LECTIN–SUGAR INTERACTIONS: THE BINDING OF 1-*O*-METHYL-DI-*N*-TRIFLUOROACETYL-β-CHITOBIOSIDE TO WHEAT GERM AGGLUTININ

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1. Introduction

Wheat germ agglutinin (WGA), a plant lectin, can agglutinate various types of animal cells, malignant cells and some protease-treated cells [1–3]. These reactions can be inhibited by *N*-acetylglucosamine and its β 1–4 oligomers [2,4]. WGA, at neutral pH, is a dimer of M_r 36 000, bearing 4 carbohydrate-binding sites [5–7]. Physicochemical analyses of the binding have been devoted to the thermodynamics of fixation [8–11] and topography of the binding site [4,10–12].

Nuclear magnetic resonance (NMR) methods are well suited for these studies since they can provide local information on the groups involved in the binding and on their immediate environment. However, NMR is not a very sensitive technique and WGA is sparingly soluble at physiological pH. For significant determination of affinity constants and kinetic parameters, the ligand concentration range should extend above and below the protein concentration. Taking into account the binding constants, it is easy to see that the methyloside ^1H resonances will often be masked by the unsolved protein lines.

Fluorine magnetic resonance offers an attractive possibility of solving this problem. In fact, fluorine-labelled saccharides have been used to study interactions with lysozyme [13] and concanavalin A [14].

Here we report results concerning 1-*O*-methyl-di-*N*-trifluoroacetyl-β-chitobioside ($(CF_3)_2$CB). We used the easily obtained methyloside to simplify the analysis of binding experiments.

2. Materials and methods

WGA, prepared as in [15], was purchased from IBF-Réactifs Pharmindustrie. Succinylated WGA was obtained from WGA by two succinylation steps [16]. The degree of succinylation was evaluated as 96% by titrating free NH_2 groups with 2,4,6-trinitrobenzenesulfonic acid [17]. The di-*N*-trifluoroacetylchitobioside was prepared from the corresponding di-*N*-acetylchitobioside [11] in two steps. 1-*O*-Methyl-di-*N*-acetyl-β-chitobioside was *N*-deacetylated by anhydrous hydrazine, at 105°C for 72 h. The chitobioside was then reacted with *S*-ethyltrifluorothioacetate, in anhydrous methanol at room temperature for 24 h [18]. $(CF_3)_2$CB can then be crystallized from acetone–ether. Its melting point and optical rotations are 275–278°C and $[\alpha]^{25}_{546} = -41.3°$, $[\alpha]^{25}_{589.5} = -23.9°$ (c = 0.23, water), respectively. *O*-(4-methylumbelliferyl)-*N*-Acetyl-β-D-glucosaminide was a gift of Dr Delmotte [19].

Emission spectra were recorded at 25°C with a Fica MK II spectrofluorimeter as in [16]. The intrinsic protein fluorescence was excited at 295 or 305 nm, so that the absorbance of the sample remained <0.05. The label fluorescence was excited at 338 nm, the isosbestic point of WGA–*O*-(4-methylumbelliferyl) glucosaminide mixtures [10].

Fluorine NMR spectra were recorded at 84.66 MHz with a Bruker WH 90 Fourier transform spectrometer. The field was locked to the D_2O resonance. Stock solutions were prepared by dissolution of freeze-dried protein or saccharide in an H_2O–D_2O (7:3), 0.15 N NaCl, 0.05 M phosphate buffer (apparent pH 7.2). The fluorine chemical shifts were measured relative to a separate sample of trifluoroethanol in H_2O–D_2O (7:3). Samples (~430 μl) were contained in a microcell (Wilmad 529E-10). A typical spectrum was recorded using 4000 points and a spectral width of 600 Hz. Depend-

ing on the concentration, 1000–5000 transients were accumulated. A digital filter was used, resulting in a 0.5 Hz broadening.

3. Results

3.1. *Intrinsic fluorescence of WGA*

The fluorescence of WGA, when excited at >290 nm, is due to 4 tryptophan residues, each of these chromophores is located in a binding site [12]. When $(CF_3)_2CB$ binds to the lectin, this fluorescence is partly quenched. When the ligand is in rather low concentration (10^{-3}–10^{-5} M), the interaction is very well described by a binding to 4 equivalent and independent sites. The parameters are:

$$K_a = (6.94 \pm 1.8)\ 10^4\ \text{l/mol} \quad F_\infty/F_0 = 0.63 \pm 0.01$$

However, as the ligand concentration increases, further sugar fixation occurs (or some other process, such as protein aggregation, takes over). A Scatchard representation of a titration is shown in fig.1. Furthermore, the course of the binding depends on the protein concentration.

