# Effect of Substituents on the Thermodynamics of D-Galactopyranoside Binding to Winged Bean (*Psophocarpus tetragonolobus*) Basic Lectin†

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ABSTRACT: Isothermal titration calorimetric measurements of the binding of deoxy, fluorodeoxy, and methoxy derivatives of D-galactopyranoside (α-D-Gal) to the basic lectin from winged bean *Psophocarpus tetragonolobus*, WBA I, have been carried out. Each of the ligands binding to WBA I displayed the same stoichiometry of one per subunit (29 kDa) of WBA I. The binding enthalpies for various derivatives are essentially independent of temperature and show complementary changes with respect to binding entropies. Replacement of the hydroxyl group by fluorine or hydrogen on C3 and C4 of the galactopyranoside eliminates binding to the lectin, consistent with C3-OH and C4-OH acting as hydrogen bond donors. The affinity for C2 derivatives of galactose decreases in the order GalNAc > 2MeOGal > 2FGal ≅ Gal > 2HGal, which suggests that both polar and nonpolar residues surround the C2 locus of galactose, consistent with the observed high affinity of WBA I toward GalNAc where the acetamido group at C2 position is probably stabilized by both nonpolar interactions with the methyl group and polar interactions with the carbonyl group. The binding of C6 derivatives follows the order Gal > 6FGal > D-Fuc ≫ 6MeOGal ≅ L-Ara, indicating the presence of favourable polar interactions with a hydrogen bond donor in the vicinity. On the basis of these results the hydrogen bond donor−acceptor relationship of the complexation of methyl-α-D-galactopyranoside with the primary combining site of WBA I is proposed.

Specificity of protein−ligand interactions is a central attribute of several biological phenomena. Among proteins that specifically recognize carbohydrates and regulate biological events, lectins constitute a distinctive group and as such provide a paradigm for such interactions (*1*, *2*). At a molecular level, these remarkable selectivities are an outcome of interplay between different forces involved in the interactions between protein receptors and their carbohydrate ligands (*3*, *4*). While the nature of these forces is fairly well understood, their magnitudes and relative contributions have been explored only in a few cases (*5*). The current dogma is that the hydrogen bonding interactions and van der Waal's interactions impart specificity to ligand binding with little contribution to binding energy, whereas hydrophobic interactions constitute the major driving force (*6*). In the case of protein−carbohydrate interactions, however, the hydrogen bonding interactions are predominant because of the participation of the sugar hydroxyl groups. Thus in legume lectins, there are 5−9 hydrogen bonds between a monosaccharide and protein residues or bound water molecules that together determine their carbohydrate specificities. Studies directed toward quantification of contributions of individual forces, especially hydrogen bonds, in conjunction with structure determination of protein−ligand complexes should allow a better appreciation of the molecular specificities of these interactions (*7*).

Binding studies with specifically modified but sterically conserved ligands such as the deoxy and fluorodeoxy sugar analogues can be successfully used in determining the nature of their hydrogen bonding interactions with proteins (*7*, *8*). Fluorodeoxy sugars, where a hydroxyl group is replaced by electronegative and isosteric fluorine atom, provide information about the existence and nature of specific hydrogen bonds as they can only accept a hydrogen bond from a proximal hydrogen bond acceptor and cannot function as a hydrogen bond donor (*9*). Similarly, the contribution of hydrophobic interaction can be obtained by the binding behavior of derivatives in which the hydroxyl hydrogen is replaced with an apolar substituent (*10*). Only a few studies with fluorodeoxy and other substituted sugar analogues with an enzyme (*7*, *11*), an antibody (*12*), and a sugar transport protein (*13*) have so far been reported. We have recently reported the effect of substituents on the thermodynamics of binding of D-glucopyranosides to three mannose-specific legume lectins (*8*).

