PROTEIN–SUGAR INTERACTIONS. ASSOCIATION OF WHEAT GERM AGGLUTININ (LECTIN) AND *O*-(4-METHYL-UMBELLIFERYL)-GLYCOSIDES

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1. Introduction

Wheat germ agglutinin (WGA) is a lectin which agglutinates erythrocytes and other types of cells [1–3]. It has been shown that *N*-acetyl-D-glucosamine and its β-(1→4) linked oligomers specifically inhibit the agglutination reaction [2,4] and induce fluorescence spectral changes upon binding to WGA [5,6]. The number of binding site(s) per polypeptide chain has been reported to be *one* by LeVine et al. [7], Greenaway and LeVine [8] and *two* by Nagata and Burger [9] on the basis of equilibrium dialysis experiments with *N*-acetyl-D-glucosamine. Nagata and Burger [9] did not exclude the possibility of having determined two subsites as separate binding sites. In a previous paper [10], we reported the presence of two binding sites per polypeptide chain on the basis of equilibrium dialysis experiments with reduced-*N*, *N'*, *N''*, *N'''*-tetraacetyl chitotetraose. Recently, Dean and Homer [11] used *O*-(4-methyl-umbelliferyl) -α-D-mannoside to investigate carbohydrate-concanavaline A interactions, and found that the fluorescence of this glycoside was quantitatively quenched upon binding to the lectin. In contrast, Delmotte et al.[12] showed that the fluorescence of *O*-(4-methyl-umbelliferyl)-*N*, *N'*,*N''*-tri-acetyl-β-chitotrioside was enhanced upon binding to lysozyme. It was of interest to investigate the behaviour of *O*-(4-methyl-umbelliferyl)-glycosides of *N*-acetyl-β-D-glucosamine and of its β-(1→4) linked oligomers upon binding to WGA. We report here that the fluorescence of these glycosides are completely quenched upon binding to WGA, and that WGA has two independent binding sites per polypeptide chain for *O*-(4-methyl-umbelliferyl)-glycosides.

2. Materials and methods

WGA was purified as previously described [6]. A molecular weight of 23 500 g per polypeptide chain was used for calculations [4, 10, 13, 14]. Freeze-dried WGA was dissolved in a 0.05 M Tris–HCl buffer containing 0.2 M NaCl, pH 7.2. The concentration was determined by absorbance measurements at 280 nm using $E^{1\%}_{280} = 15.0$ in a Cary 14 spectrophotometer, using a 1 cm path length cell. Solutions were passed through millipore filters (HWAP, 0.45 μ) just before use, using a Swinnex-13 millipore adapter.

The *O*-(4-methyl-umbelliferyl)-glycosides of 2-acetamido-2-desoxy-β-D-glucopyranose: MUF-G1cNAc, *N,N'*-diacetyl-β-chitobiose: MUF-$(G1cNAc)_2$ and *N,N',N''*-triacetyl-β-chitotriose: MUF-$(GlcNA)_3$ were prepared as previously described [12]. The *O*-(4-methyl-umbelliferyl)-glycosides of β-D-glucopyranose and of 2-acetamido-2-desoxy-β-D-galactopyranose were purchased from Koch–Light. Concentrations of *O*-(4-methyl-umbelliferyl)-glycosides were determined using a molar absorbance of: 12 800, at 316 nm [12]. Fluorescence intensities were measured at the maximum wavelength of the emission spectrum (375 nm). Emission spectra were recorded with a Jobin and Yvon spectrofluorimeter. The spectrofluorimeter was equiped with a XBO-250 W Xenon lamp, two prism monochromators and a 9558 EMI photomultiplier. Part of the excitation beam was focused on a Rhodamine B quantum counter whose fluorescence was measured with a 6097 EMI photomultiplier. The ratio of sample and reference beam intensities was recorded. Thus any variation in lamp intensity was corrected for. Emission spectra were corrected for the wavelength

dependence of photomultiplier response and monochromator transmission. The solutions were contained in 5 mm quartz cells (Hellma) maintained at 25°C.

Two parallel experiments were done with protein solutions and with *N*-acetyl-tryptophan-methyl ester (or buffer alone) solutions having identical absorbance at 320 nm. The fluorescence quantum yield of the *O*-(4-methyl-umbelliferyl)-glycoside bound to WGA was obtained by extrapolating a plot of $F_0/F_0\text{-}F$ *vs.* $1/C_p$ at $1/C_p = 0$ [11], where F is the measured fluorescence of the MUF-glycoside solution in the *presence* of WGA, F_0 is the measured fluorescence of the MUF-glycoside solution in the *absence* of WGA and C_p is the total protein monomer concentration. The absorption spectrum is not changed upon binding to WGA of MUF-glycosides. In order to obtain a better accuracy, independent experiments were also done by adding MUF-glycoside solution into both protein solution and buffer alone.

