Group Epitope Mapping by Saturation Transfer Difference NMR To Identify Segments of a Ligand in Direct Contact with a Protein Receptor

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Received January 2, 2001. Revised Manuscript Received March 13, 2001

Abstract: A protocol based on saturation transfer difference (STD) NMR spectra was developed to characterize the binding interactions at an atom level, termed group epitope mapping (GEM). As an example we chose the well-studied system of galactose binding to the 120-kDa lectin *Ricinus communis* agglutinin I (RCA_{120}). As ligands we used methyl β-D-galactoside and a biantennary decasaccharide. Analysis of the saturation transfer effects of methyl β-D-galactoside showed that the H2, H3, and H4 protons are saturated to the highest degree, giving evidence of their close proximity to protons of the RCA_{120} lectin. The direct interaction of the lectin with this region of the galactose is in excellent agreement with results obtained from the analysis of the binding specificities of many chemically modified galactose derivatives (Bhattacharyya, L.; Brewer, C. F. *Eur. J. Biochem.* **1988**, *176*, 207−212). This new NMR technique can identify the binding epitope of even complex ligands very quickly, which is a great improvement over time-consuming chemical modifications. Efficient GEM benefits from a relatively high off rate of the ligand and a large excess of the ligand over the receptor. Even for a ligand like the biantennary decasaccharide with micromolar binding affinity, the binding epitopes could easily be mapped to the terminal β-D-Gal-(1−4)-β-D-GlcNAc (β-D-GlcNAc = *N*-acetyl-D-glucosamine) residues located at the nonreducing end of the two carbohydrate chains. The binding contribution of the terminal galactose residue is stronger than those of the penultimate GlcNAc residues. We could show that the GlcNAc residues bind "edge-on" with the region from H2 to H4, making contact with the protein. Analysis of STD NMR experiments performed under competitive conditions proved that the two saccharides studied bind at the same receptor site, thereby ruling out unspecific binding.

Drug−receptor interactions, signal transduction processes, and cellular recognition are only a few examples of binding processes where a profound understanding is indispensable. Hence, there is a large demand for new methods to characterize the nature of these binding interactions. Most studies dealing with the specificity of interactions rely on individually testing large amounts of ligands with different properties. Specific characterization of which parts of a ligand are in direct contact to a protein is currently left largely to X-ray analyses of cocrystallized ligand−receptor complexes.

NMR spectroscopy has been utilized to detect binding of low-molecular-weight compounds to large biomolecules for almost 30 years.[1,2] When choosing a NMR-based method to study binding, one wishes to gather information not available by other techniques, since the raw speed of high-throughput screening (HTS) robots is faster. However, in particular the past 5 years have produced many new NMR-based screening methods to characterize binding processes, e.g., transferred nuclear Overhauser enhancement (trNOE) spectroscopy, by which details on the three-dimensional structure of the ligand in the bound state can be obtained,[3−5] or structure−activity relationship (SAR) by NMR, which provides information about the binding site of the protein.[6]

Certain situations do not allow the analysis of single compounds, such as when studying substances extracted from natural sources or from a combinatorial library. Our recently presented saturation transfer difference (STD) NMR method offers several advantages over normal methods to detect binding activity (Scheme 1). First, the binding component can usually be directly identified, even from a substance mixture, allowing it to be utilized in screening for ligands with dissociation constants K_D ranging from ca. 10^{-3}−10^{-8} M. Second, the building block of the ligand having the strongest contact to the protein shows the most intense NMR signals, enabling the mapping of the ligand's binding epitope. And third, very important for a NMR-based detection system, its high sensitivity allows using as little as 1 nmol of protein with a molecular weight >10 kDa.[7−11] One should also point out that this method is not restricted to the detection of carbohydrate ligands. Our

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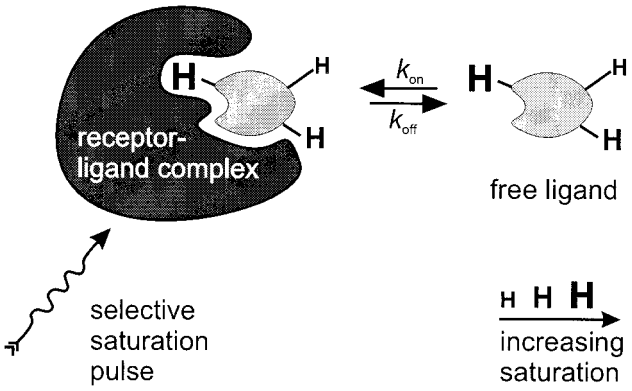
10.1021/ja0100120 CCC: $20.00
Published on Web 06/02/2001

Scheme 1. Illustration of Group Epitope Mapping (GEM) for a Ligand in Fast Exchange between the Bound State and the Free State[a]



[a] Irradiation of the protein at a resonance where no ligand signals are present leads to a selective and very efficient saturation of the entire protein by spin diffusion. Saturation is transferred to the binding parts of the ligand by intermolecular saturation transfer. Here, groups represented by the large proton are in close contact with the protein, while the medium-sized proton symbolizes a group with less interaction. The smallest proton represents a group with almost no contact with the protein, thus receiving minimal saturation. Therefore, the degree of saturation of the individual protons of a small ligand molecule reflects the proximity of these to the protein surface.

group was able to demonstrate that peptides, glycopeptides, and typical representatives of drug candidates such as aromatic and heterocyclic compounds can also be easily detected.[12,13]

Here we show the use of STD NMR to obtain information about the binding specificity at an atom level. As an example we use *Ricinus communis* agglutinin, which is a 120-kDa tetramer consisting of two As−sB dimers attached to each other *non*covalently. The B-chain contains the lectin domain with binding affinity for terminal galactoses. The utility of lectins is closely related to their specificity toward a given saccharide, i.e., characterization of oligosaccharides, or isolation of glycoproteins and oligosaccharides by affinity chromatography. With the RCA_{120} lectin, a variety of methods such as hemagglutination inhibition assays,[14,15] affinity chromatography,[16,17] equilibrium dialysis, microcalorimetry,[18] or, in the past few years, surface plasmon resonance[19,20] have been used.

Scheme 1 shows the different steps involved in the STD NMR experiment. STD NMR relies on the transfer of saturation from the protein to the ligand. For this to work, the protein must be selectively saturated. This is possible because protein resonances have significant intensity even in the negative ppm region or in the downfield region above 10 ppm—outside of the spectral window of low-molecular-weight substances. Therefore, even if one wishes to study peptides, there are no difficulties in selectively saturating the protein in the negative ppm region. Very efficient intramolecular transfer of magnetization within the protein (so-called spin diffusion) then leads to fast saturation of the entire protein and not only of the few directly saturated resonances.[21]

