PROTEIN–SUGAR INTERACTIONS

A nuclear magnetic resonance investigation of the binding of *O*-methyl-di-*N*-acetyl-β-chitobioside to wheat germ agglutinin (lectin)

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1. Introduction

Wheat germ agglutinin (WGA) is a plant lectin which can agglutinate various animal cells, particularly malignant cells and normal protease treated cells [1–3]. Agglutination is inhibited by *N*-acetyl-glucosamine and its β-1–4-linked oligomers [2–4]. Under physiological conditions, WGA is a dimer. In acidic media, it dissociates in 2 subunits mol. wt 18 000 [5,6]. The dimer binds 4 ligands containing the *N*-acetylglucosamine moiety with equal affinity. In other words, each protomer binds saccharides at 2 spatially different but chemically equivalent locations [5,7,8].

Lectin–sugar interactions have been studied by fluorescence [9–13], absorption [13] and circular dichroism [14] techniques. Although nuclear magnetic resonance (NMR) has been used extensively to study the interactions of sugars with lysozyme (see ref in [15]) and concanavalin A [16], only one report so far has been devoted to WGA [17]. The binding constant of *N*-acetyl glucosamine was determined [17] and showed that the methyl group resonance was strongly shifted upon binding. In this communication, we present results of an ^1H NMR investigation of the binding of 1-*O*-methyl-di-*N*-acetyl-β-chitobioside ($CBOCH_3$) and of 1-*O*-methyl-tri-*N*-acetyl-β-chitotrioside ($CTOCH_3$) to WGA. The use of methyloside derivatives, in effect removing the anomeric equilibrium, greatly simplifies the analysis.

Abbreviations: NMR, nuclear magnetic resonance; WGA, wheat germ agglutinin; $CBOCH_3$, 1-*O*-methyl-di-*N*-acetyl-β-chitobioside; $CTOCH_3$, 1-*O*-methyl-tri-*N*-acetyl-β-chitotrioside; DSS, sodium 2,2-dimethyl-2-silapentane-sulfonate

2. Materials and methods

WGA, prepared as in [18], was purchased from Industrie Bioliogique Française (Gennevilliers). D_2O (99.85%) was obtained from Commissariat à l'Energie Atomique (Saclay). $CBOCH_3$ and $CTOCH_3$ (fig.1) were prepared by acetolysis of chitin and isolation of the per-*O*,*N*-acetyl chitobiose and per-*O*,*N*-acetyl chitotriose by silicagel column chromatography [19]; then the acetochloro-derivatives [20] were reacted with methanol [21]. *O*-Acetyl groups were removed by catalytic methanolysis with 0.01 M sodium methoxide in methanol at 4°C for 24 h. $CBOCH_3$ and $CTOCH_3$ were crystallized from their methanolic solution. The melting point and optical rotations of the 1-*O*-methyl-di-*N*-acetyl-β-chitobioside were: m.p. 259–260°C, dec; (Lit.: freeze-dried glycoside; 287–288°C, [22]); $[\alpha]^{25}_{546} = -33°C$; $[\alpha]^{25}_{589} = -27°C$ (ca. 0.5, H_2O). The values for 1-*O*-methyl-

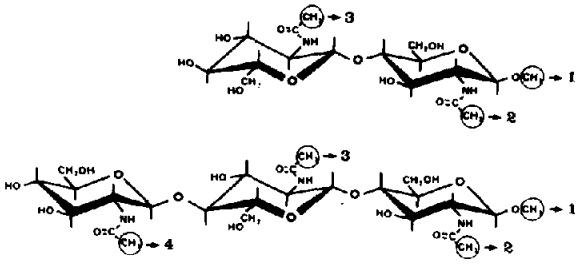

Fig.1. The structure of the two ligands: 1-*O*-methyl-di-*N*-acetyl-β-chitobioside ($CBOCH_3$) (upper panel) and 1-*O*-methyl-tri-*N*-acetyl-β-chitotrioside ($CTOCH_3$) (lower panel). Numbers refer to the protons of the methoxy (1) and acetamido (2, 3, 4) residues.

tri-*N*-acetyl-β-chitotrioside were: m.p. 330°C, dec; (Lit., freeze dried glycoside : 306–310°C, [22]); $[\alpha]_{546}^{25} = -37°C$; $[\alpha]_{589}^{25} = -30°C$ (ca. 0.6, H_2O). Stock solutions were prepared by dissolution of freeze-dried saccharide or WGA in a D_2O buffer containing 0.05 M sodium phosphate and 0.1 M sodium chloride. DCl or NaOD were added to bring the pH meter reading to 7.2.

