Topography of the combining region of a Thomsen–Friedenreich-antigen-specific lectin jacalin (*Artocarpus integrifolia* agglutinin)

A thermodynamic and circular-dichroism spectroscopic study

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Thermodynamic analysis of carbohydrate binding by *Artocarpus integrifolia* (jackfruit) agglutinin (jacalin) shows that, among monosaccharides, MeαGalNAc (methyl-α-N-acetylgalactosamine) is the strongest binding ligand. Despite its strong affinity for MeαGalNAc and MeαGal, the lectin binds very poorly when Gal and GalNAc are in α-linkage with other sugars such as in A- and B-blood-group trisaccharides, Gal-α1-3Gal and Galα1-4Gal. These binding properties are explained by considering the thermodynamic parameters in conjunction with the minimum energy conformations of these sugars. It binds to Gal-β1-3GalNAcαMe with 2800-fold stronger affinity over Galβ1-3GalNAcβMe. It does not bind to asialo-G$_{M1}$ (monosialoganglioside) oligosaccharide. Moreover, it binds to Galβ1-3GalNAcαSer, the authentic T (Thomsen–Friedenreich)-antigen, with about 2.5-fold greater affinity as compared with Galβ1-3GalNAc. Asialoglycophorin A was found to be about 169333 times stronger an inhibitor than Galβ1-3GalNAcαSer. The present study thus reveals the exquisite specificity of *A. integrifolia* lectin for the T-antigen. Appreciable binding of disaccharides Glcβ1-3GalNAc and GlcNAcβ1-3Gal and the very poor binding of β-linked disaccharides, which instead of Gal and GalNAc contain other sugars at the reducing end, underscore the important contribution made by Gal and GalNAc at the reducing end for recognition by the lectin. The ligand-structure-dependent alterations of the c.d. spectrum in the tertiary structural region of the protein allows the placement of various sugar units in the combining region of the lectin. These studies suggest that the primary subsite (subsite A) can accommodate only Gal or GalNAc or α-linked Gal or GalNAc, whereas the secondary subsite (subsite B) can associate either with GalNAcβMe or GalβMe. Considering these factors a likely arrangement for various disaccharides in the binding site of the lectin is proposed. Its exquisite specificity for the authentic T-antigen, Galβ1-3GalNAcαSer, together with its virtual non-binding to A- and B-blood-group antigens, Galβ1-3GalNAcβMe and asialo-G$_{M1}$ should make *A. integrifolia* lectin a valuable probe for monitoring the expression of T-antigen on cell surfaces.

INTRODUCTION

Artocarpus integrifolia agglutinin (jacalin) is a tetrameric (M_r 40000) carbohydrate-binding protein isolated from the seeds of jackfruits (Suresh Kumar *et al.*, 1982), consisting of two distinct types of polypeptide chains (Roque-Barriera, 1985; Khan *et al.*, 1988). It has two binding sites for the complementary sugars (Appukuttan & Basu, 1985*a*,*b*; Sastry *et al.*, 1986). The mechanism of sugar binding to the lectin has recently been elucidated by lineshape analysis of ^{13}C- and ^{19}F-labelled sugar by n.m.r. spectroscopy (Sastry *et al.*, 1988). The lectin has recently been crystallized and characterized with (Dhanraj *et al.*, 1988) and without (Basu *et al.*, 1988) the bound ligand. *A. integrifolia* lectin has attracted much interest with regard to its biological activities. Particularly well studied among these are its potent and selective mitogenic effects on distinct T- and B-cell functions (Bunn-Moreno & Campos-Neto, 1981) and its unique binding to IgA$_1$ from human serum (Roque-Barreira, 1985; Kondoh *et al.*, 1986). Analysis of carbohydrate

specificity carried out by us revealed that it is very specific for Thomsen–Friedenreich antigen (T-antigen; Galβ1-3GalNAc; Sastry *et al.*, 1986), a tumour-associated antigen of non-oncofetal origin. Thus *A. integrifolia* lectin, unlike peanut (*Arachis hypogaea*) agglutinin does not bind to conformationally related disaccharides such as lactose, N-acetyl-lactosamine and Galβ1-3GlcNAc (Pereira *et al.*, 1976; Neurohr *et al.*, 1980, 1982; Sastry *et al.*, 1986). Despite the inability of peanut lectin to discriminate between T-antigen and other closely related sugars, it has been widely used in immunohistochemical studies for monitoring the expression of T-antigen, and any structure reacting with it has been routinely defined as T-antigen. In contrast with the consensus that only the Galβ1-3GalNAcα structure is the cancer-associated Thomsen–Friedenreich antigenic determinant (Springer *et al.*, 1979), anti-(T-antigen) monoclonal antibodies poorly discriminate between Galβ1-3GalNAcα and Galβ1-3GalNAcβ structures (Clausen *et al.*, 1986, 1988). Our initial studies on the specificity of *A. integrifolia* lectin gave indications of its potential as an exquisitely

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specific anti-(T-antigen) probe. Since T-antigen is an important marker in human carcinoma (Anglin et al., 1977) and the fact that peanut agglutinin as well as anti-(T-antigen) monoclonal antibodies are not very specific for T-antigen (Clausen et al., 1988), we have examined the carbohydrate-binding activity of this lectin with a number of synthetic saccharides in an attempt to explore its carbohydrate specificity at the molecular level.

These studies have revealed that A. integrifolia lectin is specific for Thomsen–Friedenreich antigen (Gal-β1-3GalNAcα1Ser). It does not recognize Galβ1-3-GalNacβMe and asialo-G_{M1} oligosaccharide, making it a highly discriminating, yet easily available, probe for studying the expression of T-antigen on cell surfaces. Ligand-induced changes in the molar ellipticities in the tertiary structural region of the protein allowed us to define the arrangement of subsites in the lectin molecule.

Part of this work has been presented at the International Union of Biochemistry Congress held at Amsterdam, on 25–31 August 1985, at Interlec 9 held at Cambridge (U.K.) on 27–31 July 1987, at a Group Monitoring Workshop of the Department of Science and Technology, Government of India, at New Delhi, on 10–11 February 1988, and at the Preparatory Meeting of the Indian Society for Microcirculation at Bangalore from 31 October to 1 November 1988.