The concentrations of MUF-G1cNAc and MUF-$(G1cNAc)_2$ were varied between 3.4 and 55 X 10^{-6} M, 1.9 and 30 X 10^{-6} M respectively. With MUF-$(G1cNAc)_3$ the concentration range was 1 to 34 X 10^{-6} M, with the larger concentration of MUF-$(G1cNAc)_3$ the average number of occupied sites per molecule of the lectin polypeptide chain was only 0.6, but no larger concentration of MUF-$(G1cNAc)_3$ could be used because the solution became turbid.

3. Results and discussion

The fluorescence intensities of the *O*-(4-methyl-umbelliferyl)-glycosides MUF-$(G1cNAc)_{1,2,3}$ were smaller in the presence than in the absence of WGA. Plots of $F_0/F_0\text{-}F$ versus $1/C_p$ are straight lines whose *y*-axis intercepts are equal to 1 thus demonstrating that the fluorescence quenching yield of MUF-glycosides bound to WGA is zero, (fig. 1). When the same experiments were done with MUF-GalNAc in the same concentration range, fluorescence quenching of bound MUF-GalNAc was also total as shown in fig. 1. On the contrary, using MUF-Glc in the same concentration range, no fluorescence quenching was detected. In all cases, the fluorescence quenching was reversed upon addition of free G1cNAc or its oligomers. These results indicated that MUF-$(G1cNAc)_{1,2 \text{ and } 3}$ and MUF-GalNAc were selectively bound to WGA and that the carbohydrate moiety of MUF-glycosides is responsible for the specificity of binding.

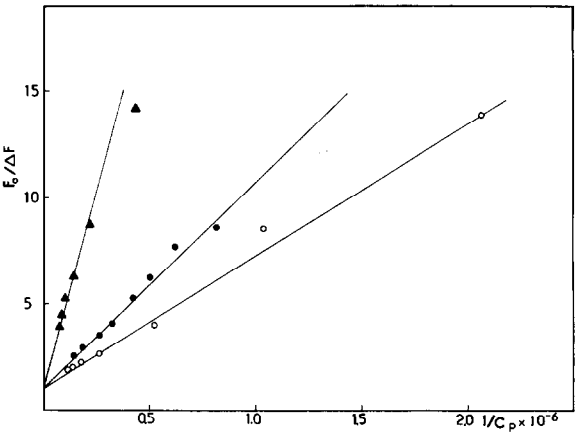

Fig. 1. Fluorescence intensity changes of *O*-(4-methyl-umbelliferyl)-glycosides upon addition of wheat germ agglutinin. Aliquots of a solution of WGA (0.80 X 10^{-4} M) were added to a solution of MUF-glycosides (8 X 10^{-6} M). After correction for dilution, the data were plotted. (o---o) MUF-$(G1cNAc)_2$, (•---•) MUF-G1cNAc, (▲---▲) MUF-GalNAc.

With concanavalin A, Dean and Homer [11] showed that the fluorescence quantum yield of MUF-α-D-mannose was zero when bound to the protein, and that the fluorescence quantum yields of MUF-α-D-glucuronide and MUF-α-D-glucuronide and MUF-α-(and β-)glucosides were unchanged upon addition of the protein. Delmotte et al. [12] showed that the fluorescence quantum yield of MUF-$(G1cNAc)_3$ was increased upon binding to hen-egg lysozyme. Thus, although WGA shares with lysozyme similar sugar affinities, and similar fluorescence behaviours upon binding of free sugars [5,6], the binding sites for the 4-methyl-umbelliferyl group appears quite different. From fluorescence intensity measurements at various concentrations of MUF-glycosides in the present and in the absence of protein, the concentration of bound (C_b) and free (C_f) MUF-glycosides, r/C_f were calculated, and data were plotted according to the representation of Scatchard [15] as shown in fig. 2, where $r = C_b/C_p$. The calculated points determine straight lines which intercept the *x*-axis near 2, and the *y*-axis at $r/C_f = 9 \times 10^4$ M^{-1} and 17×10^4 M^{-1} for MUF-G1cNAc and MUF-$(G1cNAc)_2$, respectively. The free energies