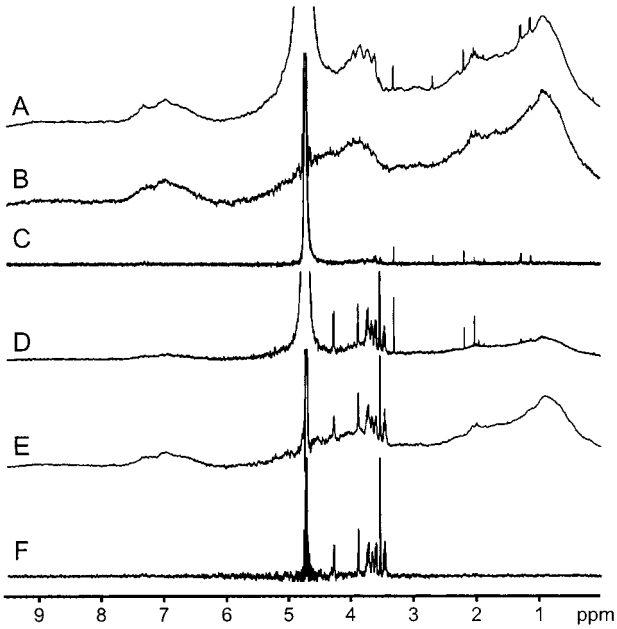


Figure 1. (A) Reference 1D NMR spectrum of the 120-kDa lectin RCA_{120} (50 μM in binding sites), displaying the very broad lines normal for a protein this size. The few sharp resonances arise from low-molecular-weight impurities. (B) Corresponding STD NMR spectrum showing that, by irradiating at −2 ppm, the entire protein is saturated uniformly and can therefore be efficiently used for the STD NMR technique. One can also see that the impurities contained in the spectrum are effectively subtracted and therefore do not give rise to signals in the difference spectrum. (C) 1D NMR spectrum recorded with a $T_{1\rho}$ filter, consisting of a 30-ms spin-lock pulse, to eliminate the broad resonances of the protein. Only those resonances of the low-molecular-weight impurities remain in the spectrum. (D) Reference 1D NMR spectrum of RCA_{120} (40 μM in binding sites) in the presence of 1.2 mM β-GalOMe, without the $T_{1\rho}$ filter. (E) Corresponding STD NMR spectrum showing that β-GalOMe yields signals and therefore binds to the receptor. (F) STD NMR spectrum as in (E) but with the $T_{1\rho}$ filter eliminating all protein background signals.

Figure 1A shows a normal ^1H NMR spectrum of RCA_{120}, revealing the broad resonances typical for a protein of this size. The few sharp resonances are due to low-molecular-weight impurities. Spectrum B in Figure 1 corresponds to the STD NMR spectrum of the same sample recorded with off-resonance irradiation at 30 ppm—resulting in no saturation of the protein—and on-resonance irradiation at −2 ppm. The protein envelope is identical to that shown in Figure 1A, which proves that intramolecular spin diffusion is, in effect, able to transfer the saturation over the entire protein. We have also obtained a series of STD spectra with saturation times ranging from 250 ms to 2.5 s. No influence on the STD spectra of the receptor could be observed (not shown). This proves that even at short irradiation times, the entire receptor is efficiently saturated. Additionally, low-molecular-weight impurities observed in spectrum A are

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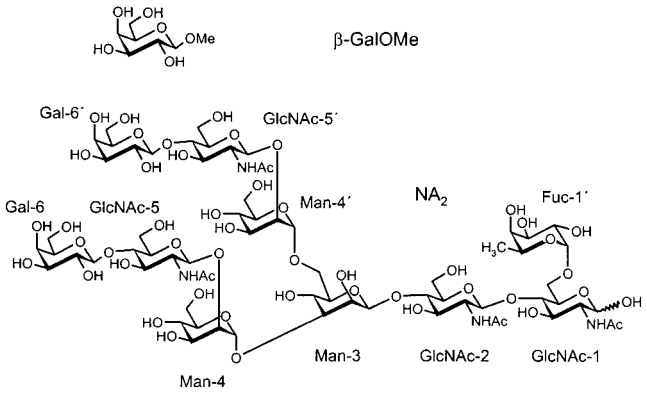
Scheme 2. Structures of β-GalOMe and NA_2



no longer detectable in spectrum B, verifying efficient subtraction. Spectrum C shows a normal NMR spectrum recorded with a $T_{1\rho}$ filter consisting of a 30-ms spin-lock pulse prior to acquisition. No protein resonances can be detected, since they have relaxed within this period, and only the impurities contained in the buffer remain visible.

A small ligand which is not directly affected by the saturation pulse, here methyl β-D-galactopyranoside (β-GalOMe, Scheme 2), was added to the sample as shown in the reference spectrum (Figure 1D). Saturation is exclusively transferred to molecules bound to the protein. Therefore, the difference spectrum contains only signals of molecules with binding affinity. This is shown here in the STD NMR spectra in Figure 1E,F, recorded without and with the protein suppression filter, respectively. Clearly, only NMR signals of the ligand β-GalOMe remain.

These spectra prove that we are able to (1) use RCA_{120} as a receptor, (2) detect binding substances, and (3) effectively suppress protein signals. Since neither the ligand nor the protein needs to be isotopically labeled or modified for binding to be detected, the method is applicable to the majority of problems involving protein−ligand interactions.

Methods and Materials

All NMR experiments were performed on a Bruker Avance DRX 500-MHz spectrometer equipped with a 5-mm inverse triple-resonance probe head at 300 K. NMR samples were prepared in 500 μL of 99.9% D_2O buffer containing 20 mM NaCl, 10 mM phosphate buffer at pH 7 (not corrected for D_2O), and 0.04% NaN_3. Solute exchange was achieved by ultrafiltration of the 120-kDa RCA_{120} (Vector Laboratories) with a Centricon (Millipore) membrane having a cutoff value of 50 kDa. Protein concentrations in the NMR samples were in a range between 20 and 50 μM, determined using UV absorbance at 280 nm of $A^{1\%,1\ cm} = 11.8$ for the monomer.[22] β-GalOMe was purchased from Sigma-Aldrich. The asialo complex type decasaccharide NA_2 (Scheme 2) was isolated from fibrinogen.[23,24] Addition of the saccharides to the protein NMR sample took place from concentrated stock solutions; therefore, dilution effects were minimal.

The pulse scheme of the 1D STD NMR spectrum corresponds to a modified 1D NOE difference pulse sequence and is displayed in Figure 2. Subtraction of the 1D STD spectrum was performed internally via phase cycling after every scan to minimize artifacts arising from temperature and magnet instability. For samples with higher concentrations of H_2O, the water suppression by gradient tailored excitation (WATERGATE) scheme for suppression of the residual HDO signal

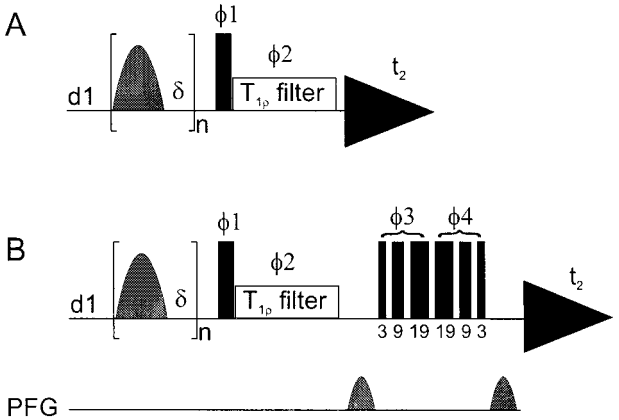


Figure 2. Pulse sequence for the 1D STD NMR spectra recorded in D_2O (A) and with an additional WATERGATE step for H_2O samples (B). The subtraction is performed after every scan via phase cycling. The on- and off-resonance frequency of the selective pulse is therefore switched between 30 and −0.4 ppm after every scan. The length of the selective pulse was set to 50 ms, and the delay δ between the pulses is 1 ms; the duration of the presaturation period was adjusted by the number of pulses n (typically $n = 40$). $d1$ is an additional short relaxation delay which was here set to 100 ms. The intensity of the selective saturation Gauss pulses corresponds to a strength of 86 Hz. Phases in (A) are $\phi_1 = (x, -x, -x, x, y, -y, -y, y, -x, x, x, -x, -y, y, y, -y)$; $\phi_2 = 2(y, -y), 2(-x, x)$; and $\phi_{rec} = 2(x), 2(-x), 2(y), 2(-y), 2(-x), 2(x), 2(-y), 2(y)$. Phases in (B) are $\phi_1 = (x, -x)$; $\phi_2 = (y, -y)$; $\phi_3 = 2(x), 2(y), 2(-x), 2(-y)$; $\phi_4 = 2(-x), 2(-y), 2(x), 2(y)$; $\phi_{rec} = 2(x), 2(-x)$. The two PFGs have equal intensity and sign. Water suppression is achieved here by a binomial 3-9-19 pulse sandwich, resulting in inversion of all signals except the HDO signal at the carrier frequency. The delay between the 3-9-19 pulses is 0.2 ms each (cf. Methods and Materials)

