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THE BINDING OF MONOSACCHARIDES TO WHEAT GERM AGGLUTININ: FLUORESCENCE AND NMR INVESTIGATIONS

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The synthesis of *N*-acetyl- and *N*-trifluoroacetyl-glucosaminides was reported. The interaction of these compounds with wheat germ agglutinin, a plant lectin specific for *N*-acetyl-glucosamine and sialic acid, was investigated by two complementary approaches : ^{1}H and ^{19}F NMR, and fluorescence spectroscopy. This last technique relies on the existence of a competitive equilibrium involving the protein, the ligand and *O*-(methylumbelliferyl)-*N*-acetyl-glucosaminide, a fluorescent saccharide. The binding constants and the chemical shifts in the complex were determined and were related to the protein structure.

Sugar protein interactions are involved in many biological phenomena and are currently under intense study. Sugar binding proteins, called lectins (1), have been identified in all living organisms, from viruses to mammals, and from bacteria and fungi to plants (for recent reviews, see 2-6). Lectins are proteins, or glycoproteins, devoid of enzymatic activities which selectively bind simple carbohydrates, polysaccharides and the sugar moieties of glycoconjugates.

We have investigated the specific interaction of *N*-acetyl-glucosamine and of several derivatives with wheat germ agglutinin (WGA). This lectin can agglutinate malignant cells and protease treated cells (7,8). It is used to purify glycoconjugates by affinity chromatography (2) and to fractionate cell populations (9-12). Two complementary spectroscopic methods have been utilized in this work : proton and fluorine nuclear magnetic resonance and fluorescence spectroscopy. Fluorine NMR is potentially a very useful tool in the field of

Abbreviations : WGA : wheat germ agglutinin ; αMeGlcNTFA : *O*-methyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranoside ; βMeGlcNTFA : *O*-methyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside ; βMeGlcNAc : *O*-methyl-2-deoxy-2-acetamido-β-D-glucopyranoside ; βMUFGlcNAc : *O*-(4-methylumbelliferyl)-2-deoxy-2-acetamido-β-D-glucopyranoside ; 6-deoxyGlcNAc : 2,6-dideoxy-2-acetamido-D-glucose ; DSS : sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

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sugar-protein interactions (13,14). Since saccharides can be labeled specifically at a single site, spectra will be very much simpler than with proton NMR. Furthermore, the ^{19}F nucleus has a spin of 1/2 (and therefore desirable magnetic properties) and has a sensitivity almost equal to that of ^{1}H. Last, the fluorine atom is not very bulky, so that replacing a CH_3 group by CF_3 should not seriously alter the binding mechanism.

Since saccharides are not by themselves luminescent, and because the effect of monosaccharide binding on the protein intrinsic fluorescence is weak, we have resorted to competition with a fluorescent derivative, *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide (MUFGlcNAc). In this paper, we describe the synthesis of substrates, equilibrium investigations using either NMR or fluorescence spectroscopies, the derivation of binding constants and finally we discuss our results.

MATERIALS AND METHODS

a) Synthesis of *O*-methyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranoside (αMeGlcNTFA). This compound is obtained using the following steps. Methanolysis of 2-deoxy-2-acetamido-glucopyranose according to (15,16) yields methyl-2-deoxy-2-aminoglucose as a mixture of anomers. These may be separated by chromatography on an anionic ion exchange column, as previously described (17). The α anomer is then trifluoroacetylated by action of *S*-ethylthiotrifluoroacetate (18).

b) Synthesis of *O*-methyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (βMeGlcNTFA). Glucosamine is trifluoroacetylated as described in (a). The resulting 2-deoxy-2-trifluoroacetamido-D-glucose is peracetylated. The α-anomer is isolated by chromatography on silicagel and is brominated. 1-bromo-2-deoxy-2-trifluoroacetamido-3,4,6-tri-*O*-acetyl-α-D-glucopyranose is reacted with methanol, in the presence of freshly prepared silver oxide. *O*-methyl-2-deoxy-2-trifluoroacetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside is purified by chromatography on silicagel and de-*O*-acetylated using sodium methanolate.

