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The interaction of *N*-trifluoroacetylgalactosamine and its derivatives with winged bean (*Psophocarpus tetragonolobus*) basic agglutinin reveals differential mechanism of their recognition: a fluorine-19 nuclear magnetic resonance study

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Abstract Here, we show the binding results of a leguminosae lectin, winged bean basic agglutinin (WBA I) to *N*-trifluoroacetylgalactosamine (NTFAGalN), methyl-α-*N*-trifluoroacetylgalactosamine (MeαNTFAGalN) and methyl-β-tifluoroacetylgalactosamine (MeβNTFAGalN) using ^{19}F NMR spectroscopy. No chemical shift difference between the free and bound states for NTFAGalN and MeβNTFAGalN, and 0.01-ppm chemical shift change for MeαNTFAGalN, demonstrate that the MeαNTFAGalN has a sufficiently long residence time on the protein binding site as compared to MeβNTFAGalN and the free anomers of NTFAGalN. The sugar anomers were found in slow exchange with the binding site of agglutinin. Consequently, we obtained their binding parameters to the protein using line shape analyses. Aforementioned analyses of the activation parameters for the interactions of these saccharides indicate that the binding of α and β anomers of NTFAGalN and MeαNTFAGalN is controlled enthalpically, while that of MeβNTFAGalN is controlled entropically. This asserts the sterically constrained nature of the interaction of the MeβNTFAGalN with WBA I. These studies thus highlight a significant role of the conformation of the monosaccharide ligands for their recognition by WBA I.

Keywords Lectin · Winged bean basic agglutinin · 19 F NMR

Abbreviations

NMR	Nuclear Magnetic Resonance
WBA I	Winged bean basic agglutinin
NTFAGalN	*N*-trifluoroactylgalactosamine
MeαNTFAGalN	Methyl-α-*N*-trifluoroacetylgalactosamine
MeβNTFAGalN	Methyl-β-tifluoroacetylgalactosamine
PNA	Peanut agglutinin

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Introduction

Lectins, the carbohydrate-specific proteins, are distributed ubiquitously in nature [1–4]. Recognition of carbohydrate receptors by lectins is implicated in numerous biological processes such as protein folding and targeting to cellular organelles, homing and sequestration of lymphocytes, host pathogen interactions, metastases, fertilization *etc*.[2, 3, 5–10]. The specificity and dimension of lectin–carbohydrate interactions have been determined using immunochemical and spectroscopic methods [1–3, 11–17]. However, these methods have limitations. Since most naturally occurring carbohydrates do not possess significant absorption spectra, for these studies sugars are utilized tethered to either a chromophore or fluorophore. However, this may interfere with the specificities of interactions. Such interactions can be investigated by Nuclear Magnetic Resonance (NMR), which does not require introduction of bulky substituents or other external molecules [18]. The ^{19}F and ^{13}C nuclei, because of their large chemical shifts, yield unambiguous spectra and are more useful than the commonly used ^{1}H NMR for characterizing such interactions [19–21].

Winged beans contain two lectins, which are of almost identical molecular masses (M_r~58,000) and monosaccharide specificities, but differ with respect to their erythrocyte binding properties and isoelectric points [20–25]. The WBA I (pI~10.8) recognizes A1 blood group antigen strongly, followed by A2 and the B antigen, but not the H determinant [11]. Its acidic counterpart, WBA II, is specific towards the H-antigen

and its related substances [15]. To characterize the dynamics and mechanism of WBA I- saccharide interaction, we report here the binding of *N*-trifluoroacetylgalactosamine (NTFAGalN) anomers, methyl-α-*N*-trifluoroacetylgalactosamine (MeαNTFAGalN) and methyl-β-trifluoroacetylgalactosamine (MeβNTFAGalN) to lectin by ^{19}F NMR spectroscopy. We find that, while both the sugar anomers bind with equal propensity to the lectin in a reaction that is controlled mostly by the activation enthalpy term, the slower second order rate constants for the binding of MeβNTFAGalN are due to unfavorable activation entropy.