was employed.[25] Here, the hard pulse WATERGATE version was used since it gave better results than the soft pulse version. The strength of the 3-9-19 pulses in the pulse sandwich was set to $(\gamma/2\pi)B_1 = 6944$ Hz. The π pulse at this power level corresponds to a length of 26 in the 3-9-19 notation. The delay between the 3-9-19 pulses determines the next frequency with zero intensity, $\Delta\nu_{next\ null}$ (Hz) $= 1/2d$. The delay d was set to 0.2 ms, resulting in the next frequency with zero intensity at $\Delta\nu = 2500$ Hz.

The on-resonance irradiation of the protein was performed at a chemical shift of −0.4 ppm unless otherwise stated. Off-resonance irradiation was applied at 30 ppm, where no protein signals were present. 1D STD NMR spectra were multiplied by an exponential line-broadening function of 1−3 Hz prior to Fourier transformation. The irradiation power of the selective pulses in all STD NMR experiments was set to $(\gamma/2\pi)B_1 = 86$ Hz. Selective presaturation of the protein was achieved by a train of Gauss-shaped pulses of 50-ms length each, separated by a 1-ms delay. The number of selective pulses n determines the presaturation period, and the standard value was 40 pulses, leading to a total length of the saturation train of 2.04 s. The additional delay $d1$ was set to 100 ms in all STD experiments. If ligands with very long relaxation times are studied, this delay should be increased as relaxation artifacts may arise. The total number of scans was 256 or 512, and 16 dummy scans were applied, typically using 12 ppm spectral widths for the 1D STD NMR spectra. All 1D STD spectra of samples containing ligands were recorded with a 30-ms spin-lock pulse, or so-called $T_{1\rho}$ filter, after the $\pi/2$ pulse with a strength of $(\gamma/2\pi)B_1 = 4960$ Hz, which eliminates the background protein resonances to facilitate analysis. As has been pointed out in our previous publication,[7] the spin-lock pulse also results in a slightly decreased STD signal intensity of the ligand. To compensate for this effect, the reference NMR spectra were also recorded with a spin-lock pulse of the same length. Integration of the 1D and 2D STD NMR spectra was achieved by creating region

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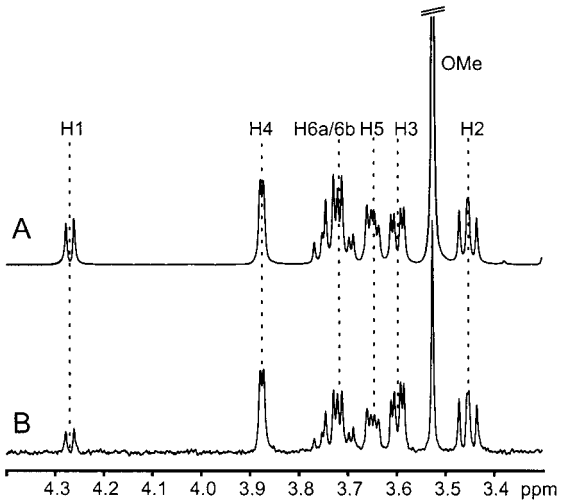

Figure 3. (A) Reference WATERGATE NMR spectrum of a mixture of RCA_{120} (40 μM binding sites) and β-GalOMe (4 mM) in a ratio of 1:100. (B) WATERGATE STD NMR spectrum of the same sample. Prior to acquisition, a 30-ms $T_{1\rho}$ filter was applied to remove residual protein resonances. From the STD spectrum, one can characterize the binding epitope by using relative integral intensities of the signals in spectrum B (cf. Figure 4).

files for the individual spectra to guarantee identical boundaries. In competition and titration experiments, the individual 1D STD NMR experiments were acquired and processed identically.

For the group epitope mapping analysis, the STD integrals of the individual protons of the two saccharides are referenced to the strongest STD signal in each spectrum, which is assigned to a value of 100%. The differential STD effects within one saccharide then yield information on the proximity of the individual protons to the protein surface.

2D STD total correlation spectroscopy (TOCSY)[26] spectra were recorded with 32 or 40 scans per t_1 increment. A total of 256 t_1 increments were collected in an interlaced mode for the on- and off-resonance spectra, respectively. Prior to subtraction, both spectra were processed and phased identically. An MLEV-17 mixing time[26] of 100 ms was applied in all TOCSY spectra. The acquisition times for the 2D experiments were typically around 13 h. All spectra were processed on Silicon Graphics O_2 workstations with Bruker Xwinnmr 2.5 software. 2D spectra were multiplied with 90°-shifted squared sine bells in both dimensions and zero-filled two to four times.

Results

The binding interactions of β-GalOMe and NA_2 with *Ricinus communis* agglutinin were studied by STD NMR spectroscopy (cf. Scheme 2). Samples of (I) RCA_{120} with β-GalOMe, (II) RCA_{120} with NA_2, and (III) RCA_{120} mixtures of both carbohydrates were prepared and analyzed.

1. Group Epitope Mapping (GEM). A. Analysis of β-GalOMe. Figure 3 displays (A) the reference NMR spectrum of β-GalOMe with RCA_{120} in a 100:1 ratio and (B) the corresponding STD NMR spectrum. The STD spectrum proves that β-GalOMe binds to the RCA_{120} receptor, since large signals of the ligand can be observed. In addition, protons of the ligand which are nearest to protons of the protein can easily be identified from the STD NMR spectrum, because they are saturated to the highest degree. The different signal intensities of the individual protons are best analyzed from the integral values in the reference and STD spectra, respectively. The integral value of the largest signal of β-GalOMe, the H3 proton, was set to 100%. Figure 4 displays the relative degree of saturation for the individual protons. The H2, H3, and H4 ring protons all have similar STD intensities between 87% and 100%.

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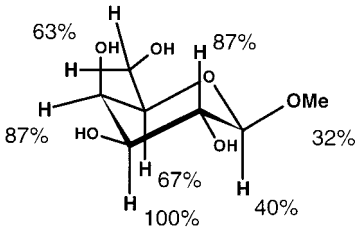

Figure 4. Structure of β-GalOMe and the relative degrees of saturation of the individual protons normalized to that of the H3 proton as determined from 1D STD NMR spectra at a 100-fold excess. The concentration of RCA_{120} was 40 μM and that of β-GalOMe 4 mM.