Proton magnetic resonance spectra were recorded at 90 MHz, in the Fourier mode. A modified WEFT method [23] was used to attenuate the residual HDO signal. In this modification, the decoupler is gated to provide a selective 180° pulse at the HDO frequency. The 90° measuring pulse is applied when the HDO magnetisation almost vanishes. Under our conditions, this method proved to be more effective and versatile than the original WEFT prescription. The spectrometer frequency was locked on the D_2O resonance and sodium 2,2-dimethyl-2-silapentane-sulfonate (DSS) provided an internal reference signal. A typical spectrum was recorded using 8192 data points and a spectral width of 900 Hz. Depending on the concentrations, 200–2000 transients were accumulated. Digital filtering was applied, resulting in an equivalent broadening of 0.5 Hz. All the resonance shifts reported in this paper are upfield. They are differences between the resonance frequencies of saccharide protons in the presence and in the absence of WGA.

3. Results

Figure 2 shows part of the NMR spectra of $CBOCH_3$, free and in the presence of varying amounts of WGA. Three lines are prominent in the spectrum of free CB. They are assigned to the methoxy group (1) (3.49 ppm), to the 'reducing end' acetamido group (2) (2.02 ppm), and to the second acetamido residue (3) (2.07 ppm) respectively [22,24]. The chemical shifts and linewidths of these resonances are temperature dependant, for given WGA and CB concentrations. The linewidths decrease with increasing temperature. The behavior of the *O*-methyl chemical shift is exemplified in fig.3. We interpret these results as showing that fast exchange (on the NMR time scale) sets in above 300°K. On the other hand, above 320°K, the protein probably begins to denature,

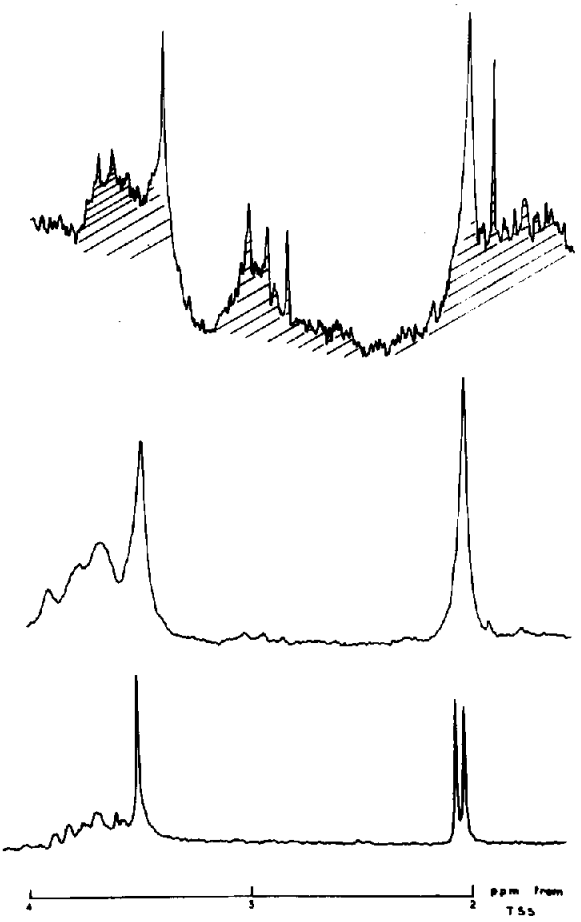

Fig.2. Partial ^1H NMR spectra. Lower trace: free $CBOCH_3$, conc. 1.1×10^{-2} M. Middle trace: $CBOCH_3$ (3.8×10^{-3} M) in the presence of WGA (4.6×10^{-5} M). Upper trace: $CBOCH_3$ (5.4×10^{-4} M) with WGA (6.9×10^{-5} M). Solutions in a D_2O buffer containing 0.05 M sodium phosphate and 0.1 M sodium chloride. The pH was 7.2 and the temp. 320°K.