Materials and methods

Materials

2-Amino-2-deoxygalactose, *S*-ethyl-trifluorothioacetate and D_2O were procured from Sigma, St. Louis USA. All the other reagents used were of analytical grade.

Purification of WBA I

WBA I was purified [23–25] by affinity chromatography and its concentrations were determined spectrophotometrically using $\varepsilon^{1\%,\ 1cm}_{280nm}$=9.37 [26].

Synthesis of NTFAGalN, MeαNTFAGalN and MeβNTFAGalN

The synthesis of NTFAGalN and of MeαNTFAGalN and MeβNTFAGalN were carried out as described in [27, 28].

NMR measurements

NMR samples were prepared in phosphate buffer saline, pH 7.2. The resultant protein concentration in the samples was 1.45 mM (WBA I monomer). The concentration of NFTAGalN was varied from 2.30-9.09 mM, MeαNTFAGalN from 0.75–8.72 mM and MeβNTFAGalN from 9.09–18.09 mM. All samples contained 0.03 % azide for preventing bacterial growth and D_2O for field frequency locking of the spectrometer [20]. ^{19}F spectra were recorded in tubes of 5 mm diameter on a Bruker WH 400 MHz spectrometer. Trifluoroethanol was used as the internal reference for obtaining ^{19}F spectra. Spectra with good signal-to-noise ratio were obtained with 1 h of accumulation and a 3 s delay time. Experiments were carried out at different temperatures using a Bruker variable temperature unit. Line broadening of resonance was measured at half of its height.

Results

Trifluoroacetylated galactosamine derivatives bind specifically to WBA I

The ^{19}F NMR spectra of NTFAGalN, MeαNTFAGalN and MeβNTFAGalN in the absence and presence of WBA I are shown in Figs. 1 and 2. In NTFAGalN anomeric mixture, resonance to the upfield was assigned for the β-anomer (β-NTFAGalN) and one at the downfield to the α-anomer (α-

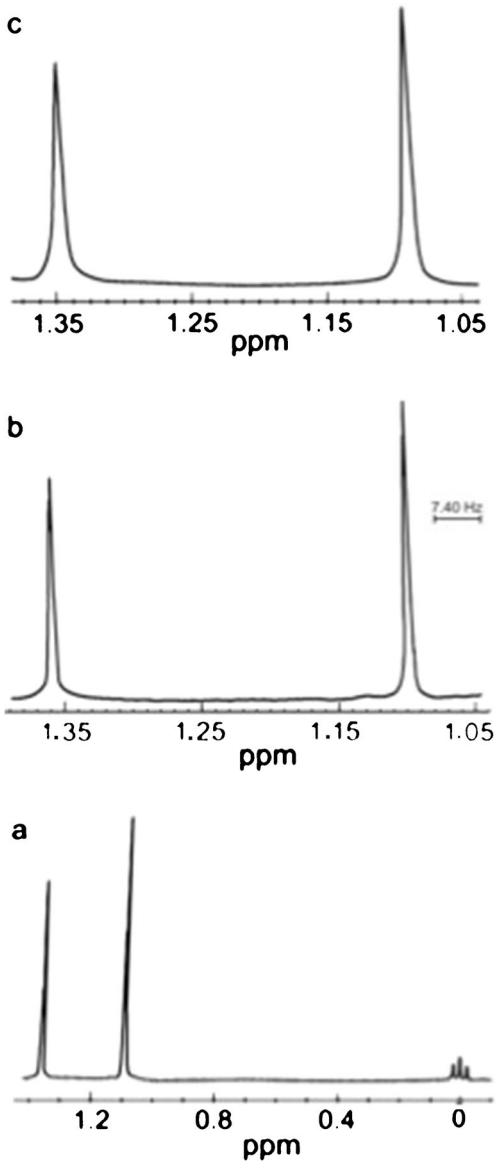

Fig. 1 Spectra of the anomers of NTFAGalN in the absence (**a** and **b**) and in the presence of WBA I (**c**). A 2.30 mM concentration of NTFAGalN was used, and the protein concentration was 1.45 mM as monomer. The ^{19}F spectra corresponding to the anomers of NTFAGalN is expanded in (**b**). Linewidth at half height corresponds to 1.09 Hz for free sugar (**b**) and 1.64 Hz and 1.61 Hz for α and β anomers, respectively, in the bound form (**c**). Triplet at high field in (**a**) is due to trifluoroethanol internal standard. Chemical shifts were listed assigning the middle resonance as 0.0