On the other hand, H5, H6a, and H6b have smaller STD intensities, ranging from 63% to 67%. The lowest intensities correspond to the H1 proton and the protons of the *O*-methyl group, which reach values of only 40% and 32%, each. Thus, a clear distinction between protons with a strong contact to the protein, i.e., H2, H3, and H4, and the others can be made. These results correlate well with the known binding specificity of RCA_{120} for the nonreducing end of terminal galactoses.[22,27]

For the titration, competition, and saturation time experiments, we introduce the STD amplification factor. This factor allows a better assessment of the absolute magnitude of the STD effect. The STD amplification factor is the fractional saturation of a given proton multiplied by the excess of the ligand over the protein. In effect, the STD amplification factor is the relative intensity of the STD signal compared to that of a signal of the protein; i.e., an STD amplification factor of 22 means that the STD signal has an intensity 22 times higher than the intensity of a protein proton. The STD amplification factor provides an easy measure to quantify the amplification of the protein information observed in the STD signals of the ligand. Obviously, the STD amplification factor can be significantly larger than 1 only if the residence time of a ligand is significantly shorter than the saturation time and if the concentration of the ligand is higher than that of the protein. Therefore, the STD amplification factor can be used to compare the STD effect of corresponding resonances of the two sugars in the competition and titration experiments, even if the protein concentration is not identical in the corresponding NMR samples. In the equation below, $(I_0 - I_{sat})/I_0$ is the fractional STD effect, expressing the signal intensity in the STD spectrum as a fraction of the intensity of an unsaturated reference spectrum; i.e., a value of 0.32 as displayed for the first point of the titration shown in Figure 5A would be equivalent to a saturation of 32% of the ligand's signal intensity. Obviously, the fraction of ligands which are saturated is continuously reduced when the ligand excess is increased. By multiplication of the ligand excess with this fractional STD effect, the STD amplification factor is obtained; e.g., at the first data point in Figure 5, the fractional STD effect is 0.32 and the ligand excess 6.25, resulting in a STD amplification factor of 2.0. Figure 5B shows the curve which is obtained by plotting the STD amplification factor against the ligand concentration. As expected, the curve flattens at higher ligand concentrations, in this case reaching a STD amplification factor of ca. 10.

$$\text{STD amplification factor} = \frac{I_0 - I_{sat}}{I_0} \times \text{ligand excess}$$

In this equation, I_0 is the intensity of one signal in the off-resonance or reference NMR spectrum, I_{sat} is the intensity of a

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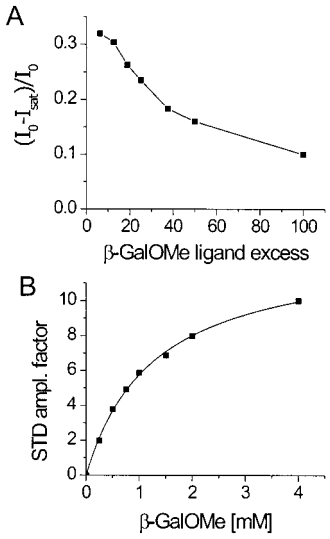

Figure 5. (A) Diagram showing the fraction of the H4 signal of β-GalOMe which is saturated at a given ligand excess. The concentration of RCA_{120} was 40 μM and the saturation time 2 s. (B) Display of the same data in terms of the STD amplification factor. This second plot shows that even though the fraction of ligand which is saturated decreases at a higher ligand excess, the absolute STD signal intensity increases in the form of a saturation curve. The STD amplification factor is obtained by multiplying the fractional STD effect shown in (A) with the ligand excess and is proportional to the signal intensity.

signal in the on-resonance NMR spectrum, and $I_0 - I_{sat}$ represents the intensity of the STD NMR spectrum.

By introducing the STD amplification factor, it is also possible to quantify the "active" ligand concentration labeled in the STD experiment, which therefore allows an estimation of the amount of protein needed for a STD experiment. If a specific resonance of the ligand has a STD amplification factor of 10, then, a protein concentration of 50 μM results in a labeled ligand concentration of 500 μM. Therefore, knowledge of the STD amplification factor will directly translate into the signal intensity which is obtainable from a given amount of protein.

The time course of saturation is obtained by plotting the STD amplification factor against the saturation time T_{sat}. Figure 6 shows the saturation profile for two selected resonances of β-GalOMe at three different ligand concentrations. The squares correspond to the H3 proton and the circles to the protons of the *O*-methyl group. In all three diagrams, the STD amplification factor of the H3 proton is larger than that of the *O*-methyl group. However, one can clearly observe that the larger the excess of β-GalOMe is, the more pronounced are the differences between the STD effects of the protons with strong contacts and those that have only weak interactions. This means that it is of advantage to use a higher excess when mapping the ligand's epitope. Additionally, one can observe that longer saturation times are required to reach the maximum observable STD effect at high excess. At a 0.5 mM concentration, a 2-s saturation time is more than sufficient, while at a 4 mM concentration, even a saturation time of 5 s is not long enough to reach the maximum STD effect. The relative STD intensities, which were specified for β-GalOMe in Figure 4, are therefore specific for a given ligand concentration and do not represent absolute values. Another advantage arises from the fact that absolute signal intensities grow with increasing concentrations. The maximal STD amplification factor for the H3 proton at a 0.5 mM concentration reaches only values of ca. 4, while at a 4 mM concentration it reaches values of ca. 18, making the STD experiment highly sensitive.

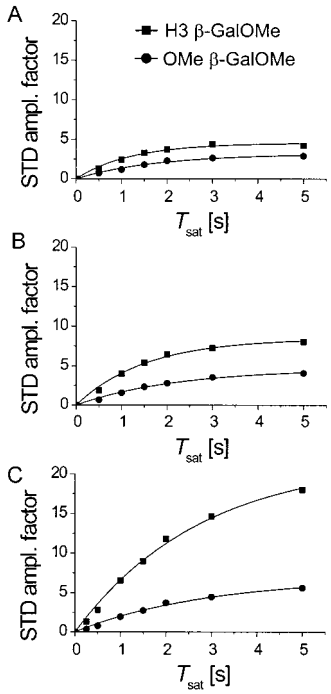

Figure 6. Observed STD amplification factors of two resonances of β-GalOMe plotted against the saturation time T_{sat} at three different ligand concentrations (■, H3 proton; ●, OMe protons). STD amplification factors at concentrations of (A) 0.5 mM, (B) 1 mM, and (C) 4 mM β-GalOMe in the presence of 40 μM binding sites of RCA_{120}. A large ligand excess yields larger STD intensities and better discrimination between strongly and weakly binding groups.

Effective group epitope mapping (GEM) within one residue or building block should be possible if the dissociation rate constant k_{off} is greater than or of the same magnitude as the intermolecular saturation transfer rate within the bound ligand. Effectively, the ligand has to leave the binding site before all magnetization has been equally distributed among all spins in the ligand. This condition is fulfilled for ligands with a high turnover number. Since β-GalOMe is a weakly binding ligand with a dissociation constant of 2.6×10^{-4} M, the turnover number, or dissociation rate constant, is high enough to map the interactions within the monosaccharide to the individual protons.