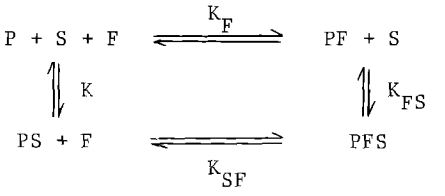
c) Other glucosamine derivatives were prepared according to established procedures : *O*-methyl-*N*-acetyl-β-D-glucosaminide, 1,3-di-*O*-methyl-*N*-acetyl-α-D-glucosaminide (19), *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide (20). *N*-acetyl-6-deoxyglucosamine was a gift from Prof. N. Sharon.

d) Wheat germ agglutinin, prepared as in (21), was purchased from Pointet-Girard IBF-Reactifs (Villeneuve-la-Garenne, France) and used as received. Stock solutions were prepared in 0.15 M NaCl, 0.05 M phosphate buffer, pH 7.2. Samples were filtered (Millipore filter : HAWP 0.45 μm pore diameter) before each experiment.

e) Fluorescence spectra were recorded on a Fica MK II spectrofluorimeter. The excitation wavelength was 338 nm, the isosbestic point of WGA-MUFGlcNAc mixtures, and the fluorescence was detected at 380 nm. The optical density of sample at the excitation wavelength never exceeded 0.05. NMR spectra were recorded in the Fourier transform mode with a Bruker WH 90 spectrometer. This equipment was also used for fluorine work, at a nominal frequency of 84.66 MHz. Samples for ^{1}H spectroscopy were lyophilized and dissolved in 99.8 % D_2O. To further reduce the ^{1}H ^{2}HO signal, a selective 180° pulse (0.1 sec duration) was applied, using the decoupler at the water frequency, followed 2-4 seconds later by a non-selective 90° measure pulse. ^{1}H chemical shifts are reported relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). So-

lutions for ^{19}F NMR must contain ^2H_2O to provide a lock signal. However, because fluorine chemical shifts depend on the solvent isotopic composition (22), the $^2H_2O/H_2O$ ratio was kept constant (1:2, v/v). We have used trifluoroethanol as an internal reference.

f) Processing of results. It has been shown conclusively (23-25) that WGA comprises four sugar binding sites for a Mr ca. 36000. We have used a non linear least-squares program to compute equilibrium constants and limiting chemical shifts (or fluorescence intensities) and their possible errors. Competitive binding experiments were performed using a program and then a graphical method (26) to provide a convenient appraisal of the quality of the fit. Letting P stand for a protein site, S for a nonfluorescent sugar and F for a fluorescent sugar, the model assumes the following reaction scheme, which also defines the various binding constants.



It can be shown (26) that a plot of

$$\left[\frac{P_0}{[PF] + [PFS]} - 1\right] \cdot \left[1 + K_{FS}[S]\right] \cdot [F]$$

versus [S] will be a straight line, of slope K/K_F, intercepting the x and y axes respectively at distances K_F^{-1} and $-K_S^{-1}$ from the origin.

We assume specifically in the following that WGA comprises four independent and equivalent sites. Ligands are in fast exchange, on the NMR time scale, between bound and free environments. The observed chemical shifts are then averages of values corresponding to the two environments (27).

RESULTS

a) Binding of MUFGlcNAc to WGA

Results obtained by us and others for this ligand are given in Table 1. The values of the affinity constant agree within experimental errors. The re-

Table 1 - Binding constants derived from competition experiments with MUFGlcNAc

Ligand	K_S	$K_F \times 10^{-4}$	K_{FS}	Ref.
MUFGlcNAc		2.67		(13)
		1.98		(25)
		2.86		(28)
1,3-di-*O*-methyl-α-GlcNAc	18	2.60	0	this work
6-deoxy-GlcNAc	785	2.76	0	"
βMeGlcNAc	445	2.72	0	"
βMeGlcNTFA	330	2.85	0	"
αMeGlcNTFA	420	2.63	50	"

