Determination of the Carbohydrate-binding Properties of Peanut Agglutinin by Ultraviolet Difference Spectroscopy*

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The anti-T lectin from peanuts was purified on a new affinity matrix, and the number of carbohydrate binding sites was determined by equilibrium dialysis with [14C]Lactose to be four per tetramer. Methyl-α- and methyl-β-D-galactopyranoside and lactose were found to perturb the UV spectrum of the lectin in the aromatic region and their association constants were determined by UV difference spectroscopy to be 1.8, 1.0, and 1.3 x 10^3 M^-1, respectively, at 25°C. Thermodynamic parameters were also obtained for the two galactosides from measurements at several temperatures. For the α anomer, ΔH° = -42 kJ mol^-1 and ΔS° = -78 J K^-1 mol^-1; for the β anomer, ΔH° = -43 kJ mol^-1, and ΔS° = -86 J K^-1 mol^-1. Analysis of the lectin for metal atoms disclosed 0.98 mol of Ca²⁺ and 0.78 mol of Mg²⁺/subunit, while manganese was present in trace amounts only. The results of the present study indicate that recent improvements in instrumentation should make UV difference spectroscopy more widely applicable to studies of protein-ligand interactions.

Lectins have proved invaluable for studies of the nature, distribution, and mobility of cell surface carbohydrate species, as well as their modification during cell differentiation and malignant transformation (1). The recognition sites of many of the lectins used in cell studies, however, are still characterized incompletely. The relative binding of simple sugars by many lectins has been examined by hemagglutination inhibition, but accurate association constants and the thermodynamics of sugar binding have rarely been determined. To date, the carbohydrate-binding properties of only one lectin, concanavalin A, have been studied in detail (2). In a few cases, association constants have been determined by equilibrium dialysis, but suitable radiolabeled compounds are often not available or the association constants are too low for equilibrium dialysis experiments to be performed, i.e. less than 10^3 M^-1.

The present investigation shows that UV difference spectroscopy has become practical for the determination of association constants and the thermodynamics of lectin-sugar interactions. We have used this method successfully to characterize the carbohydrate-binding properties of peanut agglutinin, a lectin specific for terminal D-galactosyl residues (3). PNA, having a specificity similar to that of serum T-agglutinin (4), is being used widely for monitoring the distribution of the T-antigen on erythrocyte surfaces (5, 6) and as a probe for studying T lymphocyte subpopulations (7-11). Moreover, this lectin is a useful tool for the separation of mature and immature murine and human thymocytes (12, 13), permitting the study of in vitro maturation of immature thymocytes into immunocompetent cells and providing better characterization of lymphocyte subpopulations with regard to their surface properties (14). PNA receptors have also been established as a new cell surface marker in studies of in vitro differentiation of teratocarcinomas (15, 16). Despite the usefulness of this lectin, only semiquantitative hemagglutination inhibition data on its carbohydrate-binding properties have been reported (3, 17, 18).

EXPERIMENTAL PROCEDURES

Methyl-α-D-galactopyranoside and methyl-β-D-galactopyranoside were obtained from Plantinell Inc., Waukegan, IL, and were recrystallized twice from ethanol. Lactose monohydrate was from Ana-chem Ltd., Montreal, Quebec. Purity was checked by paper chromatography in pyridine/ethyl acetate/water (2:5:5). [1-14C]-Lactose (specific activity 57.7 mCi/mmol) was from Amersham Corp., Oakville, Ontario. P-nitrophenyl-β-D-lactoside was a gift from Dr. D. R. Bundle, National Research Council of Canada, Ottawa. Peanuts (Arachis hypogaea) were obtained from a local foodstore. N-Hydroxysuccinimide-derivatized agarose beads (19), Affi-Gel 10, was from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario. The glucose-binding lectin from the pea (Pisum sativum) was prepared by affinity chromatography on Sephadex (20).

Preparation of Affinity Matrix—Three hundred milligrams of p-nitrophenyl-β-D-lactoside were dissolved in 100 ml of methanol and 15 ml of water, and the compound was hydrogenated catalytically for 1 h with Pd/C (35 mg). The catalyst was removed by filtration and the methanol solution of p-aminophenyl-β-D-lactoside evaporated to dryness. The compound was redissolved in 20 ml of 0.1 M NaHCO₃ buffer, pH 8.0 at 4°C, and coupled to the Affi-Gel 10, according to the manufacturers' revised procedure.