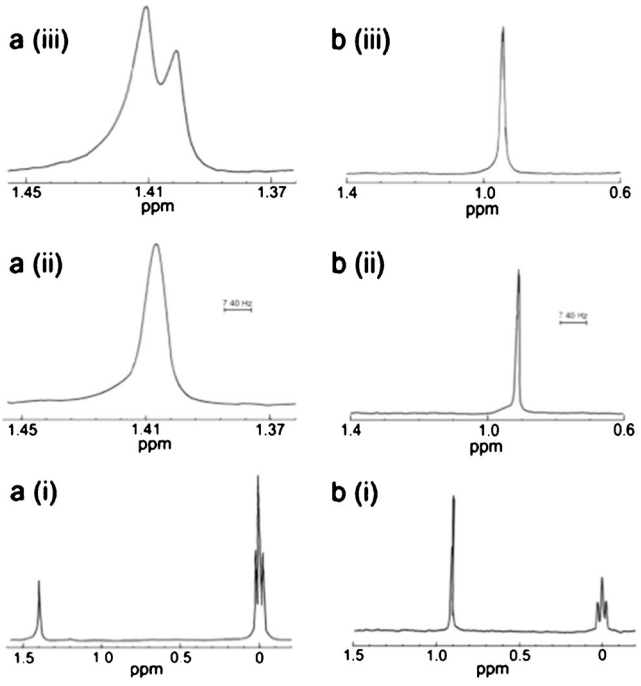

Fig. 2 ^{19}F spectra of MeαNTFAGalN (**a**) and MeβNTFAGalN (**b**) in the absence [**a** (i), **a** (ii), **b** (i) and b (ii)] and in the presence of WBA I [a (iii) and b (iii)], respectively. A 7.07 mM MeαNTFAGalN and 9.09 mM MeβNTFAGalN were used, and the protein concentration was 1.45 mM as monomer. Linewidth at half height corresponds to 4.32 Hz and 0.548 Hz for free sugar [**a** (ii) and **b** (ii)], and 4.38 Hz and 0.822 Hz for the bound form, MeαNTFAGalN [**a** (iii)] and MeβNTFAGalN [**b** (iii)]. Triplet at a high field in (**a**) is due to trifluoroethanol internal standard

NTFAGalN) [20, 29]. In presence of the WBA I, the resonance of sugars showed line broadening. The chemical shifts of NTFAGalN anomeric mixture and MeβNTFAGalN did not change while that of MeαNTFAGalN shifted downfield by 0.01 ppm. Line broadening increased progressively as the molar ratio of the ^{19}F saccharides to protein was decreased and was abrogated in the presence of a 100-fold excess of MeαNTFAGalN. Moreover, diethylpyrocarbonate-modified WBA I, an inactive form of the protein, even at the highest concentration tested (5 mM), failed to elicit broadening of the sugar resonance. Thus, the line broadening of sugar resonance occurs due to its specific binding to WBA I and this rules out increase in viscosity or the metal ions non-specifically associated with the protein as being responsible for the observed line broadening.

Trifluoroacetylated galactosamines are in slow exchange with WBA I

For a molecule undergoing chemical exchange between two sites *i.e.* between a free state and bound to a molecule, the spin-spin relaxation rate is given by [30, 31]

$$1/T_{2P} = f/(T_{2m} + \tau_m) \quad (1)$$

where, f is the fraction of small molecule bound to the macromolecule, τ_m is its residence time in the binding site, and T_{2m} is the spin–spin relaxation time in the bound environment. In the fast exchange limit ($T_{2m} >> \tau_m$), average width of the free and bound saccharide is observed. In slow exchange limit ($\tau_m >> T_{2m}$), line broadening is governed by the exchange rate $1/\tau_m$, which is equal to the dissociation rate constant k_{-1} of the complex. These two processes can be distinguished by using the temperature dependence of the line broadening. The resonance of all the sugars anomers broadened with increasing temperature, indicating that they are in slow exchange with the protein (Fig. 3a). The ratio of individual anomers in the mixture was evaluated by measuring the area under the respective peak for which the equilibria under consideration are given by Eq. (2, 3)

