CHEMBIOCHEM

Supporting Information

CHEMBIOCHEM

Supporting Information

for

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

So-Young Shin, Heiko Gäthje, Oliver Schwardt, Gan-Pan Gao, Beat Ernst, Soerge Kelm, and Bernd Meyer*

Saturation Transfer Difference NMR (STD NMR)

Analysis of receptor ligand inter-actions is possible by Saturation Transfer Difference NMR (STD NMR) [1-3]. The protein is selectively saturated and via spin diffusion the saturation is spread over the entire protein and also transferred to binding ligands. The efficiency of the saturation transfer depends on the proximity of the ligand hydrogen atoms to the protein. Therefore, resonances of ligands interacting with the protein appear with reduced intensity in the spectrum (c.f. Figure S1). Subtraction from a reference spectrum, where irradi-ation occurs outside the spectral window of the protein yields the STD spectrum, showing only ligand signals. Thus it is possible to use STD to screen for ligands. Likewise it allows the determination of the K_D value by a plot of the STD amplification factor against the concentration [2]. STD NMR can also be used to map the binding epitope of the ligand because large STD values indicate a close contact between ligand atom and protein.

Materials and Methods

Protein: Transmembrane proteins cannot be handled easily in aqueous solutions because they are prone to aggregation. Therefore, it is more convenient to use a solu-

ble chimeric MAG system. In this case, a dimeric MAG construct comprising the Fc-unit of an immunoglobulin and the first three domains of MAG, FcMAGd_{1-3} was employed [4].

Saccharides: The trisaccharides were synthesized through an enzymatic sialidation of chemically synthesized disaccharide acceptors with sialyltransferase ST3Gal III [5, 6] whereas the tetrasaccharide was obtained by a full chemical approach[7].

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 ^{1} experiments were performed on a Bruker Avance DRX 700 MHz spectrometer with a 5 mm Cryoprobe^{TM} at 285 K. NMR samples were prepared in 600 µL D_2O (99.9%) buffer containing 140 mM NaCl, 10 mM phosphate buffer, 3 mM KCl and 6 mM NaN_3 at pH 7 (not corrected for D_2O). Protein concentrations in the NMR samples were in a range between 8 and 14.9 µM. Saccharides were added to the protein NMR sample from a concentrated stock solution (10 mg/mL) neglecting dilution effects due to small amounts. 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. Since the spin lock reduces STD signal intensity the reference spectra were also recorded with a spin lock pulse. The total number of scans for 1D STD NMR spectra was between 2048 and 8192 with 16 dummy scans and typically 12 ppm sweep width. The spectra were multiplied by an exponential line broadening function of 1-3 Hz prior to Fourier transformation. For STD HSQCs 96 or 112 scans per increment with a total of 512 increments for the tetrasaccharide and 208 scans per increment with 256 increments for the trisaccharides were acquired.

Increments were collected in an interleaved mode for on- and off resonance spectra. Both spectra were processed and phased identically and then subtracted. All STD HSQC spectra were multiplied by an exponential line broadening function of 1-5 Hz in F2 and 35 or 70 Hz in F1 and zero-filled two times. Spectral width was 6 ppm in F2 and 100 ppm in F1. All spectra were processed on Silicon Graphics octane workstations with Bruker Topspin 1.3 software.

Binding epitope: The carbohydrate signals of the ligands cluster in the region between 3.2 and 4.0 ppm in the proton spectrum. This leads to a severe overlap of signals. Therefore, it is very difficult to fully interpret these 1D STD spectra. To overcome this problem we used STD HSQC spectra that use the carbon chemical shifts to achieve a higher resolution of the proton signals (c.f. Figure 4, main paper) [1, 8]. Therefore we were able to analyze most of the signals by integration with the program Topspin. The strongest STD signals were assigned a value of 100%, respectively. The binding epitope was usually determined with a sample with a high ligand excess to diminish the possibility of rebinding and thus to have the highest possible discrimination within the ligands and to gain a better signal to noise ratio.

