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BINDING OF 4-METHYLUMBELLIFERYL-GALACTOSIDES TO PEANUT AGGLUTININ

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SUMMARY : The binding of 4-methylumbelliferyl-α-D-galactopyranoside, -β-D-galactopyranoside and -D-Galβ(1→3)DGalNac to peanut agglutinin was studied by fluorescence. Peanut agglutinin quenched the fluorescence intensity of 4-methylumbelliferyl-α-D-galactopyranoside but enhanced that of the two 4-methylumbelliferyl-β-galactosides. For α-D-galactopyranoside, the association constants measured at 4 and 25°C were 3.4×10^3 and 1.7×10^3 M^{-1} respectively, and for D-Galβ(1→3)DGalNac, 1.5×10^5 and 3.3×10^4 M^{-1}. The binding enthalpies estimated from these values are consistent with the existence of extended sugar binding sites in the peanut agglutinin molecule.

Peanut agglutinin (PNA)[1], the lectin extracted from the groundnut seed, *Arachis hypogea*, is a protein able to agglutinate neuraminidase-treated human erythrocytes (1). Inhibition of such agglutination by saccharides showed PNA to be specific for galactosides; the most effective inhibitor in this respect was the disaccharide D-Galβ(1→3)DGalNac (2,3). Because of this specificity, PNA has been widely used as a cell surface probe and as a marker for T-lymphocyte subpopulations and immature circulating cells (4). The interaction between PNA and its ligands has been studied by ultraviolet difference spectroscopy (5) and ^{13}C nuclear magnetic resonance (6). However, the magnitude of the signals recorded by these techniques appeared rather low.

Sugars labelled with 4-methylumbelliferyl (MeUmb) have proved very useful and sensitive probes for investigating the sugar-binding parameters of lectins (7-9). These compounds have not yet been used to study PNA, because of the latter's limited cryosolubility (10), and of the difficulty of obtaining MeUmb-D-Galβ(1→3)DGalNac.

This paper reports the interaction of PNA with three galactosides having MeUmb as their probe : α-D-galactopyranose, β-D-galactopyranose and particu-

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[1] Abbreviations : PNA, peanut agglutinin; Meumb, 4-methylumbelliferyl; MeUmb-D-Galβ(1→3)DGalNac, 4-methylumbelliferyl-2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-β-D-galactopyranoside.

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larly the disaccharide D-Galβ(1→3)DGalNac, whose association parameters have not been described so far.

MATERIALS AND METHODS

PNA was purified as described previously (10) by a modified version of the procedure of Sutoh *et al.*(11) from seeds of *Arachis hypogea*, var. *virginia*. The purified preparation was shown to be homogeneous by polyacrylamide gel electrophoresis at pH 7.5 in the presence and absence of sodium dodecyl sulfate and by analytical ultracentrifugation. Before each experiment, PNA was filtered through a Biogel P200 column to remove any saccharide breakdown products of the affinity column (10). The PNA concentration was determined by absorbance at 280 nm using A = 9.6 (12).
MeUmb-α-D-galactopyranoside and MeUmb-β-D-galactopyranoside were from Sigma (St Louis, U.S.A.). MeUmb-D-Galβ(1→3)DGalNac was a generous gift from Dr. K.L. Matta (Roswell Park Memorial Institute, Buffalo, U.S.A.). The MeUmb-galactoside concentrations were determined at 318 nm using $\varepsilon = 1.36 \times 10^4$ $M^{-1}cm^{-1}$ (9).
MeUmb fluorescence intensities were titrated with a Jobin et Yvon JY3 fluorometer coupled to a Hewlett-Packard 9815 A calculator and 9862 A plotter. Excitation of MeUmb-galactosides was at 318 nm (2 nm slit) and emission was measured at 378 nm (10 nm slit). A ligand solution (0.7-1.7 μM) was titrated with a PNA solution ($2.4-8.5 \times 10^{-5}$ M). Experiments were performed in thermostated cuvettes (1x1x4.5 cm) under permanent magnetic stirring. All data were corrected for dilution and for a blank titration without MeUmb, using the PNA at the same concentration. To preclude PNA insolubility, titrations were performed in 0.05 M Tris-HCl, pH 6.9, containing 0.5 M $MgCl_2$ (10), 0.01 M $CaCl_2$ and 0.01 M $MnCl_2$. Results are plotted as $|F_o/F_o-F|$ *versus* $1/C_p$ (8). C_p is the total PNA monomer concentration, F_o and F are the respective ligand fluorescences measured alone and in the presence of the lectin at concentration C_p. The value of the association constant, K_a, is calculated from :

$$K_a = (F_o/F_o-F_{max}) \; / \; (d(F_o/F_o-F)/d(1/C_p))$$

where F_{max} is the value of fluorescence at infinite protein concentration.
Fluorescence polarization was measured with a T format SLM XE 450 apparatus equipped with a thermostated cell holder. Excitation was at 318 nm (1 nm slit) and the light emitted was measured at 378 nm through Schott narrow band interference filters (5.5 nm bandwidth).
All solutions used for fluorescence and polarization measurements were passed through 0.45 μm Millipore filters.