MATERIALS AND METHODS

Materials

A. integrifolia lectin was purified by affinity chromatography on cross-linked guar gum (Appukuttan et al., 1977).

Saccharides

Asialo-G_{M1} oligosaccharide was prepared from asialo-G_{M1} by the procedure of MacDonald et al. (1980). Gal-α1-4Gal, Galα1-3GalαMe and Galβ1-3GalβMe were obtained from Carbohydrate International, Arlöv, Sweden. Blood-group-A and -B trisaccharides were obtained from Biocarb Chemicals, Lund, Sweden. Fetuin from fetal-calf serum was a product from Sigma Chemical Company, St. Louis, MO, U.S.A. GlcNAcβ1-3Gal was prepared by the method of Augé & Veypieres (1977). Galα1-3Gal was prepared from blood-group-B trisaccharide by defucosylation by mild acid hydrolysis at pH 1.5 at 100 °C for 2 h (Pereira et al., 1978) and the product purified by t.l.c. Galβ1-3GalNAcαMe and Galβ1-3GalNAcβMe were synthesized by the method of Flowers & Shapiro (1965), except that MeαGalNAc and MeβGalNAc respectively were used instead of GalNAc. MeαGalNAc required for the synthesis of Galβ1-3-GalNAcαMe was prepared by the method of Preston & Preston (1986), whereas MeβGalNAc required for the synthesis of Galβ1-3GalNAcβMe was prepared by the procedure of Neuberger & Wilson (1971). ^{1}H n.m.r. spectra of the two compounds were taken and the acetamido CH_3 protons were observed at δ (chemical shift) 1.97 p.p.m. The OCH_3 proton resonated at δ 3.31 p.p.m and δ 3.45 p.p.m. in Galβ1-3GalNAcαMe and Galβ1-3GalNAcβMe respectively. For Galβ1-3-GalNAcαMe the anomeric proton resonances were as follows. H-1(Gal) was observed as a doublet at δ 4.45 p.p.m. with a J value of 9 Hz, whereas H-1(GalNAc) was observed at δ 4.76 p.p.m. (J 4 Hz) in accordance

with the configuration respectively of the two glycosidic linkages. For Galβ1-3GalNAcβMe the anomeric protons resonated at δ 4.45 (H-1, Gal) and δ 4.39 p.p.m. (H-1, GalNAc) and both were doublets with spacing (J) of approx. 8.5–8.8 Hz, showing the presence of the β-linkage of the glycosidic bond between the two monosaccharide units as well as the β-linkage of the glycosidic OCH_3 bond.

Synthesis of Glcβ1-3GalNAc was carried out by Koenigs–Knorr coupling of the two monosaccharide units by the procedure outlined by Flowers & Shapiro (1965). For the synthesis of Glcβ1-3GalNAc, tetra-O-acetylglucosyl bromide was coupled with 1-O-benzyl-4,6-O-benzylidene-2-acetamido-2-deoxygalactose in the presence of nitromethane and mercuric cyanide. The coupled product was treated with acetic acid to remove the benzylidene group, and the pure hexa-acetate was obtained by acetylation followed by chromatography of the crude product. Deacetylation of the product followed by catalytic hydrogenation in the presence of 10 % palladium on charcoal/acetic acid in dry methanol at normal pressure for 24 h gave the title disaccharide. The product was pure, as shown by paper chromatography with $AgNO_3$ staining. The compound was found to have an R_F value of 0.55, with glucose having an R_F of 1.0, when the solvent system butanol/acetic acid/water (8:4:1, by vol.) was used. The ^{1}H n.m.r. spectrum of the compound showed the acetamido CH_3 protons at δ 2.02 p.p.m., as expected, and the anomeric proton doublet at δ 4.42 p.p.m. (H-1, Glc) with a J value of 8 Hz proved the presence of a β-glycosidic linkage.

C.d. measurements

C.d. spectra were recorded on a Jasco J-500A spectropolarimeter. The spectrum for secondary-structure determination was recorded by using a protein concentration of 0.878 mg/ml in 0.02 M-phosphate buffer, pH 7.2, and a path length of 0.1 mm. The secondary-structure determination was done by using the EMBL-DA05 computer program of S. W. Provencher (Provencher & Glockner, 1981), and the region from 190 to 240 nm was scanned. The data were expressed in terms of mean residue ellipticities [θ] in degrees·cm^2·dmol^{-1}, taking 110 as the mean residue weight.

C.d. titrations of the protein with sugars were done by using a 5 mm-path-length cell and the wavelength region scanned was from 250 to 330 nm. Ligand-induced changes in the molar ellipticities of the lectin at 284 nm were employed to find the association constant (K_a) for binding of sugars to the lectin according to the method of Chipman et al. (1967), using the following relationship:

$$\log(S - P_b) = -\log K_a + \log\left(\frac{\theta_0 - \theta_c}{\theta_c - \theta_\infty}\right)$$

Where θ_0 is the mean residue ellipticity of the protein in the absence of sugar, θ_c is the observed volume-corrected mean residue ellipticity upon titration with the sugar, $(S - P_b)$ is the free-sugar concentration and θ_∞ is the mean residue ellipticity value where no further changes occur upon addition of sugar. θ_∞ was obtained by extrapolating from the experimental data by plotting $\theta_0/(\theta_0 - \theta_c)$, against $(1/S)$, where S is the total sugar concentration. The plot of $\log[(\theta_0 - \theta_c)/(\theta_c - \theta_\infty)]$ against $\log(S - P_b)$ gives $-\log K_a$ as the intercept from which the association constant K_a was obtained.

Fluorescence measurements

The thermodynamic parameters for the association of sugars to the lectin were evaluated from the ligand-induced changes in the intrinsic fluorescence intensity of the protein. A Union Giken FS 501 A fluorescence polarizer equipped with photon-counting photomultipliers was used for fluorescence measurements. Samples were excited as 282 nm with 7 nm slit width, and emission was monitored by means of a band-pass metal interference filter ($\lambda_{\frac{1}{2}} = 7$ nm) centred at 332 nm along with a 332 nm cut-off filter. Measurements were done under constant stirring by the addition of small defined aliquots of sugar to a fixed volume of protein (A_{280} 0.07). Readings were recorded after an interval of 2 min. The fluorimeter was controlled by a microprocessor that allowed averaging of several measurements. Typically the average of ten measurements was taken and the S.D. was less than 0.55%. Temperature was maintained within ± 0.1 °C of the desired temperature by means of a Lauda water bath. The association constants (K_a) were determined by the method of Chipman et al. (1967):

$$\log [S]_f = -\log K_a + \log\left(\frac{F_c - F_0}{F_x - F_c}\right)$$

where ($F_c - F_0$) is the change in fluorescence intensity upon saccharide binding, F_0 is the initial fluorescence of the protein in the absence of saccharide, F_x is the fluorescence intensity upon saturation of all the sugar-binding sites and F_c is the volume-corrected observed fluorescence. $[S]_f$ represents the free-sugar concentration. The free energies for binding were evaluated by using the equation:

$$-\Delta G = RT \ln K_a \qquad (1)$$

The changes in enthalpies (ΔH) were determined from van't Hoff plots of the temperature-dependent K_a values. The change in entropy (ΔS) was evaluated from the following relationship:

$$\Delta G = H - T\Delta S \qquad (2)$$

Haemagglutination-inhibition assays

Trypsin-treated rabbit erythrocytes were prepared by incubating ten parts of standard erythrocyte suspension (4%) with one part of 1% trypsin solution at 37 °C for 1 h. The cells were washed several times by resuspension in saline (0.9% NaCl) and subsequent centrifugation and finally remade as a standard suspension. Glycophorin A was purified from pooled human erythrocytes by the method of Furthmayr & Marchesi (1983). Asialoglycophorin A and asialofetuin A were prepared by hydrolysis of glycophorin A and fetuin respectively by heating in 0.05 M-H_2SO_4 for 1 h at 80 °C. Haemagglutination-inhibition assays were performed in duplicate by 2-fold serial dilutions of the inhibitors in phosphate-buffered saline (0.02 M-phosphate/0.15 M-NaCl, pH 7.4), in 8 × 12 U-well serological microtitre plates. To 50 μl of the inhibitor solution in each well was added 50 μl of the protein (1.7 μg/ml) and the mixture incubated for 2 h at 4 °C. Erythrocyte suspension (50 μl) was added subsequently and scoring done after 2 h.