Isolation of PNA—An extract containing PNA was prepared from peanuts following the procedure of Lotan et al. (3) and applied to a column (1.6 x 13 cm) of the affinity medium, in PBS. Unbound proteins were washed out with PBS (approximately 800 ml) at a flow rate of approximately 100 ml/h, and the lectin was specifically eluted with 0.1 M D-galactoside in PBS. Lectin-containing fractions, as detected by UV absorbance at 280 nm, were pooled and dialyzed extensively against PBS to remove the eluting sugar. The capacity of the above affinity matrix was 10 mg of protein/ml of gel. The homogeneity of the product was established by polyacrylamide gel electrophoresis. PNA concentrations were determined from absorbance at 280 nm, using A280 = 7.7 (3). Protein solutions were stored at 4°C, with 0.02% NaN₃ added.

Mental Analysis—Samples of PNA were analyzed by atomic absorption spectroscopy, either directly or after dialysis against 0.01 M EDTA, pH 7.2, followed by dialysis against 0.5% NaCl in the presence of Chelex 100 resin (Bio-Rad Laboratories Ltd.).

Equilibrium Dialysis—Solutions of PNA (3.8 mg/ml) and of lactose-buffered saline (0.01 M phosphate, 0.15 M NaCl, pH 7.2; Con A, concanavalin A.
Binding Properties of Peanut Agglutinin

A solution containing [1-14C]lactose, was made up in PBS. Each chamber of the dialysis cell received 100 μl of the protein or a lactose solution. After equilibration for 2 days at 4°C, 80-μl aliquots were taken from the chambers and counted in 10 ml of scintillation fluid (Aquasol, New England Nuclear, Montreal, Quebec) on a Beckman LS-7000 scintillation counter.

UV Difference Spectroscopy—UV difference spectra were recorded on a Cary 219 spectrophotometer in masked semimicro cells, of 1-cm pathlength. This instrument is capable of reading absorbance to 0.0001 unit. Aliquots of 1 ml of PNA solution (approximately 2 mg/ml in PBS) were added to both sample and reference cells and the baseline was recorded into the instrument’s memory unit to be subtracted automatically from subsequent spectra. Small aliquots (1 μl) of a solution of the sugar under study was added to the sample cuvette, while the reference cuvette received the same amount of PBS buffer. The difference spectrum was recorded after thermal equilibration had been re-established, as indicated by a thermocouple in the cell holder. The total volume of ligand solution added was 10 to 15 μl, rendering concentration corrections for dilution unnecessary. Control experiments were conducted with methyl-α-D-glucoside, which is not an inhibitor of PNA (3).

Equilibrium Dialysis—PNA has been shown to be a tetramer of identical subunits (3, 22), but its valency was reported as only 2 (Ref. 23, no experimental details given). Since valency cannot be determined directly by UV difference spectroscopy when the protein is the absorbing species, the number of carbohydrate binding sites on the PNA tetramer was determined by equilibrium dialysis, using [1-14C]lactose. The data are shown in Fig. 1, plotted according to the Scatchard equation

\[ r/c = K(n-r) \]

where \( r \) is the number of moles of lactose bound per mol of PNA, \( c \) is the free sugar concentration, \( n \) is the number of binding sites per mol of PNA, and \( K \) is the association constant. The molecular weight of PNA was taken to be 110,000 (3). The number of binding sites, obtained from the x intercept in Fig. 1, was found to be 3.7 ± 0.3. As PNA is a tetramer at neutral pH (3, 22), our data indicate one binding site per subunit of 27,500 molecular weight. The apparent association constant for lactose at 4°C was found to be 1.1 × 10^4 M^-1 (see Table I). Since mutarotation of the lactose will be occurring under the conditions of this experi-

![Fig. 1. Equilibrium dialysis data for the binding of [1-14C]lactose to peanut agglutinin at 4°C.](image)

![Fig. 2. Ultraviolet difference spectra at site saturation (solid lines) of peanut agglutinin with methyl-α-D-galactoside (A), methyl-β-D-galactoside (B), and lactose (C) at 25°C. In Panel A, the dotted line is the spectrum obtained with 1 × 10^{-4} M sugar, approximately 64% saturation. PNA concentration was 1.9 mg/ml.](image)
methyl-a-D-galactoside. The association constants obtained; from 0.021 decreased with increasing temperature. Thus, the shape of the difference spectrum was found, but its magnitude as a function of the total sugar concentration for methyl-a-D-galactoside (●), methyl-b-D-galactoside (▲), and lactose (■) at 25°C.