RESULTS

When MeUmb-galactosides were excited at 318 nm, the wavelength of fluorescence emission maximum was maintained at 378 nm (uncorrected spectra) in the presence of PNA. Under these conditions, the fluorescence intensity of MeUmb-α-D-galactopyranoside was progressively quenched,but the fluorescence of MeUmb-β-D-galactopyranoside and MeUmb-D-Galβ(1→3)DGalNac was enhanced. These fluorescence changes were reversed by addition of 0.1 M lactose, showing that they were due to sugar-specific binding.

Fluorescence titrations

The titration experiments were performed at 4 and 25°C and at fixed excitation and emission wavelengths (318 and 378 nm respectively). Blank titrations without MeUmb gave final values amounting to < 10% of the initial fluorescence

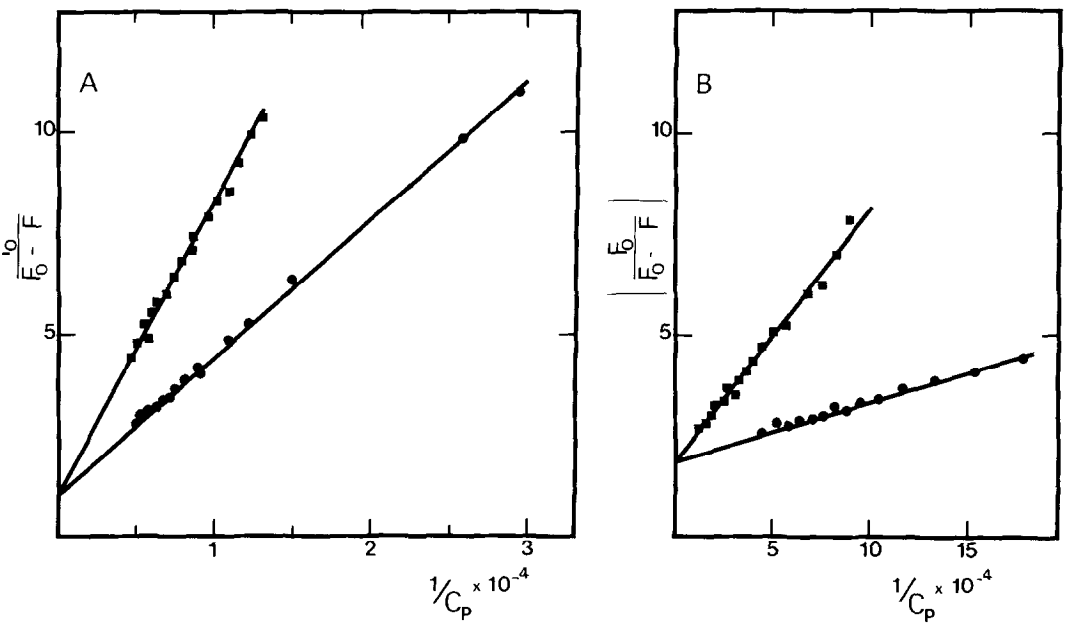

Fig. 1 : Fluorescence intensity changes of MeUmb-galactosides upon addition of PNA at 4 (●——●) and 25°C (■——■).
A : MeUmb-α-D-galactopyranose
B : MeUmb-D-Galβ(1→3)DGalNac

intensity for MeUmb-α-D-galactopyranoside and < 4% of this value for MeUmb-D-Galβ(1→3)DGalNac.

Plots of F_o/F_o-F of MeUmb-α-D-galactopyranoside *versus* $1/C_p$ (8) are straight lines with y-axis intercepts equal to 1 or a little more (Fig. 1), thus demonstrating that quenching of MeUmb-α-D-galactopyranoside fluorescence was total or almost total (> 85%) when this compound was bound to PNA.