If integration of individual signals was not possible 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 C4, C5, C7 and C8 of both sialic acids of the tetrasaccharide (**3**) are overlapped in the STD HSQC spectrum and were thus determined with the intensity method. If this method could not be applied usually due to severe overlap we provided the sum of integrals of the overlapped signals. E.g. the C2, C5 and C6 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 provided the sum for C2, C5 and C6 respectively. A further complication of the HSQC spectra was the low signal to noise ratio which was usually around 3 after three days of experiment on a Bruker 700 MHz NMR spectrometer equipped with Cryoprobe™.

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 ($^1J_{C,H} \approx 170$

[1] Heteronuclear Single Quantum Coherence

Hz) showed very low intensity or were not observable in the STD HSQC. A comparison with the 1D STD spectra provided additional STD intensities on those signals (c.f. Table 1). 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%.

STD HSQC spectra give a better resolution of the signals due to the carbon domain but the spectral resolution in the proton domain is lower than in a 1D STD spectrum. Therefore we used the 1D STD spectra whenever the HSQC data were ambiguous, as in the case of the *N*-acetyl group of the galactosamine in 2 (c.f. main text). This signal overlapped with the signal of the *N*-acetyl group of the sialic acid so strongly that it was not possible to integrate the two signals separately in the HSQC spectrum (c.f. Table 1).

The spacer at the reducing end of the terminal galactose or galactosamine in the analyzed oligosaccharides shows some STD effect but these effects are entirely based on artefacts arising from an aggregation of these hydrophobic chains. This becomes clear when the spectra of the ligands without protein in buffer solution is analyzed. Here, the spacer also shows STD signals of the same intensity.

Dissociation constants: The K_D values were obtained by titrating stock solutions of the oligosaccharides to solutions of FcMAGd$_{1\text{-}3}$ and the acquisition of STD spectra at each concentration (c.f. Figure S2). The K_D value was obtained from the analysis of the *N*-acetyl signal of the α(2-3)-bound sialic acid. This signal was chosen, because it exhibited the largest STD effect in all analyzed molecules and thus represents the part of the carbohydrate interacting most intimately with MAG. We acquired STD spectra and reference spectra and determined the STD amplification factor [2], 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.

In 1D STD NMR spectra absolute STD effects were determined by measurement of the scaling factor between STD and reference spectrum. Multiplication with the ligand excess gave the STD amplification factor which in turn was plotted against the concentration. A fit was calculated with the program origin to gain the K_D values.

Negative controls: For negative controls we acquired STD spectra of the ligands without protein. Small artefacts in these spectra arise due to aggregation and similar effects. These artefacts 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 artefacts were negligible.

Table S1: STD percent values from 1D STD spectra (relative to the N-acetate signal of the 2-3 linked sialic acid).

	Protons	Sia	Gal	Gal GalNAc	2,6 Sia
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-1	- - -	39 49 55	20 32 44	- - -
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-3a	23 27 36	- - -	- - -	- - 17
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-3e	25 39 38	- - -	- - -	- - 20
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-4	- - -	47 64 68	29 39 47	- - -
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	NHCOC***H₃***	100 100 100	- - -	- 9 12	- - 39

Table S2: ^{1}H and ^{13}C NMR chemical shifts of ligands.

		2-3-Sia	Gal	GalNAc Gal	2-6-Sia
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-1		4.415 4.252 4.236	4.206 4.273 4.258	
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-2		3.370 3.274 3.268	3.365 3.756 3.753	
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-3a	1.530 1.534 1.531	3.852 3.817 3.812	3.546 3.569 3.545	 1.416
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-3e	2.483 2.487 2.480			 2.454
Sia-Gal-Gal	H-4	3.396	3.680	3.937	

Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc		3.410 3.405	3.673 3.663	3.923 3.938	 3.398
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-5	3.584 3.594 3.589	3.419* 3.381 3.379	3.433* 3.432 3.507	 3.569
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-6a	3.344 3.346 3.340	3.451 3.500 3.476	3.451 3.479 3.688	 3.453
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-6b		3.451 3.448 3.426	3.451 3.479 3.347	
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-7	3.322 3.345 3.335			 3.315
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-8	3.609 3.624 3.645			 3.614
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-9a	3.594 3.597 3.622*			 3.589*
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	H-9b	3.353 3.409 3.375			 3.375
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	NHCOC***H*** ***3***	1.797 1.767 1.763		 1.755 1.742	 1.767
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-1	174.023 173.522 173.473	104.213 104.146 104.076	101.703 100 100	 172.919
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-2	99.757 99.103 99.039	69.710 68.418 68.742	69.710 50.377 50.194	 99.793
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-3	 39.653 38.671	75.575 74.975 74.867	82.712 80.020 79.912	 39.577
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-4	68.614 67.983 67.897	67.403 66.706 66.731	68.559 67.356 67.069	 67.577
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-5	51.663 51.051 50.946	74.880 74.130 74.026	74.800 74.124 72.394	 50.946
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-6	72.937 72.075 72.081	60.976 60.159 60.257	60.976 59.831 62.722	 71.958
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-7	68.049 67.343 67.244			 67.455
Sia-Gal-Gal Sia-Gal-GalNAc	C-8	72.040			

Sia-Gal-(Sia)GalNAc		71.319 71.107			71.107
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	C-9	62.508 61.754 61.867			61.660
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	NHCO***C***H$_3$	21.446			21.633
Sia-Gal-Gal Sia-Gal-GalNAc Sia-Gal-(Sia)GalNAc	NH***C***OCH$_3$	174.381 174.327		174.170 174.023	174.327

* pairwise assignment uncertain

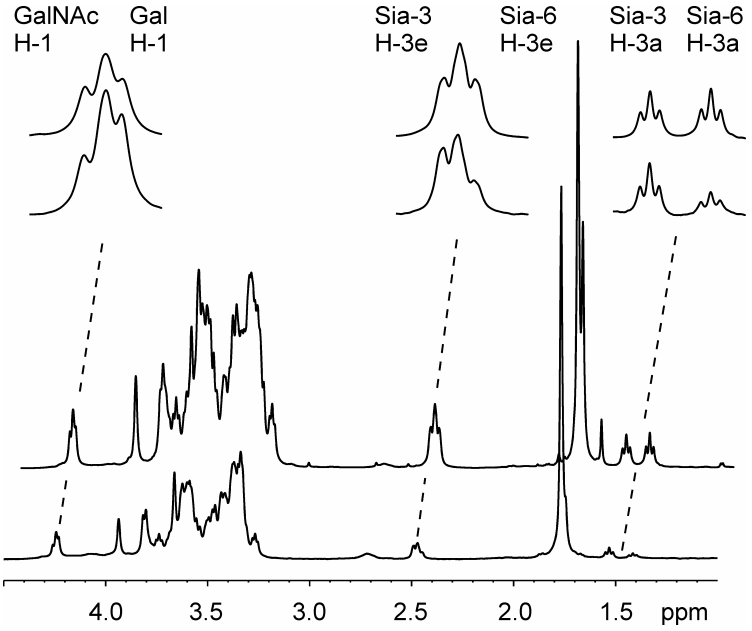

Figure S1: STD spectrum (bottom) and reference spectrum (top) of the tetrasaccharide: Siaα(2-3)Galβ(1-3)[Siaα(2-6)]Gal*N*Ac (**3**). Both spectra were acquired with 41-fold excess of ligand over protein with a 14.9 µM FcMAGd_{1-3} solution. The STD spectrum was scaled by a factor of 5 to the height of the reference spectrum. The zoomed sections are scaled by a factor of 13. The different intensities in the STD spectrum when compared to the reference spectrum are visible. The *N*-acetyl signal is the strongest in the STD spectrum. One can clearly identify from the intensities of the H3 signals that the α(2-3)-linked sialic acid has a much closer proximity to the protein. Also the *N*-acetyl signals show a marked shift in relative intensities when going from the normal spectrum to the STD spectrum.

