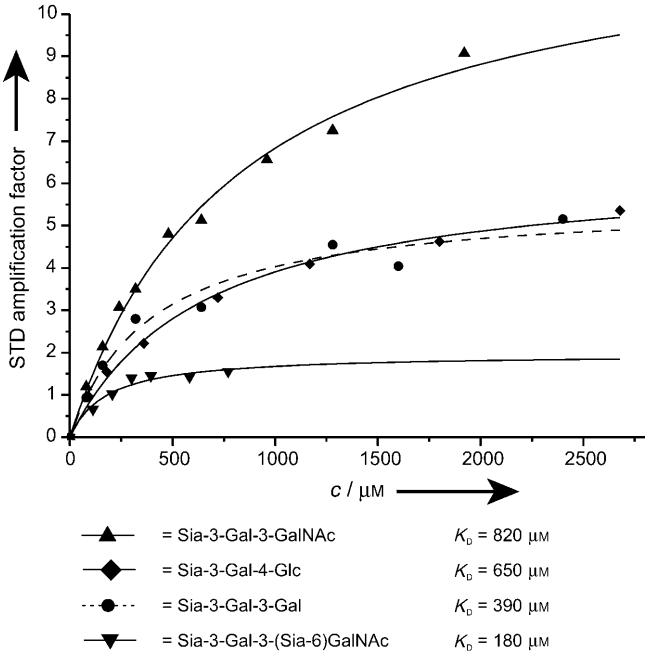

Figure 1. Titration data of the four oligosaccharides analyzed for binding constant with FcMAGd$_{1\rightarrow3}$. The binding constants for the trisaccharides are in the range 390 to 820 μM. The addition of the sialic acid at position 6 of the Gal*N*Ac residue leads to a fourfold improvement in the binding constant to 180 μM. For the analysis of the binding constant, the *N*-acetyl signal of the α(2→3)-linked neuraminic acid was used in all cases.

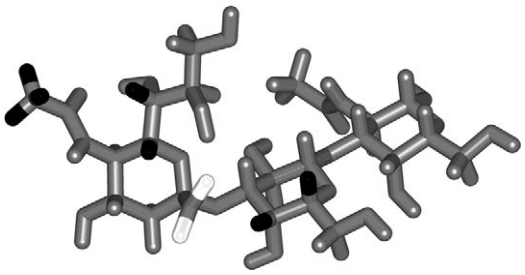

Figure 2. Binding epitope of trisaccharide ligand **2** shown in the conformation obtained by Bhunia et al.[16] The strongest signal (*N*-acetyl group) was scaled to 100% and all other signals were related to it. Only the signals with the closest proximity to the protein are highlighted here. The protons shown in black carry the dominant STD effects of 30–100%. Additionally, the carboxy group of the sialic acid residue, which was shown by other methods to participate in binding, is shown in white.

Conclusions

One of the major problems in the analysis of its binding mode is the lack of a three dimensional structure of MAG. It has been shown that Arg118[10] and Lys67 are essential for binding. Based on trNOE data and homology modeling, in the accompanying paper Ernst et al. have devised a binding mode for **3** to MAG that is in agreement with the STD NMR data presented here.[16] Combination of these NMR-based methods and modeling shows the clear advantage of arriving at validated binding modes. The relative orientation of the pharmacophoric groups of these natural ligands will help to validate homology models of MAG and will lead to new, rationally designed inhibitors.

The design and development of new ligands based on this information is under way. These new compounds might show a way to assist in the regeneration of severed nerves.

Experimental Section

A MAG chimera construct comprising the Fc unit of an immunoglobulin and the first three domains of MAG, $FcMAGd_{1-3}$, was employed.[4] The trisaccharides were synthesized through an enzymatic sialylation of chemically synthesized disaccharide acceptors with sialyltransferase ST3Gal III,[5,6] while the tetrasaccharide was obtained by a full chemical approach.[16]

NMR experiments: All titration experiments were performed on a Bruker Avance DRX 500 MHz spectrometer equipped with a 5 mm inverse triple-resonance probe head at 285 K. STD HSQC experiments were performed on a Bruker Avance DRX 700 MHz spectrometer with a 5 mm Cryoprobe at 285 K. NMR samples were prepared in D_2O (600 μL, 99.9%) buffer containing NaCl (140 mM), phosphate buffer (10 mM), KCl (3 mM), and NaN_3 (6 mM) at pH 7 (not corrected for D_2O). Protein concentrations in the NMR samples were in the range 8 to 14.9 μM. Saccharides were added to the protein NMR sample from a concentrated stock solution (10 mg mL^{-1}); dilution effects due to small volumes were neglected. Titration was performed up to a 150-fold excess of ligand over protein. For all 1D spectra, water suppression was achieved by the WATERGATE sequence with d19 = 200 μs.

For STD spectra, the on-resonance irradiation of the protein was performed for titration experiments at a chemical shift of −2 ppm and for STD HSQCs at +7 ppm. Off-resonance irradiation was applied at +40 ppm. Selective saturation of the protein was achieved by a train of Gauss-shaped pulses of 50 ms length each, separated by a delay of 1 ms. We used 40 pulses leading to an overall saturation time of 2.04 s. An additional relaxation delay of 1 s was used. The power of the $\gamma B_1/2\pi$ varied between 125 to 138 Hz. All 1D STD spectra were recorded with a 10 ms spin lock pulse to eliminate the background protein resonances.