RESULTS

By utilizing several synthetic saccharides it has been possible in the present study to deduce a considerable amount of information on the carbohydrate specificity of
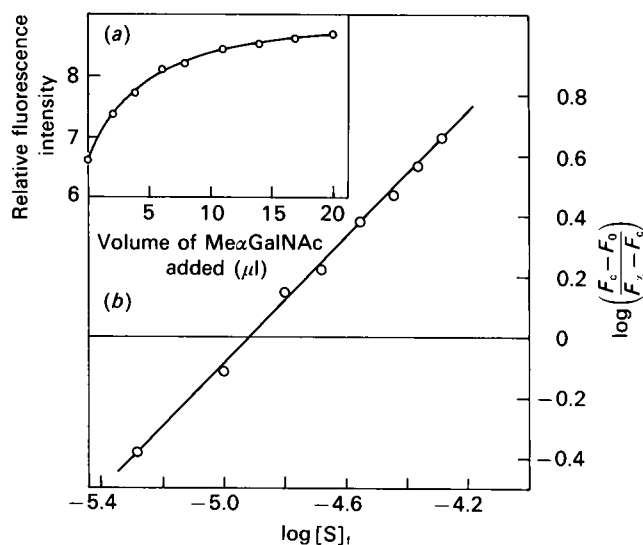

Fig. 1. Evaluation of the association constant for the binding of MeαGalNAc to A. integrifolia lectin by intrinsic fluorescence

(a) Enhancement of the intrinsic fluorescence of A. integrifolia lectin upon its titration with MeαGalNAc (3.42 mM stock) at 20 °C (for details, see the Materials and methods section). (b) Plot for the determination of association constant according to the method of Chipman et al. (1967).

the anti-(T-antigen) lectin from A. integrifolia. In a previous study we have established that the ligand-dependent changes in the intrinsic fluorescence of A. integrifolia agglutinin could be utilized to quantify the parameters for the association of saccharides to the lectin (Sastry & Surolia, 1986). A representative plot of the changes in fluorescence intensity of the lectin upon its titration with MeαGalNAc at 20 °C is shown in Fig. 1. The value of association constant determined from this plot is 7.3×10^5 M^{-1}. The slope of the straight line was unity for MeαGalNAc for binding to the lectin with an equivalent weight of M_r 20000, confirming the bivalent nature of the lectin, which has an M_r of 40000 (Appukuttan & Basu, 1985; Sastry et al., 1986). The values of K_a for several α-linked sugars thus determined are listed in Table 1 together with the values of ΔH and ΔS as determined from van't Hoff plots and eqn. (2) respectively.

It is apparent from Table 1 that MeαGal is significantly a stronger binding ligand than Galαl-3Gal, Galαl-3-GalαMe, Galαl-4Gal, Galαl-6Glc and the blood-group-B trisaccharide. Likewise MeαGalNAc is about 450-fold more potent an inhibitor than the blood-group-A trisaccharide determinant. It may be noted that Galαl-3Gal and Galαl-3GalαMe etc., which have galactose in α-linkage with another galactose residue, show very similar ΔH values (-36 to -38 kJ·mol^{-1}), indicating that these saccharides interact with the lectin in a similar fashion. Moreover, the values of ΔH and ΔS are within the range of values observed for galactose.

Table 2 lists the thermodynamic parameters for the binding of several β-linked disaccharides. It is apparent from the Table that, among the β-linked disaccharides, Galβl-3GalNAcαMe, the structure related to the

Table 1. Association constants and thermodynamic parameters for the binding of α-sugars to _A. integrifolia_ lectin

Abbreviations: A-Tri and B-Tri are blood-group A and B trisaccharides respectively.

Sugar Temp. (°C) ...	$10^{-3} \times K_a$ (M^{-1})				$-\Delta H$ (kJ·mol^{-1})	$-\Delta G^*$ (kJ·mol^{-1})	$-\Delta S$ (J·mol^{-1}·K^{-1})
	15	20	25	30			
MeαGalNAc	122.0	73.28	46.6	30.6	63.7	28.04	123.8
MeαGal†	63.0	40.0	34.0	21.0	55.0	26.46	99.0
Galα1-3GalαMe	7.48	5.75	4.46		36.8	21.36	53.61
Galα1-3Gal	7.15	5.45	4.32		38.37	21.25	59.44
Galα1-4Gal	0.841					16.13	
Mellibiose	6.76	5.31	4.365	3.31	38.05	21.11	58.32
A-Tri	0.27					13.4	
B-Tri	0.24					13.12	

* Values were calculated for 15 °C.
† Values taken from Sastry _et al._ (1986).

Table 2. Association constants and thermodynamic parameters for the binding of β-sugars to _A. integrifolia_ lectin

Sugar Temp. (°C) ...	$10^{-3} \times K_a$ (M^{-1})				$-\Delta H$ (KJ·mol^{-1})	$-\Delta G^*$ (KJ·mol^{-1})	$-\Delta S$ (J·mol^{-1}·K^{-1})
	15	20	25	30			
MeβGalNAc	0.548					15.1	
MeβGal†	0.285	0.26	0.16	0.10	42.0	13.53	98.8
Galβ1-3GalβMe	0.321					13.86	
Galβ1-3GalNAcαMe	1520.0	806.0	400.31	229.0	95.6	34.07	213.0
Galβ1-3GalNAcβMe	0.540	0.40	0.325		36.87	15.06	75.0
Glcβ1-3GalNAc	24.30	18.76	13.76		42.2	24.18	62.0
GlcNAcβ1-3Gal	11.74					22.43	
GalNAcβ1-3GalαMe	489.77	301.99	181.97		68.8	31.36	130
Asialo-G$_{M1}$ oligo-saccharide	N.B.‡						

* Values were calculated at 15 °C.
† Values taken from Sastry _et al._ (1986).
‡ N.B., no binding at 400 μM concentration.