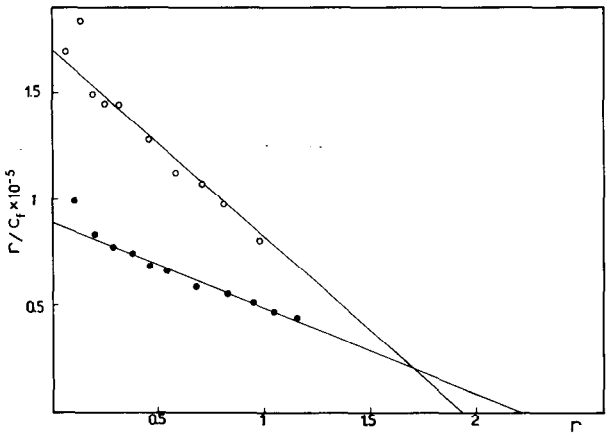

Fig. 2. Scatchard plots for binding to WGA of MUF-GlcNAc (●———●) and MUF-$(GlcNAc)_2$ (○———○).

of association and the association constants for MUF-GlcNAc and its derivatives are shown in table 1. The association constant for MUF-$(GlcNAc)_3$ was estimated from an incomplete Scatchard plot assuming that the protein monomer had two binding sites for this glycoside. Because the points of the Scatchard plot determine a straight line, for each MUF-glycoside, it can be concluded that the two binding sites are equivalent and independent. WGA shares this property with the other lectins which have two (or more) homogeneous binding sites which behave independently [16].

The association constants for the binding to WGA of MUF-GlcNAc and of MUF-$(GlcNAc)_2$ are higher than those for the binding to WGA of the corresponding free sugars. The ratio of the association constants for MUF-GlcNAc and free GlcNAc is more than 50. For MUF-$(GlcNAc)_2$ and free $(GlcNAc)_2$, the corresponding ratio is 20. On the contrary, the association constant for MUF-$(GlcNAc)_3$ is slightly smaller than that for free $(GlcNAc)_3$. The enhancement of the association constant for MUF-GlcNAc (and MUF-$(GlcNAc)_2$) might result from a particular affinity of one WGA-subsite for the 4-methyl-umbelliferyl group and from the stabilisation of the anomeric substituent in the β form. Similarly the affinity of concanavalin A for D-glucosides was shown to be dependent on the anomeric form and on the nature of the aglycone: the association constants for the binding of Glc, *O*-methyl-β-D-Glc, *O*-methyl-α-D-Glc and *O*-*p*-nitrophenyl-α-D-Glc were 5.9×10^2, 1.76×10^3, 4.94×10^3 [17] and 1×10^4 M^{-1} [18], respectively. Allen et al. [4] related a similar behaviour of the aglycone, on the basis of inhibition of the agglutinating activity of WGA by various *N*-acetyl-glucosaminides: the concentrations required for 50% inhibition were 30, 10 and 5 mM for GlcNAc, *O*-methyl-α-(or β-)D-GlcNAc and *O*-benzyl-α-D-GlcNAc, respectively. However, these authors did not observe any difference between the α and β derivatives of *O*-methyl-D-GlcNAc.

The affinity between WGA and the 4-methyl-umbelli-

Table 1
Association constants K_a, ($1 \times M^{-1}$) and free energies ΔG°_a (kcal) association of WGA with MUF-glycosides and with free glycosides

	MUF-glycosides		Free glycosides				
	$K_a \times 10^{-4}$	$-\Delta G^\circ_a$	$K_a \times 10^{-4}$			$-\Delta G^\circ_a$	
			(a)	(b)		(a)	(b)
GlcNAc	5	6.4	0.069	–	0.13 (c)	3.9	–
$(GlcNAc)_2$	8	6.6	0.45	1.3	2.04 (d)	5.1	5.6
$(GlcNAc)_3$	1	5.6	2.0	2.2	8.3 (d)	6.0	5.9
$(GlcNAc)_4$	–	–	2.3	3.6	–	6.0	6.2

(a) from Privat et al. [6], (b) from Lotan and Sharon [5] on the basis of the enhancement of tryptophan fluorescence intensities on binding glycosides to WGA, assuming *one* binding site per WGA monomer (at 25°C). (c) from Nagata and Burger [9] on the basis of dialysis equilibrium experiments with C14-labelled GlcNAc (at 4°C). (d) from Nagata and Burger [9] on the basis of inhibition of binding of C14 labelled GlcNAc in dialysis equilibrium experiments.

feryl group of MUF-$(GlcNAc)_{1,2}$ appears to be larger than in the case of the interaction between MUF-Man and concanavalin A: the ratios of the association constants for *O*-(4-methyl-umbelliferyl) and *O*-methyl-α-D-mannopyranoside is only 1.7 ([11] and [17], respectively).

Since the fluorescences of all the MUF-glycosides investigated are equally quenched, it can be proposed that the 4-methyl-umbelliferyl occupies the same subsite in the three cases, and that the whole site is limited to three subsites, confirming previous conclusions [4–6] and contrasting with lysozyme whose site is formed by 6 subsites [19].

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