Notes : 1) All binding constants are expressed as M^{-1}. 2) K_F is the binding constant for MUFGlcNAc, either alone or in competition with a nonfluorescent sugar. K_{FS} is related to the eventual formation of a ternary complex.

lative fluorescence intensity of the complex (F_{bound}/F_{free}) shows some small discrepancies. These could be due to different experimental conditions, or to small differences in the WGA samples. Furthermore, at low ligand concentrations, the Scatchard plot is no longer linear, showing that our simple binding model is probably not valid in that range.

b) Competitive binding of monosaccharides

The data concerning monosaccharides in competition with MUFGlcNAc are also collected in Table 1. Two examples of data plotted according to (26) are shown in figures 1 and 2. The binding constant of MUFGlcNAc (K_F), which is a free parameter in the computations, is in each case found close to the value obtained for this ligand alone, showing the good quality of the data. In one

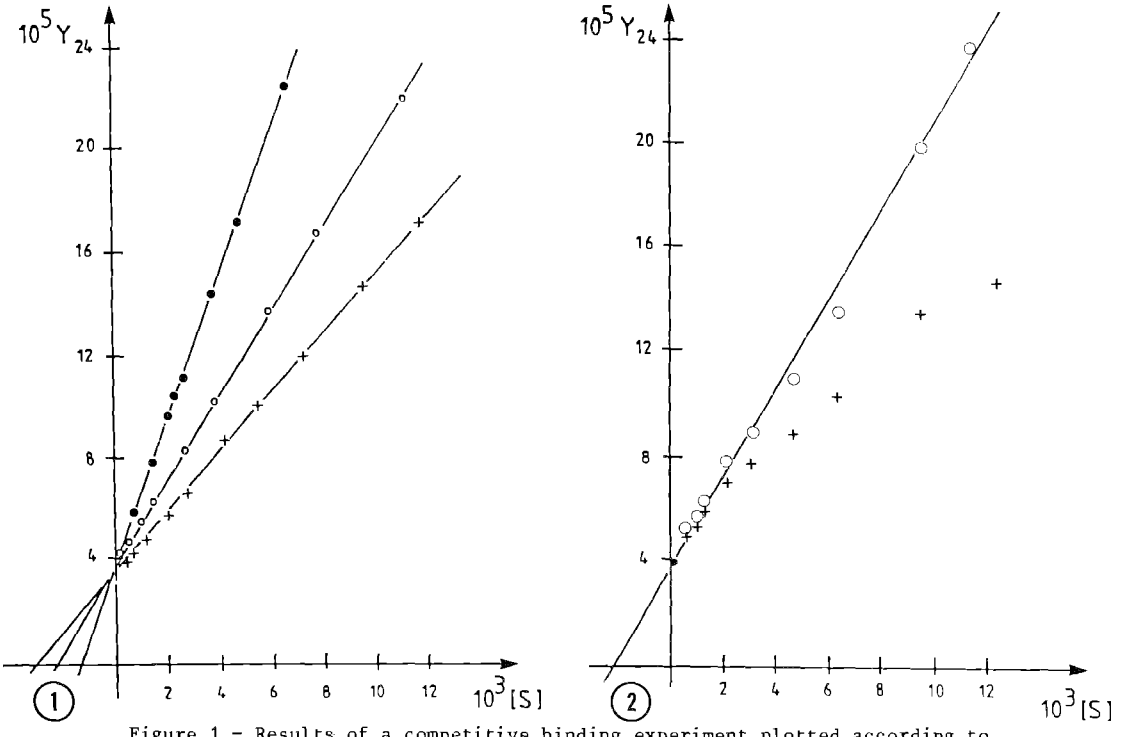


Figure 1 - Results of a competitive binding experiment plotted according to (26). The quantities

$$Y = \left[\frac{P_0}{[PF] + [PFS]} - 1\right] \cdot \left[1 + K_{FS}[S]\right] \cdot [F],$$

and [S] are given in M^{-1}. Key : -o-o- βMeGlcNAc ; -+-+- βMeGlcNTFA ; -●-●- 6-deoxyGlcNAc. The parameter K_{FS} is set to 0.