Thomsen–Friedenreich cancer-associated antigen, is the most potent ligand for binding to the lectin. Interestingly enough, the lectin binds to Galβ1-3GalNAcβMe about 2800 times more weakly than Galβ1-3GalNAcαMe does. Failure of the lectin to bind to the asialo-G$_{M1}$ oligo-saccharide (Galβ1-3GalNAcβ1-4Galβ1-4Glc), which contains the Galβ1-3GalNAc in β-linkage with other sugars, further proves that the lectin is highly specific for the tumour-associated T-antigenic structure. In this respect it differs from peanut lectin and the human pan-agglutinin associated with human anti-(T-antigen) response (Clausen _et al._, 1988), both of which are known to bind to asialo-G$_{M1}$ as well. Interestingly enough, β-linked disaccharides with a variety of hexopyranose residues at the non-reducing end showed reasonable binding to the lectin as long as Gal and GalNAc were at the reducing terminals. Thus Glcβ1-3GalNAc and GlcNAcβ1-3Gal bind to the lectin with affinities comparable with those observed for GalNAc and Gal respectively, whereas Galβ1-3Ara, like lactose etc. (Sastry _et al._, 1986), binds extremely poorly to the lectin.

Table 3 shows the results of haemagglutination-in-hibition experiments in terms of the relative potencies of the inhibitors and their concentrations required for 50 % inhibition of agglutination (IC$_{50}$). Galβ1-3GalNAc was taken as the reference compound. Galβ1-3GalNAcαSer and Galβ1-3GalNAcαMe were found to have a relative potency of 2.5 and 22.0 respectively. Asialoglycophorin

Table 3. Concentration of inhibitors required for 50 % inhibition (IC$_{50}$) of haemagglutination of _A. integrifolia_ lectin and their relative inhibitory potencies as compared with Galβ(1-3)GalNAc

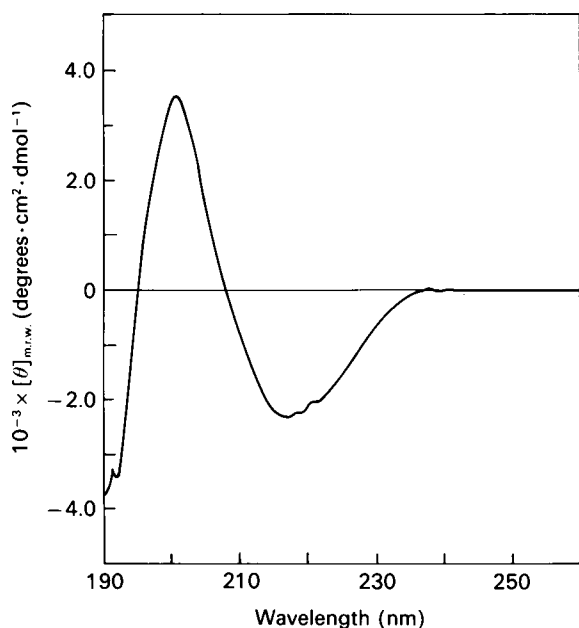| Inhibitor	IC$_{50}$ (nmol·l^{-1})	Relative inhibitory potency
Galβ1-3GalNAc	381.1	1.0
Galβ1-3GalNAcαSer	152.4	2.5
Galβ1-3GalNAcαMe	17.7	22.0
Asialoglycophorin A	0.9×10^{-3}	423444.0
Asialofetuin	8.1×10^{-3}	47049.0

Fig. 2. Far-u.v. c.d. spectra of *A. integrifolia* lectin

The cuvette path length was 0.01 cm and the protein concentration was 0.876 mg/ml in phosphate-buffered saline, pH 7.2. $[\theta]_{m.r.w.}$ is the mean residue ellipticity.

A and asialofetuin were about 423 444 and 47 049 times stronger inhibitor respectively as compared with Galβ1-3GalNAc.

C.d. in the near-u.v. region (> 250 nm) of *A. integrifolia* lectin is shown in Fig. 2. The most prominent

features of the spectrum are maxima at 267, 273 and 284 nm, and minima at 269, 279 and 288 nm and a shoulder at 293 nm. Analysis of the secondary structure of the lectin by the method of Provencher & Glockner (1981) revealed 50 % of pleated-sheet structure and the rest aperiodic structure. Only the near-u.v. c.d. spectrum of the lectin was altered by sugars (Fig. 3). Since no changes in the far-u.v. c.d. spectrum of the protein were brought about by any of the sugars studied (results not shown), conformational changes in the polypeptide backbone were ruled out. The binding equilibrium of the lectin with sugars could therefore be described by a simple single-step association reaction, viz.:

$$P + L \rightarrow PL; \quad \frac{[PL]}{[P][L]} = K_a$$
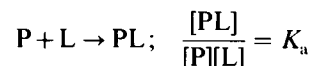
The association constants could therefore be determined by the method of Chipman *et al.* (1967). Table 4 lists the values of K_a thus determined, together with the values of changes in the free energies. The K_a values obtained by the c.d. method are in agreement with those obtained by fluorescence measurements, indicating that both techniques measure the same binding event.

More importantly, we considered the possibility that different types of saccharides studied could alter the c.d. bands in the near-u.v. region of the protein in a characteristic manner. This would then permit us to understand the arrangement of subsites in the binding region of the protein. Fig. 4 shows the ligand-induced changes in the c.d. spectrum of the protein saturated with MeαGal and MeβGal as the representative examples for the α- and β-linked saccharides respectively.
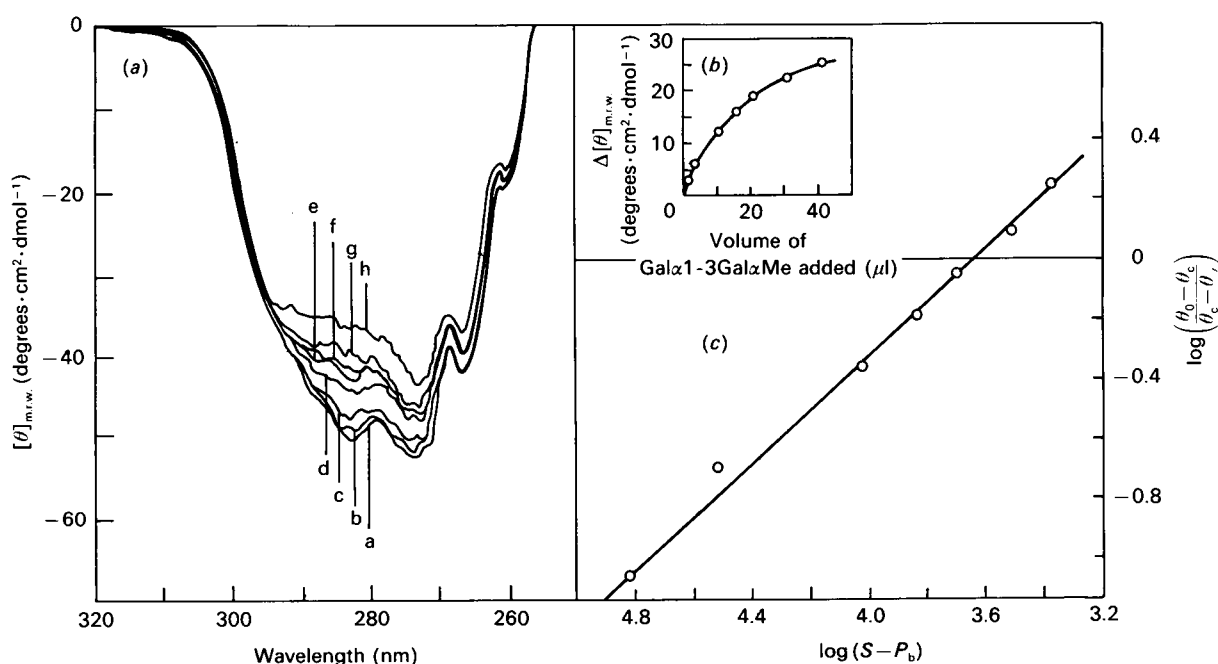
In Table 4 the changes in the mean residue ellipticities