B. Analysis of NA_2. (i) 1D STD Experiment. Figure 7 shows (A) the reference and (B) the difference spectra of a selected region of the complex type biantennary decasaccharide (NA_2, 0.55 mM; cf. Scheme 2) in the presence of RCA_{120} (50 μM). We chose this oligosaccharide as a ligand to demonstrate that epitope mapping by STD NMR is also applicable to large ligand molecules, in this case with a molecular weight of 1836 Da. Although we pointed out that it is of advantage to have a large excess of the ligand for efficient GEM, here we used only an 11-fold excess of NA_2 because the decasaccharide was not available in larger amounts. A titration curve was measured at a low protein concentration of 20 μM and a higher ligand excess. However, the signal-to-noise ratio of the STD NMR spectra was not good enough for a GEM analysis. The GEM was consequently performed at an 11-fold excess of NA_2.

As for β-GalOMe, the analysis of the relative STD intensities of NA_2 was accomplished by integrating the dispersed proton resonances and referencing them to one of the most intensive proton signals, the H4 of the galactose residue (Table 1). The 1D NMR spectra shown in Figure 7 reveal that a direct analysis of the integrals of individual proton resonances in the hump region is impeded due to severe signal overlap. Nevertheless,

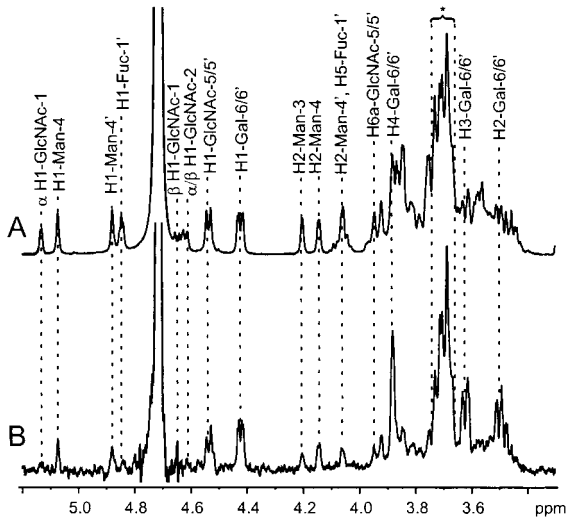

Figure 7. (A) Section of a reference NMR spectrum of a mixture of RCA_{120} (50 μM binding sites) and NA_2 (0.55 mM) in a ratio of 1:11. (B) STD NMR spectrum revealing that the directly interacting residues of NA_2 have the strongest signals. The resonances corresponding to the terminal galactoses Gal-6/6′ and the adjacent GlcNAc-5/5′ have the most intensive STD signals. The spectral region from 3.65 to 3.75 ppm marked with the asterisk shows strong STD signals which originate almost entirely from the H5 and H6a/6b of Gal-6/6′ and the H2, H3, and H4 of GlcNAc-5/5′ protons in equal parts (cf. text). H1-Fuc-1′ and α-H1-GlcNAc-1 have almost no detectable STD signal intensity. This shows that they are far away from the binding domain of the lectin.

Table 1. STD Signal Intensity of NA_2 and β-GalOMe at a Ligand Excess of 12.5- and 100-Fold, Respectively[a]

	STD signal (%)		
NA_2 (β-GalOMe) resonance	11-fold excess NA_2	12.5-fold excess β-GalOMe	100-fold excess β-GalOMe
H4-Gal-6/6′[b] (H4-β-GalOMe)	∼100	90	87
H3-Gal-6/6′[b] (H3-β-GalOMe)	∼100	100	100
H2-Gal-6/6′[b] (H2-β-GalOMe)	∼100	95	87
H1-Gal-6/6′ (H1-β-GalOMe)	70	61	40
H5-Gal-6/6′[b] (H5-β-GalOMe)	∼80	74	67
H6a/6b-Gal-6/6′[b] (H6a/6b-β-GalOMe)	∼80	72	63
H2, H3, H4-GlcNAc-5/5′[b]	∼70		
H1-GlcNAc-5/5′	52		
H2-Man-4	44		
H2-Man-4′	42		
H1-Man-4	40		
H1-Man-4′	33		
H2-Man-3	28		
NHAc-5/5	37		
NHAc-2	17		
H6-Fuc-1′	2		

[a] The intensities are normalized to the H3 proton of the respective galactose residue. [b] Values with error of ∼10% due to signal overlap.

the so-called structural reporter group spectral region corresponding to the anomeric protons is well resolved and can be used to classify the saccharide residues relevant for interaction with the lectin. Additionally, the H2, H3, and H4 resonances of the two terminal galactose residues are indicated in the STD spectrum (Figure 7B) and show large STD intensities. From the 1D STD spectra, we could determine the signal intensities of the H2, H3, and H4 protons of Gal-6/6′ to ca. 100%. For better comparison of the STD effects at a lower excess, Table 1 also shows the relative intensities for selected signals of β-GalOMe at both 12.5- and 100-fold excess. The relative STD intensities of the two saccharides are very similar at the low ligand excess. For example, the anomeric proton of β-GalOMe reaches a value of 61% in comparison to the 40% reached at a 100-fold excess. This indicates the influence of the ligand excess on the STD effects and that differentiation between direct and indirect contact of the decasaccharide probably would have been more pronounced at a higher ligand excess.

Determining the binding contribution of the *N*-acetyl-D-glucosamine (GlcNAc)-5/5′ residues is important in order to identify the binding epitope. In the 1D STD NMR spectrum, only the anomeric H1 protons and the *N*-acetyl groups of GlcNAc-5/5′ can be integrated separately. The H1-GlcNAc-5/5′ protons have an integrated mean STD effect of 52% compared to that of the H3 protons of Gal-6/6′. The resonances corresponding to the *N*-acetyl groups of GlcNAc-5/5′ have a mean value of 37%. The ring protons H2, H3, and H4 of GlcNAc-5,5′ have their resonances in the spectral region between 3.65 and 3.75 ppm. This region has a high integral in the STD experiment. Although the resonances are not dispersed and therefore no individual signals can be assigned, one can approximate the STD intensity contribution of the three GlcNAc-5/5′ ring protons in this crowded region. The signals in this section originate from different saccharide residues; however, only a few of these contribute significantly to the STD intensity.

In the spectral region from 3.65 to 3.75 ppm, we can find resonances of the H5, H6a, and H6b signals of the Gal-6/6′ residue, the H2, H3, and H4 signals of the GlcNAc residues 5, 5′, 2, and 1, the H3 and H6a signals of the Man-3 residue, the H5 proton of Man-4 residue, and two ring proton signals of Fuc-1′ (Figure 7). Even though there are signals of 23 protons in this region, only 12 of these make a significant contribution to the mean STD integral. Because the STD contributions of the signals of residues farther away from the binding site are known from the integration of the anomeric signals, we can approximate the binding contribution of the GlcNAc-5/5′ residues by subtracting the integral values of the others from the full integral over that region. No contribution of the Fuc-1′ and GlcNAc-1 signal is taken into account, since they are farthest away from the binding domain, and the other resolved signals of these residues show only minute intensities. The contribution of the H2, H3, and H4 protons of GlcNAc-2 to the mean STD integral is very small and lies around 20%, which was estimated from the STD integral of its anomeric and *N*-acetyl group signals. The contribution of the H3 and the H6a signals of Man-3 lies around 30%, and that of the H5 proton of Man-4 is about 40%. All of these proton signals make up only a small fraction of the mean integral value. The remaining STD integral value is distributed between the six H5, H6a, and H6b protons of Gal-6/6′ and the six H2, H3, and H4 protons of GlcNAc-5/5′. The STD intensity reached by the H5, H6a, and H6b protons in β-GalOMe at a corresponding ligand excess is ca. 74%. Since the STD intensity of the H1 proton is higher in the decasaccharide than in the β-GalOMe (cf. Table 1), we can approximate that the intensities of the three protons H5, H6a, and H6b are also higher by an equivalent amount. They are attributed to a mean value of 80%. By this exclusion principle, we can now approximate the mean contribution of the six GlcNAc-5/5′ protons H2, H3, and H4 to a mean value of about 70% per proton. This value is much higher than the ca. 40% reached by the protons of the Man-4/4′ residues. In conclusion, the GlcNAc-5/5′ residues make direct contact with the protein