$$P + L_\alpha \underset{k^\alpha_{+1}}{\overset{k^\alpha_{-1}}{\leftrightarrow}} P_\alpha \quad (2)$$

$$P + L_\beta \underset{k^\beta_{+1}}{\overset{k^\beta_{-1}}{\leftrightarrow}} P_\beta \quad (3)$$

where, k^α_{+1}, k^β_{+1} and k^α_{-1}, k^β_{-1} are the association and the dissociation rate constants of the α and β anomers respectively.

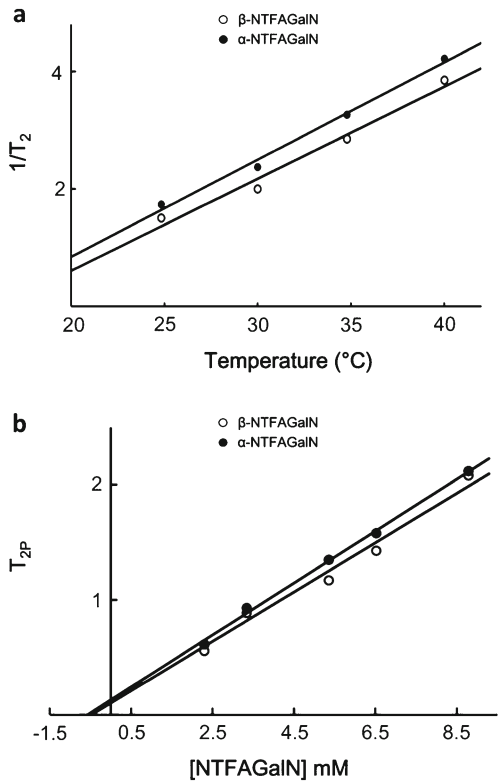

Fig. 3 **a** Line broadening of the α and β anomers of NTFAGalN as a function of temperature in the presence of WBA I (1.45 mM as monomer). **b** Plot of concentration of α and β anomers of NTFAGalN *versus* the reciprocal line broadening, T_{2p} at 25 °C

Consequently, the free protein in the sample is given as follows

$$[P]_t = [P]_f + [P_\alpha] + [P_\beta] \quad (4)$$

where, the protein bound to α and β anomer is represented by $[P_\alpha]$ and $[P_\beta]$, respectively, while $[P]_f$ is the unbound protein and $[P]_t$ is the total protein. The bound fraction (f) for the α and β anomers was expressed as in Eq. 5 and 6 respectively,

$$f_\alpha = K_\alpha[P]_t/\left(\left(1 + K_\alpha[L_\alpha] + K_\beta[L_\beta]\right)\right) \quad (5)$$

$$\begin{aligned} f_\beta &= K_\beta[P]_t/\left(\left(1 + K_\beta[L_\beta] + K_\alpha[L_\alpha]\right)\right) \\ f_\alpha &= K_\alpha[P]_t/\left(\left(1 + K_\alpha[L_\alpha] + K_\beta[L_\beta]\right)\right) f_\beta = K_\beta[P]_t/\left(\left(1 + K_\beta[L_\beta] + K_\alpha[L_\alpha]\right)\right) \end{aligned} \quad (6)$$

and the association constants of the α and β anomers can be calculated by

$$K_\alpha\left(= k^\alpha_{+1}/k^\alpha_{-1}\right) \text{and } K_\beta\left(= k^\beta_{+1}/k^\beta_{-1}\right)$$

Substituting for f_α and f_β in Eq. 1

$$1/T_{2p} = K_\alpha[P]_t/(\tau_m + T_{2m})\left(1 + K_\alpha[L_\alpha] + K_\beta[L_\beta]\right) \quad (7)$$

which, on rearrangement, as shown earlier [20, 32], gives.

$$\begin{aligned} T_{2p} &= (\tau_m + T_{2m})\left(1 + K_\alpha[L_\alpha] + K_\beta[L_\beta]\right)/K_\alpha[P]_t \\ &= (\tau_m + T_{2m})/K_\alpha[P]_t + (\tau_m + T_{2m})\left(K_\alpha + K_\beta R_s\right)[L_\alpha]/K_\alpha[P]_t \end{aligned} \quad (8)$$

where, $R_s = ([L_\beta]/[L_\alpha])$ is the ratio of anomers at the equilibrium.