Fig. 3. Near-u.v. c.d. spectrum of *A. integrifolia* lectin with Galα1-3GalαMe and without the sugar

(a) Curve a represents the spectrum due to the protein alone, and curves b–h the altered spectra obtained upon ligand binding. The concentration of the protein was 131 μM in phosphate-buffered saline, pH 7.2, and the sugar was at a concentration of 12.5 mM. [θ] is the mean residue ellipticity. (b) A plot of the ligand-dependent changes in mean residue ellipticity as observed in (a). (c) Plot of (b) for the determination of association constant according to the method of Chipman *et al.* (1967).

Table 4. Mean-residue-ellipticity ($[\theta]_{m.r.w.}$) changes upon ligand binding to *A. integrifolia* lectin and the corresponding association constants

Sugar	$10^{-3} \times K_a$ at 25 °C (M^{-1})	$\Delta[\theta]^{273}_{m.r.w.}$ (degrees·cm²·dmol⁻¹)	$\Delta[\theta]^{284}_{m.r.w.}$ (degrees·cm²·dmol⁻¹)	$\Delta[\theta]^{284}_{m.r.w.}/\Delta[\theta]^{273}_{m.r.w.}$	Percentage saturation*
MeαGal	35.0	14.6	25.1	1.71	98.0
MeβGal	0.17	2.526	2.526	1.0	56.0
Galβ1-3GalβMe†	0.19	16.3	16.3	1.0	41.0
Galα1-3GalαMe	4.26	19.1	29.5	1.54	72.0
GalNH₂	1.32	8.2	11.5	1.4	74.2
2-Deoxy-Gal	0.51	8.86	11.2	1.26	65.0
Lactose†	N.D.‡	4.2	1.6	0.38	31.0
Mellibiose	4.12	28.7	42.2	1.47	91.0
MeαGalNAc	44.0	39.8	56.1	1.40	96.5
Galβ1-3GalNAcαMe	378.51	28.5	42.0	1.47	87.0

* Calculated from K_a values and the concentration of the protein (131 μM) and the respective ligand concentrations.
† For ligands with low affinity it was not possible to exceed saturation beyond 42%.
‡ N.D., not done.

of the lectin at 274 and 283 nm brought about by various ligands and their corresponding saturation ranges are listed. It is apparent that stronger ligands, such as MeαGalNAc, MeαGal, T-antigenic disaccharide, as well as several α-linked sugars, show not only greater reduction in the ellipticity values in the near u.v.-region but also display a qualitatively different spectra when compared with those of β-linked sugars such as MeβGal, lactose, *N*-acetyl-lactosamine etc. These results are also compiled in Table 4. The thermodynamic parameters and the perturbation in the c.d. spectrum of the protein by ligands are considered in discussion for delineation of the arrangement of saccharides in the subsites of the lectin molecule.
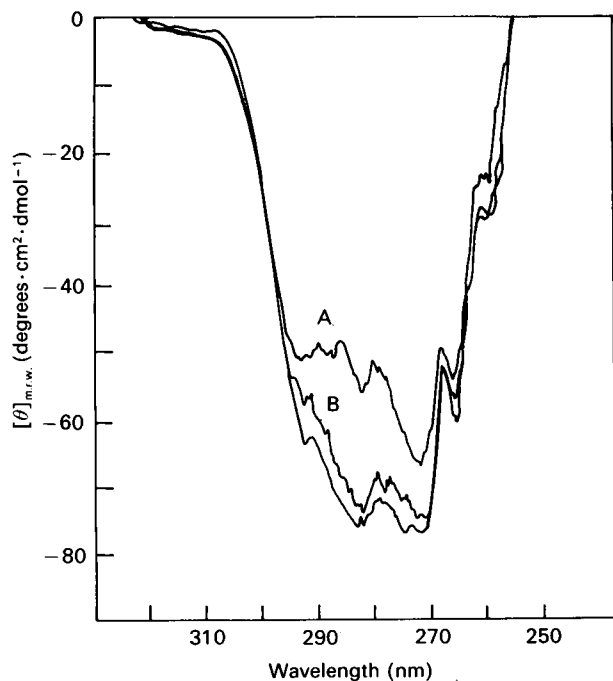

Fig. 4. Perturbation of the near-u.v. c.d. spectrum of *A. integrifolia* lectin upon binding of (A) MeαGal and (B) MeβGal as representative examples of α- and β-linked sugars

DISCUSSION

In the present study we have established the binding characteristics of *A. integrifolia* lectin in considerable detail. Our results allow interpretations of the carbohydrate-binding specificity of the lectin at the molecular level.

One of the unusual features of the lectin is that it has strong affinity for both MeαGal and MeαGalNAc, like the lectins specific for blood-group-B and -A determinants respectively (Etzler & Kabat, 1970; Goldstein *et al.*, 1981), yet it displays a remarkable specificity for binding to Galβ1-3GalNAc, the T-hapten. The present investigation resolves this dilemma and further defines the specificity of the lectin.

An especially interesting discovery was the observation that *A. integrifolia* lectin, in contrast with the widely used anti-(T-antigen) lectin from peanut, binds to the α-anomer of T-antigen (Galβ1-3GalNAcαMe) very strongly compared with its β-anomer (Galβ1-3GalNAcβMe). Lack of binding of the *A. integrifolia* lectin to asialo-G_{M1} oligosaccharide noted here further supports the fine specificity of the lectin for Thomsen–Freidenreich antigen. ΔH and ΔS calculated from our association constants are significantly different for Galβ1-3GalNAcαMe ($\Delta H = -95.6$ kJ·mol⁻¹ and $\Delta S = -213$ J·mol⁻¹·K⁻¹) and Galβ1-3GalNAcβMe ($\Delta H = -36.87$ kJ·mol⁻¹ and $\Delta S = -75$ J·mol⁻¹·K⁻¹). The strong binding of Galβ1-3GalNAcαMe is presumably due to a favourable increase in enthalpy ($-\Delta H$), which outweighs the unfavourable entropic contributions. Significant differences in the values of the thermodynamic parameters suggest differential modes for the association of these structurally related disaccharides. The value of $-\Delta H$ for Galβ1-3GalNAcβMe is comparable with that observed for the binding of MeβGalNAc or MeβGal, indicating that the former compound binds to the lectin through a single hexopyranose residue. The specific recognition of α-linked T-hapten readily explains the unique binding of *A. integrifolia* lectin to IgA₁ in serum (Roque-Barreira *et al.*, 1985; Kondoh *et al.*, 1986).