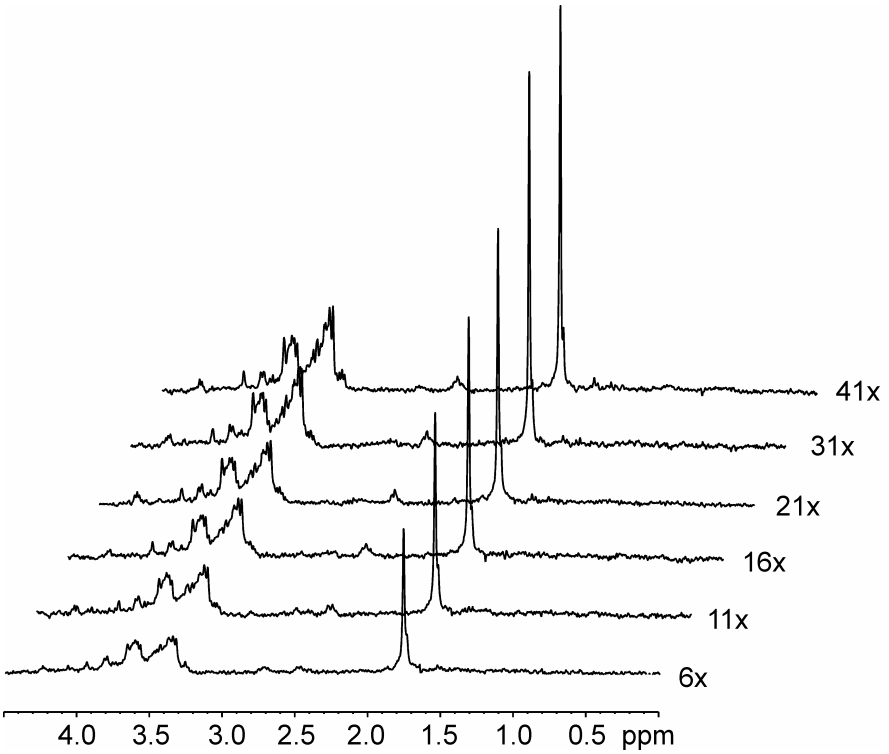

Figure S2: STD spectra of the MAG / tetrasaccharide Siaα(2-3)Galβ(1-3)[Siaα(2-6)]Gal*N*Ac (**3**) interaction with with different concentrations of the tetrasaccharide. The number next to the spectra indicate the ligand excess over $FcMAGd_{1\text{-}3}$ (7.4 μM). These data are used for the calculation of the binding constant (K_D).

References

[1] M. Mayer, B. Meyer, *Angew. Chem. Int. Ed.* **1999**, *38*, 1784.

[2] M. Mayer, B. Meyer, *J Am Chem Soc* **2001**, *123*, 6108.

[3] B. Meyer, T. Peters, *Angew Chem Int Ed Engl* **2003**, *42*, 864.

[4] S. Kelm, A. Pelz, R. Schauer, M. T. Filbin, S. Tang, M. E. de Bellard, R. L. Schnaar, J. A. Mahoney, A. Hartnell, P. Bradfield, a. l. et, *Curr Biol* **1994**, *4*, 965.

[5] G. Gao, O. Schwardt, T. Visekruna, S. Rabbani, B. Ernst, *Chimia* **2004**, *58*, 215.

[6] O. Schwardt, G. Gao, T. Visekruna, S. Rabbani, E. Gassmann, B. Ernst, *J Carbohydr Chem* **2004**, *23*, 1.

[7] O. Schwardt, G. Gao, S. Kelm, H. Gäthje, B. Meyer, S. Shin, T. Peters, A. Bhunia, B. Ernst, Synthetic Sialic Acid Containing Oligosaccharides to Explore the Binding Site of the Myelin-associated Glycoprotein (MAG), in preparation

[8] M. Vogtherr, T. Peters, *J Am Chem Soc* **2000**, *122*, 6093.