Variation of the maximum difference spectrum as a function of total sugar concentration for methyl-a-D-galactoside (●), methyl-b-D-galactoside (▲), and lactose (■) at 25°C.

UV Difference Spectroscopy—The three sugars examined, methyl-a- and methyl-b-D-galactoside and lactose, all perturbed the UV spectrum of PNA (Fig. 2). Fig. 3 shows titration curves for the binding of the three sugars to PNA, obtained from the difference maximum at 285 nm or 286 nm for various sugar concentrations. The association constants (Table I) were determined from the free sugar concentration at 50% saturation, using the relationship K = 1/[S]ₜ inval with [S]ₜ inval = [S]ₜotal - 0.5 n[P] where n[P] represents the PNA subunit concentration. With methyl-a-D-galactoside at 25°C, the difference spectrum exhibits a positive maximum at 285 nm, a smaller positive maximum at 278 nm, an isosbestic point at 273 nm and a small negative maximum at 266 nm (Fig. 2A). The binding of methyl-a-D-glucopyranoside to concanavalin A also gave a positive maximum at 285 nm (24), but no feature at 278 nm in the difference spectrum.

When the effect of temperature on the binding of methyl-a-D-galactoside to PNA was studied, no change in the overall shape of the difference spectrum was found, but its magnitude decreased with increasing temperature. Thus, the 285 nm maximum was 0.03 at 7.6°C, 0.028 at 16°C, 0.025 at 25°C and 0.021 at 35°C, respectively (corresponding to Δε values, based on PNA subunit concentration, ranging from 430 to 300 M⁻¹ cm⁻¹). Table I shows the association constants obtained; from these, thermodynamic parameters for this interaction were determined using a Van’t Hoff plot, as displayed in Fig. 4. The enthalpy and entropy changes were found to be ΔH° = −42 kJ mol⁻¹ and ΔS° = −78 J K⁻¹ mol⁻¹. Methyl-b-D-galactopyranoside gave a difference spectrum (Fig. 2B) very similar to that observed with the a anomer, exhibiting positive maxima at 286 nm and 278 nm, an isosbestic point at 274 nm and a slightly more pronounced negative maximum at 266 nm, but the magnitude of the difference spectrum was smaller. The magnitude of the absorbance change at 286 nm at sugar saturation was 0.023 at 8.6°C, 0.021 at 16.8°C, 0.017 at 25°C, and 0.015 at 35°C, respectively (corresponding to Δε values, based on PNA subunit concentration, ranging from 332 to 216 M⁻¹ cm⁻¹). Association constants were obtained again at each temperature from titration curves, and thermodynamic parameters were calculated from these constants by constructing a Van’t Hoff plot (Fig. 4); ΔH° was −43 kJ mol⁻¹ and ΔS° = −86 J K⁻¹ mol⁻¹. In the case of lactose (Fig. 2C), again positive maxima at 286 nm and at 278 nm were observed. However, the shape of the difference spectrum is different from that obtained with the monosaccharides, there being a third, smaller positive maximum at 268 nm.

Pea Lectin—To test the UV difference spectroscopy method on a system with a smaller absorbance change on binding, the pea lectin was chosen. On binding methyl-a-D-glucoside, the absorbance of a 1.3 mg/ml solution of the lectin changed by 0.01 at 7°C. The difference spectrum had a maximum at 292 nm, which may be due to tryptophan involvement in sugar-binding as chemical modification suggested (25), and a smaller maximum at 285 nm. The association constant for methyl-a-D-glucoside was 7.8 × 10⁴ M⁻¹, in agreement with the value of 8 × 10⁵ M⁻¹ obtained by equilibrium dialysis at 4°C (20).