At this stage of the investigation, it is obvious that the di-*N*-trifluoroacetylchitobioside does interact with WGA, although we cannot propose a detailed model of the binding.

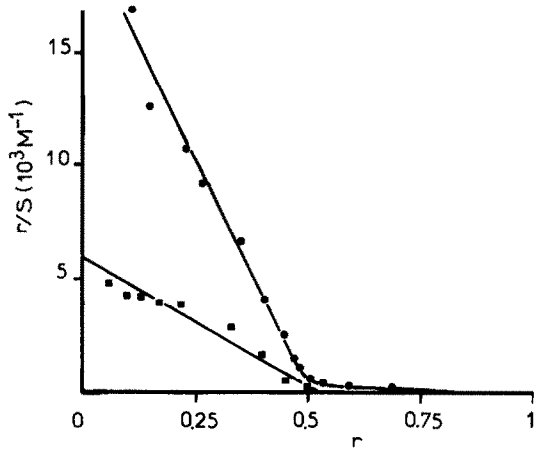

Fig.1. Scatchard plots of the binding of 1-*O*-methyl-di-*N*-trifluoroacetyl-β-chitobioside to wheat germ agglutinin for two protein concentrations (1.65 mg/ml (+) and 0.09 mg/ml (○). The binding constant reported in the text is relevant to the left-most points at low protein concentration.

3.2. *Competitive binding experiments*

O-(4-methylumbelliferyl)-*N*-acetyl-β-D-Glucosaminide (MUFGlcNAc) binds to WGA at 4 equivalent and independent sites [7], except, perhaps, at high sugar/protein ratios [20]. The binding parameters are [20]:

$$K_a = (2.67 \pm 0.1)\ 10^4\ \text{l/mol} \quad F_\infty/F_0 = 0.16 \pm 0.014$$

showing that the label fluorescence is quenched upon binding. This effect can be totally reversed by adding $(CF_3)_2CB$ to the solution showing that the two saccharides compete for the same sites. The results may be analyzed according to the following simple scheme:

$$(CF_3)_2CB + P \rightleftharpoons (CF_3)_2CB\text{-}P \qquad K_a$$

$$\text{MUFGlcNAc} + P \rightleftharpoons \text{MUFGlcNAc-}P \qquad K_f$$

where P stands for one of the lectin sites. The data were processed either graphically [21] or using a least-squares program, with similar results. The adjustable parameters were K_a, K_f, and the fluorescence yield of bound MUFGlcNAc. The fit to this model was not very good, as shown by the large error estimates. We found:

$$K_a = (1.3 \pm 1)\ 10^4\ \text{l/mol}^{-1}$$

$$K_f = (4.6 \pm 4)\ 10^4\ \text{l/mol} \quad F_\infty/F_0 = 0.06 \pm 0.1$$

A plot of our results according to [21] is shown in fig.2. Although the details of the competitive binding

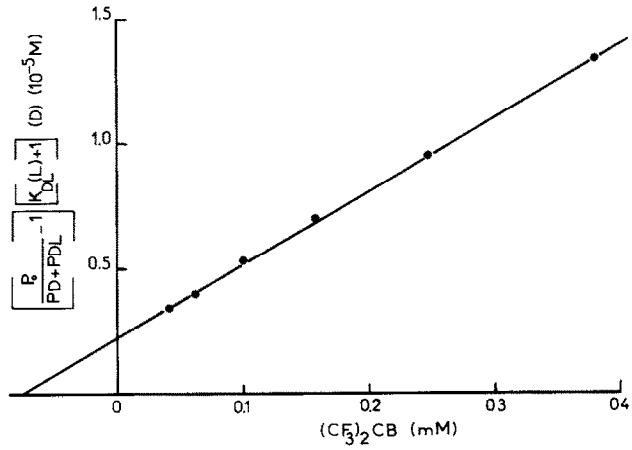

Fig.2. Competitive binding of 1-*O*-methyl-di-*N*-trifluoroacetyl-β-chitobioside and *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide to wheat germ agglutinin, plotted according to [21].

are not well understood, the phenomenon cannot be doubted. $(CF_3)_2CB$ is a specific ligand of WGA.

3.3. *Fluorine NMR spectroscopy*

The ^{19}F NMR spectrum of $(CF_3)_2CB$ shows two lines, at $\delta = 1.16$ and $\delta = 1.10$ ppm from trifluoroethanol, corresponding to the non-reducing (NR) and reducing (R) and CF_3 groups, respectively.

In order to improve sensitivity, most NMR experiments were done using succinylated WGA (SWGA), which is some 100-times more soluble than WGA. In [16], SWGA was shown, in all respects, to be quite similar to WGA.