Assuming the existence of one saccharide binding site (5) for each of the four PNA subunits (M_r 27500), the association constants and free energy values for the interaction between PNA and MeUmb-α-D-galactopyranoside measured by fluorescence are reported in Table I. The association constant at 25°C, K_a = 1.7 x 10^3 M^{-1}, was not modified by the presence of $MgCl_2$ (Decastel and Frénoy, unpublished results) and was very close to the value K_a = 1.8 x 10^3 M^{-1} determined for methyl-α-D-galactopyranoside by ultraviolet difference spectroscopy (5).

Whereas for MeUmb-β-D-galactopyranoside bound to PNA the increase in fluorescence was very small (∿ 10%) and precluded detailed investigation of binding by titration, the increase calculated for MeUmb-D-Galβ(1→3)DGalNac reached ∿ 55% of the initial value when this compound was bound to PNA. This increase was calculated from the y-axis intercepts of $|F_o/F_o - F|$ plots *versus* $1/C_p$ (Fig. 1), i.e. intercepts of approximately 1.9. Assuming the same stoichiometry of one binding site per subunit, the association constants and free energy values were calculated at 4 and 25°C (Table I).

TABLE I : Association constants and free energies for the binding of MeUmb-galactosides to PNA.

		$K_a \times 10^{-3}$	$-\Delta G$
		M^{-1}	kcal mol^{-1}
MeUmb-α-D-galactopyranoside	4°C	3.4 ± 0.4	4.4
	25°C	1.7 ± 0.3	4.4
MeUmb-D-Galβ(1→3)DGalNac	4°C	150 ± 16	6.5
	25°C	33 ± 4	6.1

Fluorescence polarization

When lectins do not markedly affect ligand fluorescence emission, the enhancement of MeUmb-glycoside polarization and/or of anisotropy provides a good index of binding to these lectins (13-15).

At 25°C, the values for polarization of free MeUmb-β-D-galactopyranoside and MeUmb-D-Galβ(1→3)DGalNac were 0.118 and 0.215 respectively. The polarization of each probe was clearly enhanced by PNA addition, i.e. 0.165 for β-D-galactopyranoside (18% saturation, percentage of the total fluorescent galactoside PNA-bound, as calculated from $K_a = 1.0 \times 10^3$ M^{-1} (5)) and 0.367 for D-Galβ(1→3)DGalNac (83% saturation). This effect was totally inhibited in the presence of 0.1 M lactose. The results show that both galactosides are bound to PNA.

Note that since MeUmb-α-D-galactopyranoside fluorescence was quenched on binding to PNA, no change in this compound's polarization could be detected in the presence of the lectin.

DISCUSSION

The fluorescence of MeUmb-glycosides is often quenched upon binding to proteins. This quenching may be partial (14) or total (7,9,16). An exception was observed for the MeUmb-chitooligosaccharides, whose fluorescence was enhanced on binding to the lysozyme (17-19) although they were quenched on binding to wheat germ agglutinin (8,20).

The present paper reports the unique case of the peanut lectin PNA, which quenches the fluorescence of MeUmb-α-D-galactopyranoside but enhances that of MeUmb-β-D-galactosides. This contrast implies that the anomeric configuration of the galactose residue bound to PNA leads to a complete change in the environment of the fluorophore.

Dean and Homer concluded that the quenching of MeUmb-α-D-mannopyranoside by Concanavalin A was due to the binding of the MeUmb moiety to an apolar site of this lectin (7). The same explanation can be proposed for the present quenching of MeUmb-α-D-galactopyranoside by PNA.

The MeUmb-β-D-galactoside fluorophores are probably located in a polar environment, but this is not sufficient to explain the fluorescence enhancement. These fluorophores might be exposed to somewhat drastic interactions altering the conformation of the ligand and consequently the quantum yield.

Association parameters were not previously reported for disaccharide D-Galβ(1→3)DGalNac binding to PNA, although this disaccharide was observed by inhibition experiments to be 20 (3) or 25 (21) times as active as methyl-α-D-galactopyranoside. At 25°C, we found that the association constant for D-Galβ(1→3)DGalNac binding to PNA was 19 times that of α-D-galactopyranoside. From the observation that binding enthalpies were independent of carbohydrate chain length, Van Landschoot *et al.* proposed that glycosides bind to Concanavalin A via a single mannopyranosyl residue (13). On the other hand, in the case of the extended lysozyme binding site, ΔH values rise with the size of the ligand (22).

Even if the van't Hoff plots obtained from two values are questionable, the ΔH calculated from our association constants at 4 and 25°C for α-D-galactopyranoside and D-Galβ(1→3)DGAlNac were significantly different, i.e. -5.4 kcal mol^{-1} and -11.8 kcal mol^{-1} respectively. These values are consistent with the existence of extended sugar binding sites already proposed for PNA (3).

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