Several monoclonal antibodies directed to the tumour-associated T-antigen have been described. However, most
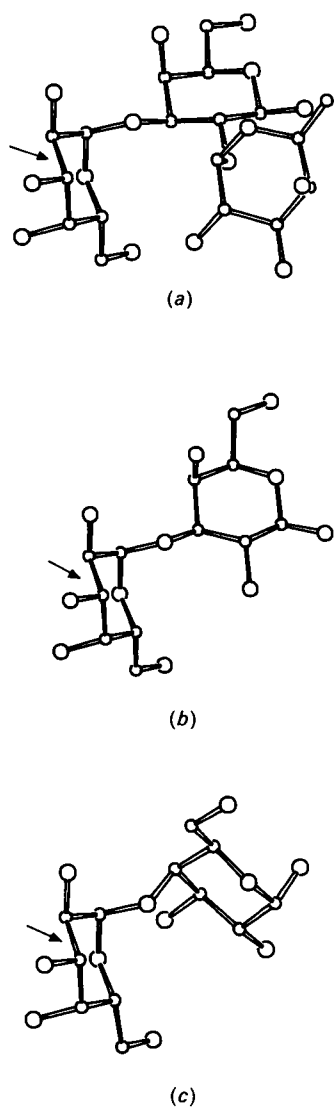
(a)

(b)

(c)

Fig. 5. ORTEP diagram (Johnson, 1965) of the minimum energy conformations of (a) blood-group-B trisaccharide Galα1-3(L-Fucα1-2)GalβOH, (b) Galα1-3GalβOH and (c) Galα1-4GalαOH

The diagrams were drawn using the program ORTEP modified to suit the HP-1000 system. Ring protons and hydroxy hydrogens are omitted in the diagram. The small and big circles represent carbon and oxygen atoms respectively. The non-reducing terminal Gal is marked by an arrow in all the three structures. The glycosidic torsion angles are defined as Φ = H1-C1-O1-CX and ψ = HX-CX-O1-C1. The conformation about the exocyclic C-5–C-6 bond is defined by the torsion angle χ, where χ = O5-C5-C6-O6. The values Φ = −65°, ψ = −50°, and Φ = 55°, ψ = 20° were used for Galα1-3Gal and L-Fucα1-2Gal fragment of blood-group-B trisaccharide respectively. These values were obtained from the hard-sphere exo-anomeric calculation of Thorgensen & Lemieux (1982). The value of χ used for both galactose units was 60°. Structure (b) was generated using the values of Φ = −50° and ψ = −35° and χ = 60° as reported by Bock et al. (1985). The reducing-end hexopyranose has been shown to be in the β-anomeric form for the structures (a) and (b) because of the known greater preference of the β- over the α-form in solution for such structures. Structure (c) has been drawn using Φ = −18° and = 33° as obtained by Svensson et al. (1986) from crystal structure determination of

of these antibodies recognize the disaccharide Galβ1-3GalNAc, irrespective of the linkage of the penultimate GalNAc residue (Clausen et al., 1986, 1988). The remarkable specificity of the lectin for T-antigen should make it a very useful reagent to detect the real T-antigen structure, which has a penultimate αGalNAc residue.

Perhaps an equally important observation is that the lectin, despite its strong affinity for MeαGal and MeαGalNAc, binds extremely poorly to A- and B-blood-group determinants and to Galα1-3GalαMe, Galα1-4Gal etc. Thus the K_a values for the binding of disaccharides in which galactose is in α-linkage with other sugars is significantly lower than that observed for MeαGal. This suggests that the replacement of the apolar methyl group of MeαGal with a hydrophilic hexopyranose residue, as in Galα1-3Gal or Galα1-3GalαMe, leads to diminished apolar contacts, resulting in their poor binding to the lectin. The values of $-\Delta H$ for their interaction with the lectin is close to the monosaccharide, galactose, indicating that they may be interacting through a single galactopyranose residue with the protein.

Galα1-3Gal and Galα1-3GalαMe can bind to the lectin in two possible orientations, i.e. through their terminal or penultimate (methylated in the case of Galα1-3GalαMe) hexopyranose residue. Since the values of ΔG and ΔH for the binding of Galα1-3GalαMe and Galα1-3Gal are identical and are consistently lower than those observed for the binding of MeαGal, it appears that the lectin interacts with these sugars through their non-reducing hexopyranose residue. Support for this suggestion also comes from the very similar thermodynamic parameters for the association of Galα1-6Glc, which, by virtue of having a single galactopyranose residue, is expected to bind through its non-reducing sugar residue alone.

It is instructive to consider the thermodynamic parameters for the binding of Galα1-3Gal and Galα1-4Gal in conjunction with their 'ball-and-stick' models (Fig. 5) plotted (ORTEP) from their minimum-energy conformations and as obtained by X-ray-diffraction studies respectively, to understand the binding behaviour of these disaccharides with the lectin. Though both the disaccharides are α-linked, their overall topographies are quite different. The reducing-end galactose residue in the α1-4-linked sugar is very much closer to the terminal galactose residue, so much so that an intramolecular hydrogen bond between O-3 of the reducing sugar with the ring oxygen atom of the non-reducing galactose has been observed in its crystal structure (Svensson et al., 1986). An examination of the three-dimensional model of Galα1-4Gal (not shown) in addition also leads to the projection of the equatorially oriented C-5 hydroxymethyl group in close proximity to the non-reducing sugar residue. Such a disposition of the reducing-end galactose residue with respect to the non-reducing galactose residue probably sterically hinders the accessibility of the terminal galactopyranose unit of this disaccharide to the binding locus of the lectin. In Galα1-

galabiose. The values of χ for both monosaccharide units has been taken as 60° owing to the 70 % occurrence of gauche-trans conformation about the exocyclic C-5–C-6 and C-5′–C-6′ bond. The reducing-end hexopyranose is shown to be in α-anomeric form because of the observed preference for α-form (55 %) in the crystalline galabiose.