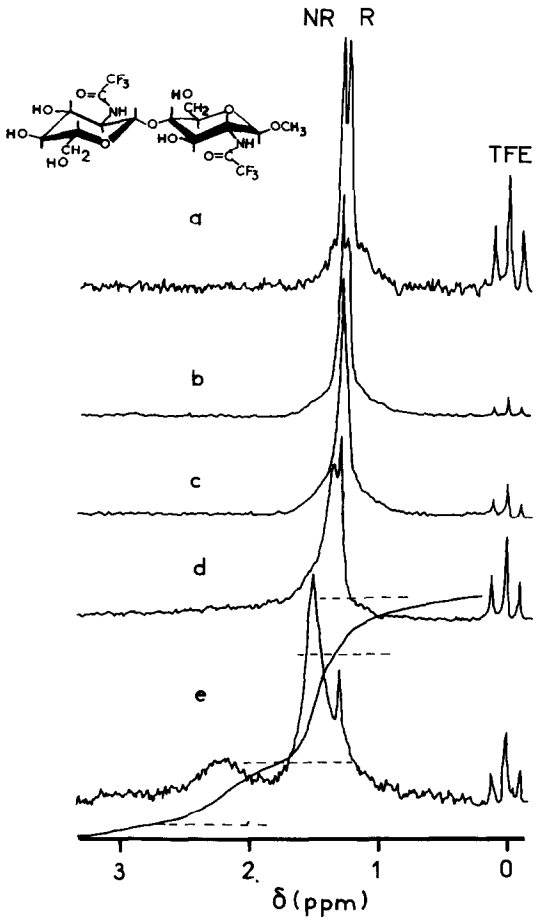

Fig.3. NMR spectra of 1-*O*-methyl-di-*N*-trifluoroacetyl-β-chitobioside in the presence of increasing concentration of succinylated wheat germ agglutinin. 0 ppm corresponds to the central resonance of trifluoroethanol. The sugar and protein concentrations were, respectively (mM): (a) 1.22, 0; (b) 4.37, 0.5; (c) 3.2, 1.075; (d) 2.8, 1.3; (e) 1.22, 2.22.

Fig.3 shows NMR spectra of $(CF_3)_2CB$ in the presence of increasing amounts of SWGA at 290 K. The signal assigned to $(CF_3)_R$ gradually shifts and broadens, as would be expected for a ligand in fast exchange between free and bound sites. Two other lines are assigned to $(CF_3)_{NR}$ in slow exchange between bulk and complex. The total integrated intensity of these two lines remains constant as the titration proceeds, and approximately equal to the area of the line assigned to $(CF_3)_R$. A complete analysis requires further experiments to better define the conditions of exchange.

When the spectra are recorded in the presence of proton broad-band noise decoupling, the low field line (bound $(CF_3)_{NR}$) vanishes (fig.4). This is an example of a negative nuclear Overhauser effect. Using the definition of [22], we find $-1 < f < -0.8$. This implies that fluorine relaxation is dominated by proton fluorine dipolar interactions. If we assume further that the CF_3 group is rigidly bound to the protein in the complex, then we conclude that the correlation time for fluorine is $>10^{-8}$ s. This can be compared to the Stokes-Einstein value of 2.8×10^{-8} s for a sphere having a volume equal to that of the WGA molecule [23]. It is likely, however, that some internal motion of the trifluoroacetyl group remains. In that case, as shown in [24], other parameters are involved.

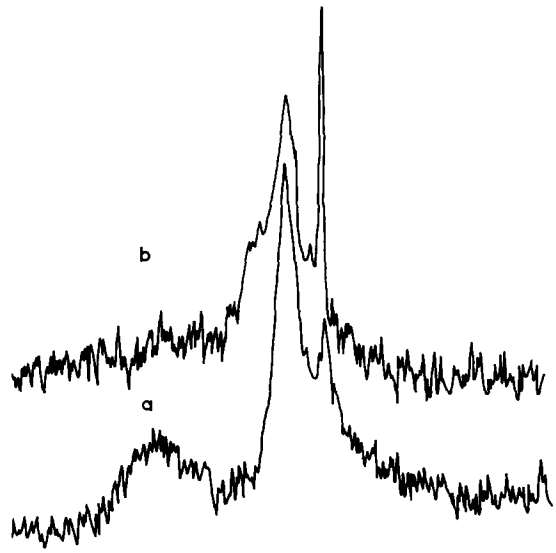

Fig.4. ^{19}F NMR of 1-*O*-methyl-di-*N*-trifluoroacetyl-β-chitobioside (0.9 mM) in the presence of succinylated wheat germ agglutinin (2.3 mM): (a) without proton decoupling; (b) with broad-band proton decoupling.

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