If integration of individual signals was not possible in the HSQC due to overlap, we first determined the sum of the integrals for the overlapped signals. Second we determined the intensities of the peaks in question and calculated their contribution to the integral. For example, the signals for the C-4, C-5, C-7 and C-8 of both sialic acids of the tetrasaccharide (**3**) are overlapped in the STD HSQC spectrum and were thus determined with the intensity method (Figure 3). If this method could not be applied, usually due to severe overlap, we provided the sum of integrals of the overlapped signals. For example, the C-2, C-5 and C-6 of Siaα(2→3)Galβ(1→3)Gal (**1**) were so strongly overlapped that it was not possible to assign them unambiguously. In these cases, we provide the sum for C-2, C-5 and C-6.

The STD HSQC spectra allowed the analysis of these complex saccharides with few limitations. The intensity of the signal depends on the $^1J_{C,H}$ coupling constant, which was set to a typical value of 145 Hz. Thus the signals for the H-3a and H-3e ($^1J_{C,H} \approx 128$ Hz) of the sialic acids and the anomeric protons of the galactoses ($^1J_{C,H} \approx 170$ Hz) showed very low intensity or could not be observed in the STD HSQC. A comparison with the 1D STD spectrum provided additional STD intensities on those signals (Table S1). The resonances of these protons were not or only slightly overlapped. Thus, it was possible to determine the relative STD values. The absolute STD values were up to 23%.

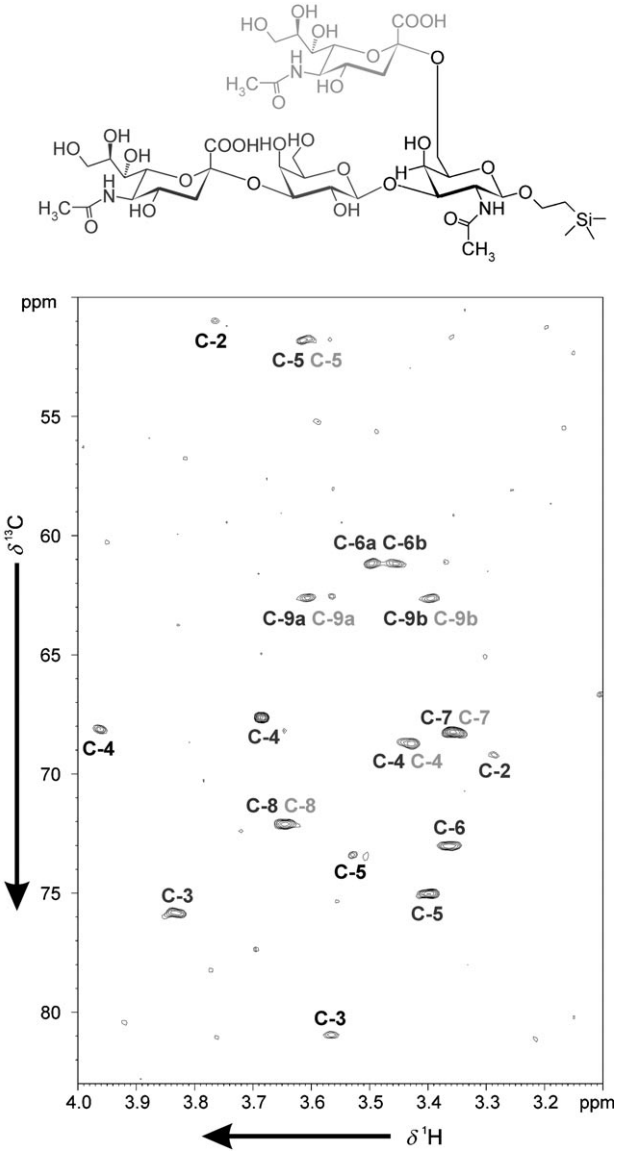

Figure 3. A section of the STD HSQC of the tetrasaccharide Siaα(2→3)Galβ-(1→3)[Siaα(2-6)]Gal*N*Ac (**3**) with $FcMAGd_{1-3}$ acquired on a 700 MHz spectrometer with a cryoprobe. Number of scans: 112 with 256 increments for both the on- and off-resonance spectra. The intensities of the signals provide highly resolved information on the binding epitope. Signals that are overlapped in the 1D spectra can largely be resolved by using the STD HSQC spectra; the exceptions are C-4, C-5, C-7, C-8 and C-9 of the two sialic acids.

K_D values were obtained by titrating stock solutions of the oligosaccharides to solutions of $FcMAGd_{1-3}$ and the acquisition of STD spectra at each concentration. K_D values was obtained from the analysis of the *N*-acetyl signal of the α(2→3)-bound sialic acid. We acquired STD spectra and reference spectra and determined the STD amplification factor,[14] which in turn was plotted against the concentration. The titration was continued as long as the STD amplification factor increased with increasing concentration of the ligand. In case of the trisaccharides a 150-fold ligand excess and for the tetrasaccharide at a 40-fold excess was used.

For negative controls, we acquired STD spectra of the ligands without protein. Small artifacts in these spectra arise due to aggregation and similar effects. These artifacts were usually lower than

0.3%, while STD spectra with protein at highest ligand concentration usually gave absolute STD effects between 1 to 9%. Thus, the artifacts were negligible.

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Keywords: axonal regeneration · carbohydrates · epitopes · myelin-associated glycoprotein · saturation transfer difference NMR

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