3Gal the reducing sugar residue is projected away from the non-reducing galactose residue, and hence no such steric hindrance of the binding of the terminal galactose residue with the lectin is observed, thus explaining why this compound has a higher affinity than Galα1-4Gal. The conformation of the Galα1-3Gal and GalNAcα1-3Gal disaccharide fragment of A- and B-blood-group trisaccharides are similar to that of Galα1-3Gal. Weak association of A- and B-blood-group trisaccharides presumably results from the steric hindrance caused by the L-fucose residue in α1-2 linkage with the reducing-end galactose residue in Galα1-3Gal and GalNAcα1-3Gal in B- and A-blood-group trisaccharides respectively.

In a previous study (Sastry et al., 1986) we demonstrated that the lectin fails to bind to lactose, N-acetyl-lactosamine and Galβ1-3GlcNAc due to a steric hindrance caused by the equatorially oriented C-3/4 hydroxy group of their reducing residue. Binding studies with Glcβ1-3GalNAc and GlcNAcβ1-3Gal not only further confirm these observations but also shed light on the role of substitutions the other way round (e.g. Galβ1-3GlcNAc changed to GlcNAcβ1-3Gal). Stronger binding of these disaccharides to the lectin as compared with lactose and N-acetyl-lactosamine supports the important role played by the axially oriented hydroxy group at C-4 in the reducing sugar for binding of the β-linked disaccharides. Moreover, the fact that the thermodynamic parameters for the association of Glcβ1-3GalNAc and GlcNAcβ1-3Gal are in the range of those for GalNAc and Gal respectively suggests that the non-reducing moieties in these disaccharides are tolerated in the binding region of the protein, though the reducing sugar contributes substantially for binding.

Precipitation of the glycoprotein–lectin complex upon interaction precluded the measurement of the affinity of binding by the method of fluorescence titration. Therefore an alternative method such as haemagglutination inhibition was employed. Galβ1-3GalNAcαSer was included in inhibition studies because it was thought to provide a good reference oligosaccharide derivative for comparison of the binding affinities of glycoproteins for the lectin, owing to the fact that the glycoproteins studied are known to have this structure as part of their carbohydrate chains. The results of these studies further confirmed the complementarity of the lectin-combining site for T-antigen. Galβ1-3GalNAcαSer, biologically the most relevant oligosaccharide derivative, was found to be a highly potent inhibitor. An even higher potency exhibited by Galβ1-3GalNAcαMe is in accord with the observation made using fluorescence measurements and, as already indicated above, arises, presumably, because of non-polar interactions of the reducing-end α-linked methyl group with the protein as shown for MeαGal in our previous study (Sastry et al., 1981). Asialoglycophorin A was found to be over 423444 times more potent than Galβ1-3GalNAc and over 169333 times more potent than Galβ1-3GalNAcαSer. Similarly asialofetuin proved to be about 47094 times more potent than Galβ1-3GalNAc and over 18814 times more potent than Galβ1-3GalNAcαSer. Asialoglycophorin A had 15 O-linked oligosaccharide chains having a Galβ1-3Gal-NAcαSer structure in addition to one N-linked oligosaccharide chain. Therefore a single asialoglycophorin A molecule could react simultaneously with more than one lectin binding site, giving rise to a dramatic rise in the binding affinity. A ligand capable of having multiple

interactions with a lectin would be expected to increase remarkably the affinity of binding, owing to the additive nature of the individual free energies (ΔG). As a consequence the resultant ΔG of such interactions would give rise to an exponential increase in the association constant. Such an explanation was offered in the case of oligosaccharide binding to Datura stramonium (thornapple) lectin (Crowley et al., 1984), where the biantennary N-acetyl-lactosamine-containing oligosaccharide was found to be 500 times more potent than N-acetyl-lactosamine, owing probably to simultaneous interaction of the biantennary sugar with both the binding sites of the lectin. This is also in accord with the reasoning forwarded by Kronis & Carver (1982) to account for the increased affinity for binding of wheat-germ agglutinin to cell-surface glycoconjugates as compared with its binding to simple sugars. Fetuin also has been shown by Spiro (1960) and Spiro & Bhoyroo (1974) to have three O-linked oligosaccharide chains in addition to having three N-linked onces. As in asialoglycophorin A, the O-linked oligosaccharide chains in asialofetuin have the Galβ1-3GalNAcαSer structure. The very high inhibitory power of asialofetuin could also arise as a result of factors similar to those implicated in the very high binding affinity of asialoglycophorin A for the lectin.

The disaccharide GalNAcβ1-3GalαMe binds very strongly to the lectin (Table 2). This disaccharide represents the terminal disaccharide fragment of globoside (GalNAcβ1-3Galβ1-4Galβ1-4Glc1-1Ser) in the α-methyl glycosidic form. From the foregoing discussion it would be apparent that this strong affinity arises largely because of the presence of the glycosidic methyl group in α-linkage, as is observed in the case of strong binding of sugars such as MeαGal, MeαGalNAc and Galβ1-3GalNAcαMe. The high affinity for GalNAcβ1-3GalαMe displayed by the lectin, however, does not detract from its potential as an anti-(T-antigen) probe, because the lectin reacts poorly with globotetrasaccharide (GalNAcβ1-3Galβ1-4Galβ1-4Glc). Evidence for this comes from haemagglutination-inhibition experiments, which reveal that globotetrasaccharide, with an IC_{50} of 2210 nmol·l^{-1} is about 12 times less reactive than Galβ1-3GalNAcαSer and about 5.8 times less reactive Galβ1-3GalNAc (A. Surolia & S. K. Mahanta, than unpublished work).

The c.d. spectrum in the near-u.v. region predominantly reflects the tertiary structure and contains absorption bands for phenylalanine, tyrosine and tryptophan. The regions between 255 and 265 nm could be due to phenylalanine as well as tyrosine side chains in the asymmetric environment. The bands at 273 nm and 284 nm together with a shoulder at 294 nm correspond to those of tyrosine and tryptophan in the asymmetric environment respectively (Jirgensons, 1973). The question of the specificity of the ligand-induced changes in the c.d. spectrum of the protein and its relationship to the arrangement of the subsites and their specificities are now discussed. Interestingly enough, all the α-linked sugars such as MeαGal, MeαGalNAc, Galα1-3Gal etc., as well as Galβ1-3GalNAcαMe, perturb strongly the 284 nm band and the shoulder region at 294 nm as well as the band at 273 nm, albeit less strongly as compared with the 284 nm band. Thus the more complementary ligands perturb the bands assigned to tryptophan as well as to tyrosine. On the other hand, most of the β-linked sugars, such as MeβGal, lactose, N-acetyl-lactosamine etc., alter principally the

region assigned to tyrosine side chain(s) (at 273 nm) alone in the protein.