From a plot of anomer concentration vs T_{2p}, the respective linewidth, the negative intercept on X-axis gives its dissociation constant K_D (=1/K_a) while the Y intercept yields the value of the residence time, namely τ_m (=1/k_{-1} and $k_{+1} = K_a$ X k_{-1} [20, 30–32] (Fig. 3b & Table 1).

From the temperature dependence of the rate constants, the activation parameters for the reaction were obtained using Eq. 9 and 10 [33].

$$E_\alpha = \Delta H^* + RT \quad (9)$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (10)$$

$$\ln(k/T) = -\Delta H^*/RT + \Delta S^*/R + \ln(k^*/h) \quad (11)$$

where ΔH*, ΔG* and ΔS* are the activation enthalpy, activation free energy and activation entropy, respectively, k is the appropriate rate constant, k' and h are the Boltzmann and the Planck's constant respectively. The activation parameters for the anomers of NTFAGalN and MeαNTFAGalN are qualitatively similar (Table 1) while those of MeβNTFAGalN are strikingly different from those of the former. This becomes quite clear by an examination of the energy diagram for MeαNTFAGalN and MeβNTFAGalN shown in Fig. 4.

Discussion

Trifluorogalactosamine's resonances broaden upon binding to WBA I

A systematic thermodynamic and kinetic analysis of the interaction of WBA I with the mixed anomers of NTFAGalN, MeαNTFAGalN and MeβNTFAGalN reported here reveals several interesting features pertaining to its ligand binding properties. The absence of chemical shift differences between the free and bound anomers of NTFAGalN and MeβNTFAGalN highlights the fact that their trifluoroacetyl group resides in a magnetically equivalent environment – a situation reminiscent of the binding of sugar anomers by *Artocarpus integrifolia* agglutinin [20] and peanut agglutinin [34] and unlike that observed for *N*-acetylglucosamine - lysozyme interaction [29, 35]. Since our studies show a pronounced increase in linewidth with increase in temperature, the line broadening effects are governed by residence time ($\tau_m >> T_m$) of the anomers at the binding site and hence the kinetic parameters for the interaction of lectin with sugar could be evaluated as per the procedures reported by Swift and Connick [30]. The values of K_a for NTFAGalN, MeαNTFAGalN and MeβNTFAGalN are in good agreement with earlier fluorescence studies [23]. The value of ΔH determined for MeαNTFAGalN is 30 % more than that obtained earlier for MeαGalN (methyl-α-*N*-acetylgalactosamine) using an indirect fluorescence assay [23]. This difference reflects a favorable contribution of the trifluoroacetyl group to the binding process.

The second order rate constants for the binding of monosaccharides to WBA I are several orders of magnitude slower than the diffusion controlled reaction indicating that the

Table 1 Kinetic and activation parameters for WBA I – trifluoroacetylgalactosaminide interaction