$CBOCH_3$ is released and the resonance frequency reverts to a value close to that of the free compound.

We analyze our results in terms of a simple equilibrium:

$$CBOCH_3 + WGA \rightleftharpoons CBOCH_3 - WGA.$$

When fast exchange obtains, the observed chemical shifts are averages of the bound and free shifts, the weights being the relative concentrations of the two species. We have used a least squares program [25] to

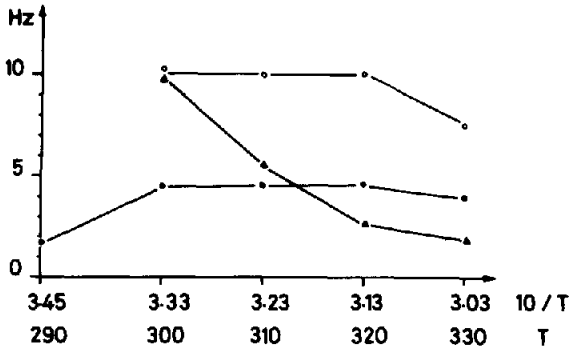

Fig.3. Temperature dependance of the shift and width of protons resonance of the methoxy group of $CBOCH_3$. Shift (○—○) and linwidth (▲—▲) for $CBOCH_3$ (5.4×10^{-4} M) in the presence of WGA (6.9×10^{-5} M). Shifts (●—●) for $CBOCH_3$ (2.9×10^{-3} M) in the presence of WGA (5.25×10^{-5} M).

fit our results to the above model. A typical analysis is summarized in table 1, where the upfield shifts of the methoxy protons are considered, at 320°K. The last column gives the computed shifts (relative to the resonance of the free compound) for the best values of the parameters, $K = 700\ M^{-1}$ and $\delta^{bound}_{OMe} - \delta^{free}_{OMe} = 81.5$ Hz. Complete results, as functions of temperature, are shown in table 2.

At low concentrations and for the lowest temperatures, the methoxy resonance is noticeably broadened. The rate constant for the dissociation of the protein–sugar complex can be found from the linewidth W by use of the following formula [26]:

$$k_d = 4\pi x_{PS} x_S^2 \frac{\Delta^2}{W - W_0}$$

Table 1
Observed and computed shifts of methoxy proton in $CBOCH_3$ in the presence of WGA at 320°K

WGA (10^5 M)	$CBOCH_3$ (10^3 M)	$\delta_{obs} - \delta_{free}$ (Hz)	$\delta_{calc} - \delta_{free}$ (Hz)
4.6	3.81	3.2	2.85
5.2	2.86	4.3	3.9
5.8	1.90	4.75	5.5
6.4	0.95	8.5	8.2
6.9	0.54	10.2	10.3

Table 2
Best values of apparent association constants and maximum shifts ($\delta_{bound} - \delta_{free}$) of methoxy protons for the binding of $CBOCH_3$ to WGA

Temp. (°K)	Association constants ($l.mol^{-1}$)	Maximum shifts
300	1100 ± 100	65 ± 5
310	950 ± 50	71 ± 5
320	700 ± 50	81.5 ± 3
330	300 ± 50	100 ± 10

where $\Delta = \delta^{bound}_{OMe} - \delta^{free}_{OMe}$ (in Hz), x_{PS} and x_S are the mole fractions of bound and free sugar respectively, and W_0 is the linewidth for the free ligand. Using previously computed values of x_{PS}, x_S and Δ, we obtain the following estimates: $k_d = 700 \pm 50\ s^{-1}$ at 300°K and $k_d = 2300 \pm 300\ s^{-1}$ at 310°K. From these values, one derives the following kinetic association constants: $k_a = 7.5 \times 10^5\ l \cdot mol^{-1} \cdot s^{-1}$ at 300°K and $k_a = 2.2 \times 10^6\ l \cdot mol^{-1} \cdot s^{-1}$ at 310°K, much smaller than the diffusion-limited value in water ($10^{10}\ l \cdot mol^{-1} \cdot s^{-1}$).