Chemical-modification studies of Appukuttan & Basu (1985a,b) revealed that lysine and tyrosine side chains are involved in the activity of *Artocarpus* lectin and that the protein is devoid of tryptophan. Our studies, on the other hand, indicate participation of both tyrosine and tryptophan in the activity of the lectin. Ligand-structure-dependent changes in the near-u.v. c.d. spectrum of the protein reflect some unique spatial disposition of the bound saccharides with respect to chromophoric groups in the combining region of the protein. Alternatively such alterations could also arise as a result of conformational changes induced upon ligand binding. However, failure to observe any detectable change in the far-u.v. c.d. spectrum of the protein, even in the presence of excess sugar, led us to preclude any significant conformational change resulting from saccharide binding. The lectin has been previously shown by us to possess an extended combining region most complementary to the T-antigenic disaccharide Galβ1-3GalNAc. However, the arrangement of the subsites in the combining region in the protein could not be established. In the light of these data it is now possible to propose the most likely arrangement of the saccharides in the combining region of the protein (Fig. 6). Since MeαGal, MeαGalNAc, galactose and GalNAc not only strongly bind to the lectin but also perturb the spectrum in nearly the same fashion, it is conceivable that MeαGal, MeαGalNAc etc., bind to the primary site designated as subsite A, where they would interact either directly with the lone tryptophan side chain in the active site of the protein or perturb it in addition to the tyrosine side chains. MeβGal and the β-linked disaccharides, which have very poor affinity for the lectin, they do not in contrast perturb the c.d. band corresponding to tryptophan but alter the region corresponding to tyrosine residue alone. This suggests that these ligands interact only with the secondary subsite designated as subsite B, which is further from the tryptophan in the active site of the lectin. In other words, the primary binding site can accommodate α-linked or free galactose or N-acetylgalactosamine, whereas the secondary site can accommodate only the β-linked monosaccharides and disaccharides. Since Galβ1-3GalNAcαMe and Galβ1-3GalNAc have high affinities for the lectin and perturb the tryptophan band in the protein also, their N-acetylgalactosamine residues would be bound in the primary site, whereas the non-reducing galactopyranose residues would be bound in the secondary subsite. According to this model other β-linked disaccharides, such as lactose, N-acetyl-lactosamine and Galβ1-3GlcNAc, can interact with the lectin by binding to the secondary subsite through their non-reducing galactopyranose residues. On the basis of these observations the arrangement of the subsites in the lectin with positions for the binding of various sugars is depicted in Fig. 6. According to this model, at least one tyrosine side chain is close to both the subsites, whereas the tryptophan side chain in the protein is in close proximity of subsite A alone. Hence binding of a saccharide to subsite A would result in the perturbation of the ellipticities of both the tyrosine and tryptophan side chains. Thus the interaction of MeαGal, MeαGalNAc, Galα1-3GalαMe and Galβ1-3GalNAc with the lectin is consistent with the observed perturbation of both these side chains in the protein. On the other hand, binding of
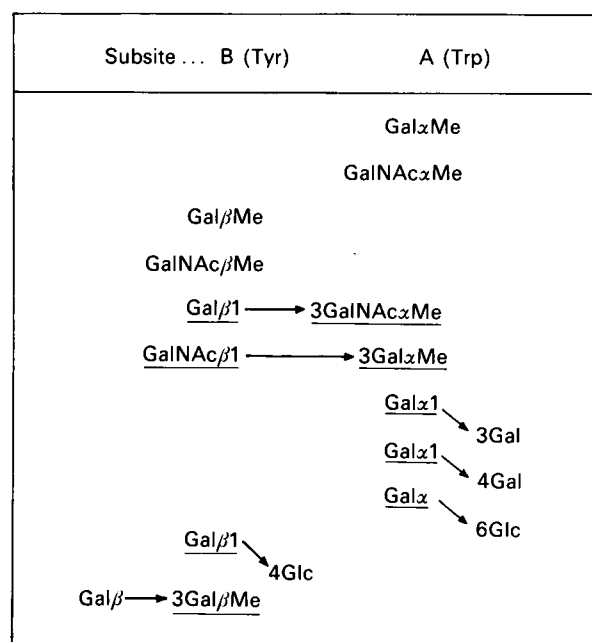

Fig. 6. A schematic representation of the combining region of *A. integrifolia* lectin

Subsite A is the primary subsite which accommodates Gal, GalNAc and their α-linked derivatives alone. In disaccharides Gal and GalNAc residues can interact with this subsite if they are in α-linkage. The tryptophan side chain(s) is presumably close to subsite A. The ratio 284/273 (Table 4) with values greater than 1 is characteristic of sugars binding to subsite A, whereas sugars interacting primarily with subsite B consistently show a value of 1 or less. Ligands associated with subsite A strongly perturb the ellipticity of tryptophan side chain(s) in addition to the perturbation of the tyrosine side chain(s). Subsite B (or the secondary subsite) is accessible only for the β-linked Gal or GalNAc residues in disaccharides or to GalβMe or GalNAcβMe, and hence they are expected to perturb tyrosine side chains only. If Gal and GalNAc are in β-linkage with sugars other than Gal or GalNAc (e.g. lactose), then the sugar at the reducing end is exposed to the medium. Galβ1-3GalβMe probably binds to the subsite B through its penultimate sugar (viz. GalβMe). Galβ1-3GalNAcβMe is also likely to bind in the same way. Binding of these disaccharides through their terminal β-linked Gal to subsite B, exposing the penultimate sugar to the medium, cannot be excluded at the moment. Strong binding of Galβ1-3GalNAcαMe is due to the fact that both residues are most complementary to the binding site. Sugars underlined in disaccharides are the ones expected to bind in the respective subsites.

MeβGal, lactose and N-acetyl-lactosamine occurs through the subsite B, and hence only the tyrosine side chains in the active site of the lectin are perturbed. Galα1-3Gal, Galα1-3GalαMe and Galα1-6Glc, which on thermodynamic considerations have been shown to bind through their terminal hexopyranose residues, also perturb the spectrum much as do Gal etc., and hence we suggest that they would associate with their non-reducing galactopyranose residue in the primary subsite, while their penultimate sugar (viz. glucose in melibiose, GalαMe in Galα1-3GalαMe, and galactose in Galα1-

3Gal) would be exposed to the medium. Glcβ1-3GalNAc and GlcNAcβ1-3Gal are expected to bind through their reducing Gal and GalNAc residues respectively, at the primary subsite in the combining region of the protein.

In conclusion, in addition to underscoring the unique binding specificity of *A. integrifolia* lectin to the Thomsen–Friedenreich antigen, our studies also provide insight into the arrangement of the sugars in the combining region of the lectin. The non-binding of this lectin to Galβ1-3GalNAcβMe and asialo-G_{M1} and its exquisite recognition of Galβ1-3GalNAcαSer makes the lectin a valuable probe for monitoring the expression of Thomsen–Friedenreich antigen on cell surfaces. A recent study from Hakomori's laboratory (Clausen *et al.*, 1988) has shown that a monoclonal antibody raised against galactosyl A antigen (precursor of type 3 A and H glycolipids, which has an internal A type structure) also reacts with T-antigen. It would, therefore, be interesting to study the reactivity of *A. integrifolia* lectin with galactosyl A antigen.

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