Amino acid sequence work on PNA has shown it has some homology to Con A (12 out of 40 residues being identical) in the region so far sequenced (26). Although the two sugar specificities are quite different, the results obtained here bear out a general similarity of PNA to Con A. One major exception is that the metal analysis points to the PNA subunits having 1 mol each of calcium and magnesium rather than the calcium and manganese of Con A. There have been two reports of other lectins with calcium and magnesium, namely, Bandeiraea simplicifolia I which had 0.5 mol of Ca2+ and 0.31 mol of Mg2+/subunit (27) and a lectin from Phaseolus vulgaris, 0.98 mol of Ca2+ and 0.63 mol of Mg2+/subunit (28). The B. simplicifolia lectin is also galactose-specific and it, PNA, and a P. vulgaris lectin also show similarities in their CD spectra, both in the far UV and aromatic regions.

There is some similarity to the behavior of Con A (24) in the form of the UV difference spectra obtained here for PNA, but of more interest are the subtle differences between the spectra given by the three sugars. The α- and β-galactosides differ by a nanometer in their maxima and in the intensity of the difference spectra they generate, while lactose, although of intermediate association constant (Table I), gives rise to a difference spectrum with an additional peak. These variations may be taken to support the view of ligand binding by the site as a mutual fit process, where the actual conformation of the site as well as the major ligand site contacts vary subtly with changes in the ligand structure (29).

In Table II, the binding constants and thermodynamic parameters obtained for PNA are listed with values from the literature for three comparable systems. The PNA values are very close to those measured by microcalorimetry for the binding of d-galactose to ricin (30). The ΔH° values for the various systems are essentially the same within experimental error, suggesting similar site interactions. The variations in the binding constants hence arise mainly from the differences in the entropy contribution.

At 25°C, the ratio of PNA binding constants for methyl-α- and methyl-β-D-galactoside is 1.8:1, and for methyl-α-D-galactoside and lactose it is 1.4:1. These ratios for the binding constants agree well with the hemagglutination inhibition data of Lotan et al. (3). From their data for the ratio of the inhibitory activity of the T-antigen to that of methyl-α-D-galactoside, and the association constant obtained here for methyl-α-D-galactoside, the association constant for the interaction of PNA with the T-antigen can be estimated to be about 1 × 1014 M−1 at 25°C, a value similar to those found for the interaction of glycopeptides with Con A, up to 2.3 × 1017 M−1 (33). These values exceed those found for anti-lactose antibodies, K = 1.6 × 106 M−1 (32) or carboxyhydrolytic enzymes such as lysozyme, which binds chitotriose with a K of 108 M−1 (34). The binding data also suggest that lactose binds more strongly than methyl-α-D-galactoside at 4°C, the reverse of the situation at 25°C.

The observed absorption changes (1 to 2%) reported here are quite small, particularly in the case of methyl-β-D-galactopyranoside, but were found to be accurately measurable with very good reproducibility. With the instrument used in the present study, even smaller total absorption changes (around 0.01 A) were sufficient for spectrophotometric titrations to be performed to determine an association constant for the pea lectin and methyl-α-D-glucoside. The value found was in good agreement with the literature value (20). Provided they have little or no UV absorbance, the interaction of more complex sugars such as glycopeptides with PNA may be

| Table II |
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| **Thermodynamic parameters for carbohydrate-binding proteins** |
| **Sugar** | **Protein** | **K_a [M]−1** | **ΔH [kJ mol]−1** | **ΔS [kJ mol]−1 K−1** |
| --- | --- | --- | --- | --- |
| Me-α-D-Gal PNA | 1.8 × 1014 | 42 | 78 |
| Me-β-D-Gal PNA | 1.0 × 1014 | 43 | 86 |
| Ricin | 3.9 × 1014 | 44 | 78 |
| Me-α-D-Man Con A | 6.4 × 1014 | 38 | 56 |
| Lac-Dyeα Anti-Lac antibody | 1.6 × 1014 | 41 | 38 |

* p-(p-Dimethylaminobenzeneazo)-phenyl-β-lactoside.

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