Figure 2 - Results of a competitive binding experiment involving αMeGlcNTFA, plotted according to (26). The quantity Y is defined in the text and in the legend of figure 1. Key : -+-+- $K_{FS} = 0$; -o-o- $K_{FS} = 50\ M^{-1}$.

Table 2 - Chemical shifts (δ) of methyl or trifluoromethyl groups in several glucosamine derivatives. Values are given in ppm, positive downfield, with respect to DSS (proton) or trifluoroethanol (fluorine)

Compounds	-ND-CO-CH_3	-NH-CO-CF_3	1-*O*-CH_3	3-*O*-CH_3
GlcNAc	2.04	-	-	-
βMeGlcNAc	2.03	-	3.50	-
1,3-di-*O*-methyl-α-GlcNAc	2.03	-	3.38	3.50
βMeGlcNTFA	-	1.12	3.52	-
αMeGlcNTFA	-	1.47	3.39	-

case (αMeGlcNTFA), it was found necessary to invoke a ternary complex (with $K_{FS} = 50\ M^{-1}$).

c) NMR of monosaccharides

The chemical shifts of proton and fluorine nuclei in methyl and trifluoromethyl groups, for the free ligands, are reported in Table 2. Although the 1-*O*-CH_3 group is not directly involved in the binding, it is a convenient reporter group, because its signal appears in a spectral region not cluttered by protein lines. When monosaccharides interact with WGA, it is found that the linewidths of methyl protons decrease with increasing temperature, as shown in figure 3. This observation supports our assumption of fast exchange. Figure 4 shows Scatchard plots of the binding of several saccharides. Complete results are collected in Table 3.

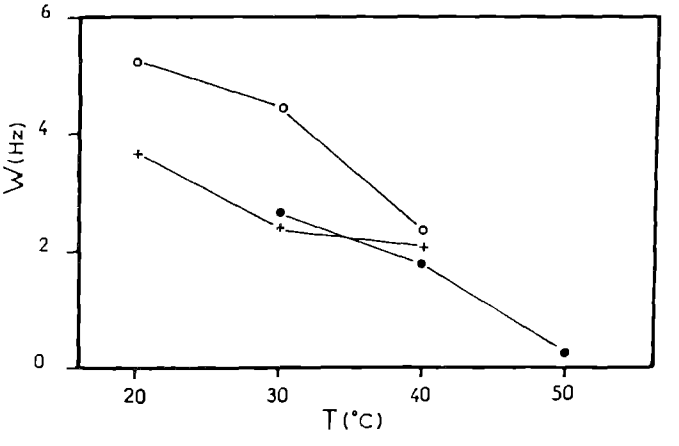


Figure 3 - Linewidth of the methoxy protons plotted as a function of temperature. The various samples and their concentrations were : -o-o- βMeGlcNTFA (6 mM), WGA (0.16 mM) ; -●-●- βMeGlcNAc (2.4 mM), WGA (0.2 mM) ; -+-+- αMeGlcNTFA (7.2 mM), WGA (0.16 mM).