Sugar anomer	T °C	K_a (M^{-1})	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	ΔG_{on} kJ mol^{-1}	ΔG_{off}	ΔG^o	ΔH_{on}	ΔH_{off}	ΔH^o	ΔS_{on} J mol^{-1} K^{-1}	ΔS_{off}	ΔS^o
α-NTFAGalN		X 10^2	X 10^3										
	25	25	2.13	8.52	48	67.6	−19.6	36.6	32.2	−26.6	−38.3	−15	−23.3
	30	22.2	3.21	14.5									
	35	19	4.06	21.38									
	40	16.6	4.85	29.33									
β-NTFAGalN	25	21.9	2.03	9.3	50	64.8	−14.8	38.6	64.8	−26.2	−33	−9.1	−23.9
	30	17.8	2.71	15.28									
	35	15.7	3.46	22.06									
	40	12.6	3.99	31.68									
MeαNTFAGalN	25	133.3	2.37	1.78	48.6	71.5	−23	27.4	68	−40.6	−17	−11.9	−59.1
	30	100	3.09	3.09									
	35	76.9	3.59	4.68									
	40	62.5	4.16	6.67									
MeβNTFAGalN	25	2.5	0.4	15.91	52.4	66.1	−13.7	3.4	25.6	−22.5	−164.7	−135.9	−28.8
	30	2.2	0.42	19.25									
	35	2	0.43	21.5									
	40	1.6	0.45	28.2									

reaction occurs through the formation of an intermediate (PL_i) [12, 16, 17, 19, 23].

$$P + L \underset{k_{+1}}{\overset{k_{-1}}{\leftrightarrow}} PL_i \underset{k_{+2}}{\overset{k_{-2}}{\leftrightarrow}} PL$$

where, $K_1=[PL_i]/\lceil P \rceil \lceil L \rceil = k_{+1}/k_{-1}$; $K_2=[PL]/\lceil PL_i \rceil = k_{+1}/k_{-1}$

Given that the first step is much faster than the isomerization step and $k_{+2} >> k_{-2}$, the residence time of the ligand on the protein is given by

$$\tau_m = 1/K_D = 1/k_{-2}$$

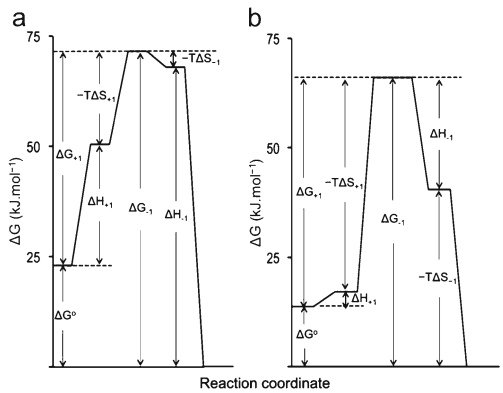

Fig. 4 Thermodynamic/Kinetic profile for the binding of (**a**) MeαNTFAGalN and (**b**) MeβNTFAGalN to WBA I

The apparent association constant can therefore be obtained as

$$[PL_i][PL]/[P][L] = K_1 + k_1k_2;\ K_1K_2 = [K_1(1 + K_2)]$$

The apparent rate constant is then expressed as

$$k_{on} = K_1K_2/k_{-2} = k_2k_1$$

The increase in the apparent rate constant with temperature for the α and β anomers of NTFAGalN and MeαNTFAGalN indicates that both k_1 and k_2 increase with increasing temperature resulting in temperature dependence of their observed rate constants. For MeβNTFAGalN on the other hand, k_2 does not increase significantly with temperature and k_1 diminishes explaining the temperature independence of the observed rate constants for it's binding to WBA I.

If $k_2 >> k_{-1}$then the formation of $[PL_i]$ can not be detected as indeed appears to be the case here. Hence, it is possible to observe only the overall reaction. These kinetic data thus can be explained as follows. As a first step, a diffusion controlled reaction would entail a loose association of the ligand (s) with the protein. The second step involves fitting of the sugars in the combining site of the protein. This step in protein-sugar interactions is limited in most cases by either energetic factors or by the requirement of a specific configuration of the reactants in some instances. WBA I-sugar interactions are limited by both of these factors and are clearly related to the structures

of the ligand and have not been noted in other lectin-sugar interactions so far [12, 16, 17, 19, 23] as would become apparent from the discussion later.