surface via their H2, H3, and H4 region. In contrast, the interactions of the H6a (at 3.946 ppm) protons of GlcNAc residues 5 and 5′ with the protein are very small, and consequently show a STD effect of only about 20%. Additionally, the interaction of the GlcNAc residues 5/5′ overall is less strong than that of the Gal-6/6′ residues. Because of the strong differentiation of the STD effects within the GlcNAc residues, it is likely that these sugars bind edge-on to the protein with the C2 to C4 region making contact and the ring oxygen and C6 pointing away from the receptor.

Exact differentiation of the binding affinity of the two branches of NA_2 by means of the H1 protons of Gal-6/6′ or of GlcNAc-5/5′ was not possible due to signal overlap. However, the individual protons have about the same intensities (cf. Figure 7), indicating identical affinities of the branches to RCA_{120}. The H1-Man-4/4′ can be integrated separately. The H1 proton of Man-4 has a value of 40% and the H1 proton of Man-4′ a value of 33%. Interestingly, the H2 protons of Man-4/4′ are both saturated to a degree of ca. 43%, again demonstrating that individual protons within one residue can show variations.

The determination of the exact size of the ligand-binding domain from STD NMR data is complex in this case, since one cannot observe a clear cutoff value at the concentration used here. The NA_2 concentration of only 0.55 mM, corresponding to an excess of 11, was not high enough for optimal group epitope mapping. However, the data clearly show that the Fuc-1′ as well as the GlcNAc-1 residues do not lie within the realms of the RCA_{120} binding pocket, since they show a degree of saturation of only about 2%. The methyl group proton resonances of the *N*-acetyl group of GlcNAc-2 and the H2 proton resonance of Man-3 are saturated to a degree of 17% and 28% relative to the H3 protons of Gal-6/6′, respectively. So the protein surface is at a large distance from these protons. For the Man-4/4′ residues, it is not straightforward to determine whether they are in direct contact with the receptor. The degrees of saturation of their H1 and H2 protons lie around a value of 33−44%, which is considerably lower than the ca. 70% reached by the H2, H3, and H4 protons of the GlcNAc-5/5′ residue. Only the terminal galactose has very strong interactions with the protein-binding domain, however with a considerable binding contribution from the neighboring GlcNAc residue. Therefore, the terminal *N*-acetyl-D-lactose (LacNAc) moiety forms the binding epitope in direct contact with RCA_{120}.

(ii) 2D STD TOCSY Experiment. The same sample was also used in a 2D TOCSY experiment. Figure 8 shows a region of the off-resonance TOCSY spectrum (A) and the corresponding region of the STD TOCSY spectrum (B). Again, one can observe how signals corresponding to residues in a near proximity to the receptor are saturated to a higher degree, resulting in more intense cross-peaks. Highlighted are the traces corresponding to the H1-Gal-6/6′ and H1-GlcNAc-5/5′ as well as the H1-Man-4 and H1-Man-4′ protons. No cross-peaks stemming from GlcNAc-1/2 or the Fuc-1′ residues can be seen due to their low degree of saturation. In agreement with the 1D STD experiment, the strongest signals are again clearly those corresponding to the terminal LacNAc moiety of the decasaccharide.

2. Titration and Competition Studies by STD NMR. A. STD NMR Titration Studies. The titration study was performed to determine the effect of the ligand excess on the STD signal intensities. Figure 9 displays the increase of the STD amplification factor for the H4-Gal NA_2 resonance. The titration plot in Figure 9 reveals that the signal intensity increase is more pronounced at low ligand concentrations and that it starts

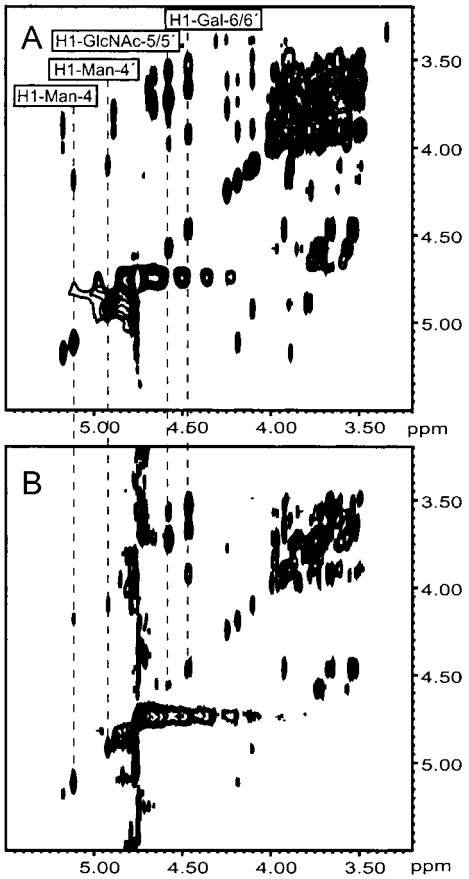

Figure 8. (A) Reference or off-resonance TOCSY spectrum of NA_2. (B) STD TOCSY spectrum obtained by subtraction of an on-resonance TOCSY spectrum from spectrum A. In the STD spectrum B, traces corresponding to the H1-Gal-6/6′ and the H1-GlcNAc-5/5′ are very strong, whereas the H1-Man-4 and H1-Man-4′ traces show reduced intensities, due to their larger distance to the binding site of RCA_{120}. GlcNAc-1/2 and Fuc-1′ cross-peaks are absent from spectrum B, proving that they have no interaction with the protein.

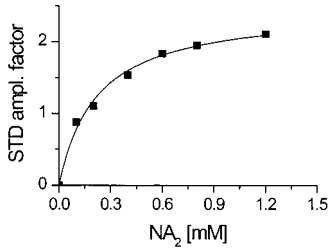

Figure 9. Titration plot of NA_2 to NMR sample containing RCA_{120} (20 μM in binding sites), monitoring the increase of the STD amplification factor of the H4-Gal-6/6′ proton versus the ligand concentration (T_{sat} = 2 s).

leveling off at higher concentrations. This is the consequence of RCA_{120} binding sites becoming saturated with ligand, but the larger effect comes from the limitation in terms of the turnover number. At a certain ligand concentration, a further increase will no longer result in a gain in signal intensity, and a maximum STD amplification factor is reached. The point at which this concentration is reached depends on the turnover number between the bound and the free states, the size of the protein, and the T_1 relaxation time of the ligand in the free state (T_{1lf}). Large saturation transfer effects will be observed for large values of k_{off} and long relaxation times T_{1lf}. Additionally, molecules exchanging slowly from the bound state to the free state reach their maximum earlier than molecules with fast