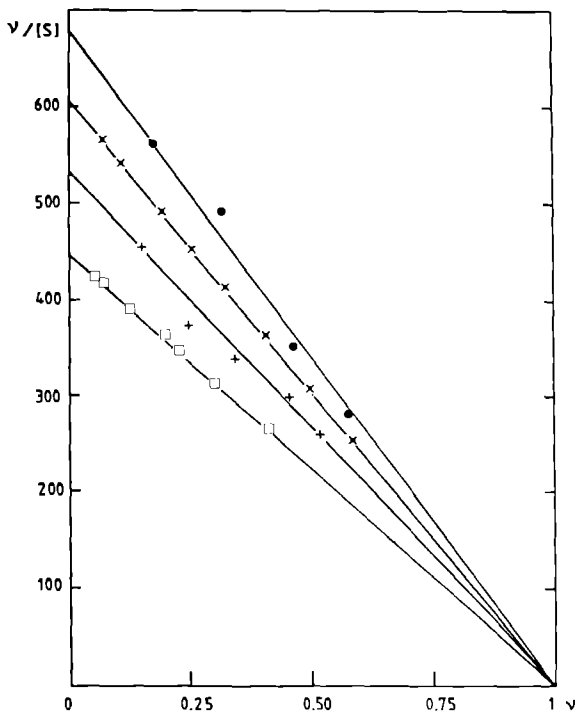


Figure 4 - Scatchard plots of the binding of βMeGlcNAc (-□-□-), βMeGlcNTFA (-+-+-), αMeGlcNTFA (-●-●-). These data are derived from the chemical shifts of 1-*O*-methyl groups. Points obtained from the chemical shifts of the NH-CO-CF_3 moiety of βMeGlcNTFA (-x-x-) are also shown.

As seen by ^{19}F NMR, αMeGlcNTFA apparently has a higher binding constant than other glycosides considered in this work. Several reasons can account for this observation. The smallness of observed shifts, along with an unfavorable signal to noise ratio means that the best parameters are not well defined. In fact, we can force a fit of our results, using for the binding constant K the

Table 3 - Chemical shifts in the complex (Δ) (in ppm, relative to the free ligand) and binding constants (K) for glucosamine derivatives at 303 K. In each case, results derived using either 1-*O*-methyl, 2-*N*-acetyl or 2-*N*-trifluoroacetyl signals are given

Ligand		K (M^{-1})	Δ
βMeGlcNAc	1-*O*-methyl	450	- 0.70
	acetamido	480	- 0.80
βMeGlcNTFA	1-*O*-methyl	535	- 0.39
	trifluoroacetamido	600	+ 0.54
αMeGlcNTFA	1-*O*-methyl	680	- 0.23
	trifluoroacetamido	2000	+ 0.26

value obtained by fluorescence measurements, i.e. K = 420 M^{-1}. The increase in the sum of squared residuals is only marginally significant. The optimal chemical shift $\Delta = \delta_{bound} - \delta_{free}$ is then 0.65 ppm.

DISCUSSION

The binding of monosaccharides to wheat germ agglutinin is well described by the simple model of four equivalents, non interacting sites. It is interesting to note that binding constants derived from NMR and fluorescence measurements agree rather well, in spite of a 10 to 100 fold difference in protein concentrations. Qualitatively, we find that the C_3-hydroxyl group is probably involved in the binding since the 3-methoxy derivative does not bind ($K=18\ M^{-1}$). A similar result was recently reported by Jordan *et al.* (14) who investigated by NMR the binding of *O*-methyl-3-deoxy-*N*-acetyl-α-D-glucosaminide and found a very little interaction. Although this experiment was conducted close to conditions where WGA dissociates in monomers ($pH_{1/2} = 4$) (29), the results confirm the fact that the 3-hydroxyl group does appear to be involved. The protein is able to discriminate between α and β anomers of large ligands (30), the β anomer being preferred. In the present case, it seems that for fluorinated glucosaminides, the binding in the site of the α anomer is probably different from that of the β anomer.

All proton shifts are to high field of the free ligand resonances. Crystallographic investigations (31-33) have shown that the main binding site of WGA is lined by a tyrosine side chain. In the crystal the aromatic ring is very close to the methyl group of the acetamido residue of bound sialic acid. We may therefore assume that all *N*-acetylated monosaccharides bind in the primary site described in the X ray work. Methoxy groups of glucosaminides also undergo sizeable high field shifts, which can also be ascribed to the ring current effect of a neighbouring aromatic side chain. The residue which is involved is not known at the present time. Several lines of evidence, derived from spectroscopic experiments in solution, point to the presence of a tryptophan side chain close to the binding site (34,35). However, the X-ray data do not support this result. Solving this discrepancy will probably require the

establishment of the protein sequence. A further disagreement between solution and solid state studies concerns the number of sites per protein molecule. We have assumed the existence of four equivalent sites, as established by equilibrium dialysis experiments, whereas only two "primary" sites are seen in the crystal (32). This behaviour can be due to the molecular packing in the solid state, which either masks the two "missing" sites, or prevents ligand molecules from diffusing towards them.

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