The acetamido resonance shifts are much smaller than those of the methoxy group. The resonance of acetamido (2) undergoes an upfield shift which is at most 3 Hz. Using the association constants given above, we compute that $\delta^{bound}_{NHCOMe} - \delta^{free}_{NHCOMe} \cong 30$ Hz. Acetamido group (3) gives rise to a signal that is strongly broadened, even at the highest sugar/protein ratios. This signal also shifts, but the poor signal: noise ratio precludes any quantitative study. One may conclude that this resonance is strongly perturbed when the ligand binds to the protein.

The line shifts observed for $CTOCH_3$ are very small, probably because fast exchange is not reached

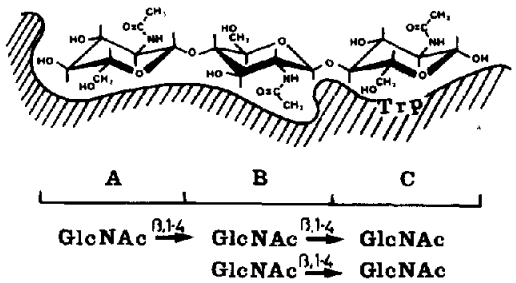

Fig.4. Proposed structure of the sugar binding site of WGA.

under our conditions. The acetamido residues of $CTOCH_3$ give 2 lines, at 2.02 ppm (3 protons, residue 2) and 2.06 ppm (6 protons, residues 3 and 4), respectively. In the presence of WGA, the intensity of the 2.06 ppm signal is drastically reduced (by half for a sugar/protein ratio of 10). One of the methyl groups 3 and 4, at least, interacts strongly with WGA.

4. Discussion

The association constants displayed in table 2 are smaller than those which where determined using fluorescence methods for the free saccharide: 4500 $l.mol^{-1}$ [9,12] and for $CBOCH_3$: 7000 $l.mol^{-1}$. Furthermore, the constants which fit the NMR results decrease with increasing temperature, while those obtained from fluorescence spectra are temperature independent. In this connection, it should be noted that fluorescence and NMR deal with very different time scales. At the concentrations used in this study, a tightly bound sugar molecule would not give an observable NMR signal. Further, one set of data is derived from the perturbation of the NMR signal of a methoxy group located on the ligand, while the other set is found from the perturbation of the fluorescence of tryptophan located in the protein.

The discrepancies between the results obtained by different methods indicate that the binding of saccharides to WGA is probably more complicated than the simple model used in both investigations. A similar situation was encountered in the case of chitobiose binding to lysozyme [27]. The limitations of the present model should not prevent us from discussing, in qualitative terms, the nature of the protein–sugar interaction, to which we now address ourselves.

Previous studies of the binding of *N*-acetyl-glucosamine and of its β-1–4-linked oligomers to WGA [3,4,10,12,28] have shown that the binding site is composed of three subsites, labelled A, B, C. Each of these may accept one *N*-acetyl-glucosamine residue. We have shown that one tryptophan residue was located in subsite C [28]. Since $CBOCH_3$ enhances the fluorescence of tryptophan, one of its *N*-acetylglucosamine residues must be found in subsite C. The other must then be located in subsite B. From the work presented here, we may conclude that acetamido group 3 (for which the methyl signal is dramatically broadened) interacts strongly with subsite B. In contrast the methyl signal of acetamido (2) is little affected, even though this residue is to be found in subsite C, presumably close to tryptophan.

Turning now to $CTOCH_3$, we find that the methyl resonance of either acetamido group 3 or 4 is extensively broadened, while other acetamidomethyl resonances are unaffected. Considering these results and those of our previous fluorescence studies, we may suggest that methyl 3 is the one involved in the binding to subsite B, just as for $CBOCH_3$.

The data reported here, together with the results of our previous investigations support the schematic drawing of the WGA binding site which we propose in fig.4.

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