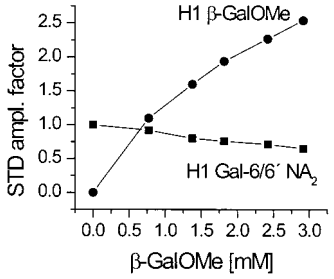

Figure 10. Diagram showing the STD amplification factors (●, H1 β-GalOMe; ■, H1 Gal-6/6′ NA_2) determined from STD spectra on titration of β-GalOMe to a sample of RCA_{120} (50 μM in binding sites) and NA_2 (0.55 mM). The STD amplification factor of the signal corresponding to NA_2 decreases from 1 to 0.66 with increasing concentration of β-GalOMe. This competition experiment gives evidence for the specificity of the RCA_{120} toward galactose-containing saccharides. The K_D of NA_2 can be calculated to be 27 μM.

exchange kinetics. This can be observed if one compares the titration curves of β-GalOMe (Figure 5B) and NA_2 (Figure 9). The ligand with a higher turnover number (I) reaches higher STD amplification factors and (II) reaches these values at higher concentrations. The intensity increases strongly as long as ligand molecules having received no saturation are available in large numbers to bind to the receptor. Hence, the STD amplification factor approaches a maximum with increasing ligand excess.[7] The stronger binder NA_2 has a maximal STD amplification factor of about 2.5, whereas the weaker binding β-GalOMe reaches a value of ca. 7 at the same concentration and saturation time. This reflects the higher k_{off} value for the monosaccharide. Very small k_{off} rates of strongly binding molecules cause the ligand to stay in the binding site for an extended period of time. This, in turn, makes the group epitope mapping very difficult. If the residence time of the ligands is longer than about 5 times the T_2 of the ligand in the bound state, one expects a spreading of the magnetization over many protons, resulting in very small differential STD effects.

B. STD NMR Competition Studies. The competition studies were performed to determine if the two ligands, NA_2 and β-GalOMe, interfere with each other at the receptor site. Determined from calorimetric titration studies at 293 K, β-GalOMe has a dissociation constant $K_D = 2.6 \times 10^{-4}$ M.[18] Surface plasmon resonance (SPR) binding data obtained for the decasaccharide immobilized on the chip's surface gave a dissociation constant K_D of 0.8×10^{-8} M, which corresponds to a very tight binding inhibitor with a dissociation constant a factor of 32 000 smaller than that of β-GalOMe.[19] There was evidence from the titration experiments that NA_2 is not a nanomolar inhibitor of RCA_{120}, since under the experimental conditions used here, only very small STD effects of the ligand would be observed. To further pursue this point, we added the weak inhibitor β-GalOMe to a sample containing RCA_{120} and NA_2. If NA_2 is, in effect, an inhibitor with a nanomolar dissociation constant, no STD signals of the monosaccharide should be observable, since it would have no opportunity to make contact with the receptor. Figure 10 displays the STD amplification factors of the H1 protons of Gal-6/6′ of NA_2 (■) and β-GalOMe (●) and shows that β-GalOMe yields large STD NMR signals when added to the sample containing RCA_{120} and NA_2. A possible explanation is that NA_2 has a weaker affinity in solution than that published for an oligosaccharide-immobilized SPR experiment. It was shown that ligand immobilization can lead to largely exaggerated K_D values, whereas protein immobilization gives binding affinities in agreement with those obtained by other methods.[28,29] We therefore performed a SPR study on a detection system where the lectin was immobilized. These results will be published elsewhere.[30] We found a K_D of 4.4×10^{-6} M for NA_2, which is in much better agreement with our STD data than the K_D of 0.8×10^{-8} M obtained by Shinohara et al.[19] The reduction of the STD effects of NA_2 by addition of β-GalOMe gives evidence for the competition for the same binding site. However, the intensities of the STD signals of different ligands cannot be directly correlated to the occupation of binding sites by analyzing just their ratio at one concentration. Since the magnitude of the STD effect relies largely on the dissociation rate constant k_{off} of the binding substances, more molecules of a ligand with a high turnover number can be saturated during the irradiation time and result in a higher signal intensity. This explains why signals of β-GalOMe grow faster than the signals of the NA_2 are reduced as a function of concentration. However, K_D values can be determined from the replacement of one ligand as a function of the concentration of another. By using the equation of Cheng and Prusoff,[31] it is possible to determine the K_D value from the IC_{50} value of a competitive inhibitor with a known dissociation constant. Here, a K_I value of 2.6×10^{-4} M for the ligand β-GalOMe was used to calculate the K_D value of NA_2 with the following equation:

$$K_D = \frac{[NA_2]K_I}{IC_{50} - K_I}$$

In this case, the concentration of NA_2 was 0.55 mM and the IC_{50} was determined to be 5.6 mM. With the above equation, this results in a K_D value for NA_2 of 2.7×10^{-5} M. Details will be presented in a separate publication.[30]

To evaluate the utility of 2D spectra for competition experiments, the STD intensities of 2D TOCSY spectra were integrated. The diagram presented in Figure 11 shows how the STD amplification factors evolve when titrating β-GalOMe to the sample containing NA_2. The RCA_{120} sample with a concentration of NA_2 of 0.55 mM was analyzed by TOCSY STD NMR spectra with concentrations of the competitor β-GalOMe of 0.0, 0.8, and 3.0 mM, respectively. Six cross-peaks with good signal-to-noise ratios were selected from the F1 traces of the H1 Gal protons of NA_2 and β-GalOMe. The values are in very good agreement with the results obtained by integrating the 1D STD NMR spectra. The plot in Figure 11 shows the increase in intensity of the selected cross-peaks of β-GalOMe, while the signal intensities of the NA_2 molecule decrease. As in Figure 10, the intensities of the signals corresponding to β-GalOMe more than double on an increase of the β-GalOMe concentration from 0.8 to 3 mM. In contrast, signal intensities of NA_2 show a decrease between 30% and 50%. The observed STD amplification factors of β-GalOMe again reached higher values compared to those of the NA_2 molecule, also in agreement with the 1D spectra. This confirms that a few cross-peaks can be sufficient to collect competition data and calculate from these K_D and/or IC_{50} values. This will be very useful if spectra are extremely crowded due to large amounts of different substances.

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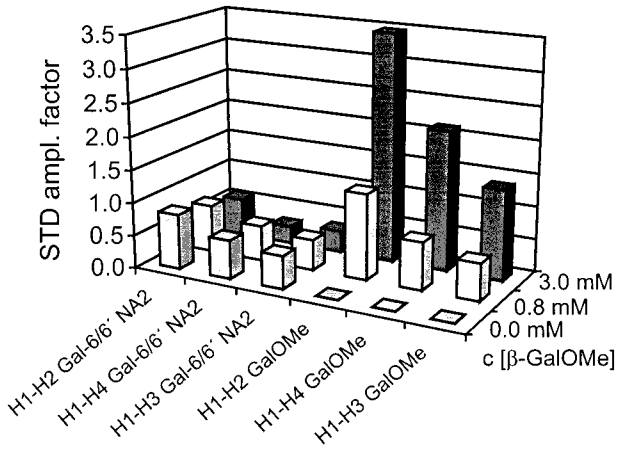

Figure 11. Diagram showing the STD amplification factors of selected cross-peak intensities of NA_2 and β-GalOMe determined from STD TOCSY spectra. The selected cross-peaks represent the F1 traces of the two H1 protons of the galactose residues. The light gray bars correspond to a β-GalOMe concentration of 0.0 mM, when only NA_2 (0.55 mM) is present in the sample containing RCA_{120} (50 μM). The open bars correspond to a β-GalOMe concentration of 0.8 mM, and the dark gray bars represent a concentration of 3.0 mM. These values are consistent with the values obtained from the 1D STD spectra (Figure 10); therefore, even a few cross-peaks can be sufficient to perform titration experiments

Discussion

The group epitope mapping (GEM) by STD NMR described here can be used to characterize at the atomic level binding interactions of ligands with a receptor. Here we demonstrate the method for the binding of two saccharides to the lectin RCA_{120}. The results from the STD NMR spectroscopic studies of RCA_{120} in the presence of the weakly binding ligand β-GalOMe clearly indicate the specificity of the RCA_{120} lectin for the nonreducing end of the saccharide. Ring protons H2, H3, and H4 form a tight contact with the protein, whereas H5 and H6a, H6b are also involved in binding but with less intense contacts to the protein. In contrast, the anomeric proton and protons of the methyl group show significantly less interactions with the protein. The titration and saturation time analysis reveals that this system is in fast exchange on the NMR time scale, since a relatively large excess of ligands can be labeled.