Activation parameters explain different modes of the binding of galactosamines

Inspection of the activation parameters for the binding of α and β anomers of NTFAGalN and MeαNTFAGalN reveal that the entropy of activation for them is small. This indicates that their association with the lectin does not involve a highly ordered transition state. Therefore, these saccharides can approach the binding pocket in several ways in contrast to the binding of the complementary monosaccharaides to peanut agglutinin, concanavalin A and *Artocarpus integrifolia* lectins [16, 20, 34]. The principal barrier for their interaction is enthalpic in nature, which reflects the expenditure of energy necessary for breaking the hydrogen bonds between the solvent and sugar and/or PNA, concanavalin A and the *Artocarpin integrifolia* lectins for recognition of their respective ligands and the formation of new ones between the sugar hydroxyls and complementary loci in these proteins. Such a situation has also been observed for the interaction of WBA I with 4-methylumbelliferyl α and β-*N*-acetylgalactosaminiside by stopped-flow fluorescence spectroscopy [17]. On the other hand, the present studies show the binding of MeβNTFAGalN is associated with a large unfavorable entropy of activation. This suggests that a highly ordered transition state is involved in the interaction of MeβNTFAGalN with WBA I, indicating that MeβNTFAGalN can approach the combining region of WBA I in a limited number of orientations only. In other words, the binding of MeβNTFAGalN to WBA I is sterically hindered.

It is instructive to consider these data in conjunction with the structure of the combining site of WBA I (Fig. 5) [36, 37]. Like all legume lectins the carbohydrate-binding site of WBA I is made up of four loops, designated as loops A to D, which come from four sequentially separate regions [36]. The observed residues in loops A and B contribute to the binding process by hydrogen bonding to a distinct pair of sugar-hydroxyls and thus, constitute the framework region of the combining site of legume lectins. These include Oδ1 and Oδ2 of Asp87 from loop A hydrogen bonding to C4 and C3 hydroxyls groups, respectively, of galactose; the backbone NH group of Gly 105 from loop B forms a hydrogen bond with the C4 sugar hydroxyls; Asn 128 ND2 from loop C, and the backbone NH group of Asp 212 from loop D forms a hydrogen bond with the C4 hydroxyl of galactose residue. Additionally, NE2 atom of His 84 from loop D forms Hydrogen bonds with C6 hydroxyl group of the galactopyranoside. A water mediated hydrogen bond links C2 hydroxyl group with ND2 atom of Asn 128 and the backbone N atom of Gly 105. Further, the galactose residue stacks against a Phe 126 side chain from loop C. The acetamido group of GalNAc experiences favorable non-polar interactions with a hydrophobic pocket lined with Tyr 106 and Trp 130. It is the variability in the size of the loop C, and more so of loop D that encodes their ultimate monosachharide specificities [36]. The length of the D loop of WBA I is the largest reported so far amongst the legume lectins [36]. The exceptionally long D loop of WBA I and especially the side chain of Ser 214 is oriented in a manner so as to sterically hinder the approach of MeβNTFAGalN, as evident from our NMR measurements. Thus, our NMR data are consistent with the orientation of the amino acid side chains in the combining site of WBA I [37] and highlights the role of substituents on carbohydrate receptors in controlling their recognition by complementary lectins.

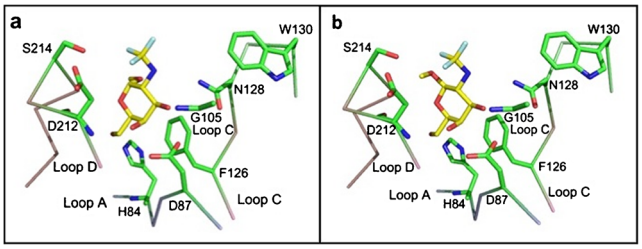

Fig. 5 Schematic representation of the interaction of (**a**) MeαNTFAGalN and (**b**) MeβNTFAGalN. Loops **a**, **b**, **c** and **d** involved in carbohydrate recognition are shown. Single letter codes for residue interaction with sugar are used while the numbers denote their position in WBA I sequence. Thus H84 and D87 loop **a**, G105 from loop **b**, F126, N128 and W130 from loop **c** and D212, S214 and G215 from loop **d** constitute the combining site residues in WBA I. Solid ball and stick models are used for representation. The interactions between the sugar hydroxyls with the combining site residues are discussed in the text but are omitted from the figure for clarity. While the methyl group in β-orientation has unacceptable steric clash with the side chain of S214, in α-orientation it is devoid of such a hindrance

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