Recently, Dalvit et al. have demonstrated that selective excitation of the HDO signal in samples containing 90% H_2O can be used to detect binding of ligands to receptors. Due to the negative NOE originating from "bound" water molecules, ligands can be detected in the water ligand observation with gradient spectroscopy (WATERlogsy) spectrum.[32] They have opposite sign compared to nonbinding molecules which only have contact to "unbound" water molecules, resulting in a positive NOE. This pathway of magnetization can be neglected in the STD experiments since the samples were measured in 99% D_2O and saturation of the protein was performed at -0.4 ppm—at more than 5 ppm from the HDO signal. Additionally, in samples with large amounts of H_2O, the irradiation of the protein does not excite the bulk water enough that it is a significant pathway for the transfer of the magnetization. This is evidenced from comparison of spectra of the same system in H_2O and in D_2O, respectively.

Other studies have concentrated on the importance of the donor and acceptor properties of the hydroxyl groups of various galactose derivatives.[14] Bhattacharyya and Brewer found that neither the C1 nor C6 hydroxyl groups of the galactose residue contribute to the binding affinity, whereas that at C2 has both acceptor and donor functions, and the C3 and C4 hydroxyl groups serve only as donors. Martin-Lomas et al. found also that C2, C3, and C4 hydroxyl groups are involved in binding. In contrast to Brewer et al., they also identified the C6 hydroxyl group as a key group for binding activity in lactose derivatives.[16] A third study conducted by the same group also classified the nature of the hydrogen-bonding interactions between the ligand and the protein into neutral or charged protein residues, depending on their free energy values.[33] It was found that the C3 and C4 hydroxyl groups interact with charged protein residues, while the C2 and C6 hydroxyl groups interact with neutral residues on the protein. These data correlate with our results obtained from STD NMR studies. The saturation received by the individual protons of the β-GalOMe show strong saturation of the H2, H3, and H4 protons, intermediate saturation of the H5 and H6 protons, and much weaker saturation of the H1 proton and the methyl group.

The time course of the STD amplification factor as a function of the saturation time shows that stronger differentiation of the binding group epitopes can be obtained at a large ligand excess. An additional advantage arises from the fact that larger concentrations also produce greater STD signal intensity, and therefore higher sensitivity. The concentration dependence of the STD amplification factors indicates relative binding strength insofar as weakly binding ligands show a strong growth even at large concentrations, whereas the STD amplification factors of stronger binding ligands level off much earlier.

Sharma et al. have shown that there is an additional binding site next to the galactose which can accommodate the next glycosyl building block of an oligosaccharide.[18] The NA_2 decasaccharide, which has a higher binding affinity than the monosaccharide, was therefore included in our study to analyze the binding of the adjacent GlcNAc and/or mannose residues. One can again directly map the terminal galactose residues of the decasaccharide to exhibit the strongest STD signals and thus be essential for binding affinity. The H2 to H4 have about 100%, H5, H6a, and H6b 80%, and H1 about 70% integral. Resonances of the neighboring GlcNAc residues also exhibit high degrees of saturation and are thus recognized by the receptor. The H2 to H4 signals have a STD integral of ca. 70%, whereas H6 shows only about 20% STD effect. The anomeric H1 has a slightly reduced STD integral of 60% compared to that of the H2 to H4 resonances. Therefore, an interaction of this residue with the protein through its H2 to H4 region is proposed. From the antepenultimate mannose residues onward, the degree of saturation continuously decreases toward the reducing end of the saccharide. This effect arises from a slow transfer of saturation through the glycosidic bonds. This is a bottleneck for the transfer of the magnetization, as only two protons, H1 and the aglyconic equivalent proton, have a distance that is usually on the order of 2.8 Å. No other protons across the glycosidic linkage come close to this distance. In addition, other protons in the sugar rings usually have multiple pathways for transferring magnetization. Another important result is the lack of preference of RCA_{120} for either the (1-3) or (1-6) linked branches of the biantennary decasaccharide. The STD intensities of the two chains are nearly equal for all analyzed protons. This also is in agreement with the results of Bhattacharyya, who found that neither the linkage to the branching mannose, nor the length of the chain, has an influence on the binding affinity.

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From the STD spectra of the mixtures of the two binding ligands, it could be clearly demonstrated that the two ligands compete for the same binding site, since the signal intensities of NA_2 decrease continuously with increasing concentrations of β-GalOMe. In addition, the STD NMR competition and titration experiments showed that NA_2 is not a nanomolar inhibitor under the conditions present in the NMR sample. The STD amplification factors reached in the titration experiment are too large for a very strongly binding ligand with slow exchange kinetics. From analysis of the competitive titration studies shown in Figure 10 and by using a one-site competition model, one obtains a dissociation constant of ca. 27 μM. This is slightly weaker than the value of 4.4 μM we determined from a SPR study.

The 2D TOCSY spectrum consolidates the data presented by the 1D STD spectra with the added advantage that more complex structures are identifiable. We could also show that integration of cross-peaks from 2D spectra can yield the data needed for titration and/or competition studies.

Conclusions

STD NMR spectroscopy can be used for analyzing binding processes, screening libraries, and mapping of ligand epitopes. The use of a high ligand excess is advantageous in two ways: first, the signal intensities are larger, making the STD experiment more sensitive, and second, the discrimination between directly interacting and nonbinding groups even within a single residue is easier. If well enough resolved, the binding epitope can be determined from integrals of the signals in 1D spectra or from 2D cross-peak integrals. For molecules as large as MW = 1836 Da, binding epitopes can be successfully assigned. Comparison of STD NMR with other methods to determine the binding epitope of non-peptide ligands shows that STD NMR provides a fast and reliable method to determine binding epitopes down to the contributions of individual groups that interact with a receptor protein. In addition, one can think of future applications combining the results obtainable by SAR by NMR concerning the binding epitope of the protein and the binding epitope of the ligand obtainable by STD NMR. By linking two weakly binding ligands via positions not crucial for binding affinity, an optimized ligand can be obtained much more quickly.

Acknowledgment. We thank Dr. S. Meyer and A. Kahrs for kindly providing the NA_2 decasaccharide. This work was generously supported by the DFG through Grants SFB470 and GRK464, and by the BMBF through Grant 1302. This paper is dedicated to Prof. Dr. Joachim Thiem on the occasion of his 60th birthday.

JA0100120