Winged bean agglutinin I (WBA I)[1] is the basic lectin (p*I* = 10) isolated from the seeds of winged bean (*Psophocarpus tetragonolobus*) that recognizes D-galactopyranosides and binds most strongly to A-pentasaccharide (*14*). Determination of the three-dimensional structure of WBA I is currently underway (*15*). However, from the high degree of sequence similarity with another Gal/GalNAc-specific legume lectin from *Erythrina corallodendron*, EcorL (*16*), it appears that the two lectins perhaps share some crucial determinants in the topology of their binding sites. Each subunit of this

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[1] Abbreviations: ConA, concanavalin A; EcorL, *Erythrina corallodendron* agglutinin; GS IV, *Griffonia simplicifolia* agglutinin IV; PNA, *Arachis hypogea* agglutinin; SBA, *Glycine max* agglutinin; WBA I, winged bean (basic) agglutinin I; ITC, isothermal titration calorimetry; L-Ara, L-arabinose; α-D-Gal, α-D-galactopyranoside; GalNAc, *N*-acetyl-D-galactosamine; FGal, fluorodeoxygalactose; D-Fuc, D-fucose; HGal, deoxygalactose; MeOGal, methoxygalactose.

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homodimeric protein consists of 239 amino acids ($M_r$ = 29 000) and possesses one sugar binding site. The thermodynamic and binding studies on WBA I have been carried out by using *N*-dansyl (*17*) and methylumbelliferyl (*18*) derivatives as indicator ligands as well as by direct measurement of heat accompanying the binding reaction by titration calorimetry (*19*). These studies showed that the association of WBA I to galactopyranosides is enthalpically driven with a small contribution from entropy changes. Furthermore, an acetamido substituent at C2 position and a hydrophobic substituent in the α-conformation at the anomeric position were shown to facilitate binding. The thermodynamic analyses of deoxy-, fluorodeoxy-, and methoxygalactopyranosides in addition to highlighting the role of individual hydroxyl groups to the binding reaction have allowed a delineation of the nature and magnitude of the forces that contribute to the interaction.

## MATERIALS AND METHODS

*Materials*. Galactose derivatives 2HGal, 4HGal, D-Fuc, 6FGal, αMeOGal, and 6MeOGal were obtained from Sigma Chemical Co. 4FGal was a kind gift from Prof. C. F. Brewer, Albert Einstein College of Medicine. All other galactose derivatives were synthesized following the methods described previously (*20*−*23*), and the purity was checked by melting point, thin-layer chromatography, and high-resolution $^{19}$F-NMR at 80 MHz on a Varian NMR spectrometer. The winged bean basic lectin, WBA I, was prepared in 20 mM phosphate buffer at pH 7.2 containing 150 mM sodium chloride as described previously (*17*). WBA I concentrations were measured spectrophotometrically using $E^{1\%,1cm}_{280nm} = 9.37$. The extinction coefficient was determined by weight method as well as from amino acid sequence data (*16*, *24*).

*Isothermal Titration Calorimetry*. Titration calorimetric measurements were made on an Omega titration calorimeter from Microcal Inc. as described previously (*19*). Briefly, 5−10-μL aliquots of the derivative solution were added via a 250-μL rotating syringe stirring at 400 rpm to the solution cell containing 1.385 mL of the lectin solution, and heat changes accompanying the additions were recorded. The total concentration of the lectin, $[P]_t$, was from 0.6 to 2.5 mM whereas the total concentration of ligand, $[L]_t$, was from 9.6 to 84 mM. The titration of ligand solution in this concentration range with the buffer solution alone gave negligible values for the heat of dilution. The time duration between the injections was at least 3 min to allow the peak to return to baseline, and the number of additions of the sugar titrant were fixed such that the area below the peak is reduced by at least 1 order of magnitude or until protein binding sites are saturated ≫85%. The heat content of a solution has been shown to be related to the total concentrations of the protein and ligand through the following equation (*25*):

$$Q_t = n[P]_t\Delta H^\circ_b V\{1 + [L]_t/n[P]_t + 1/nK_b[P]_t - [(1 + [L]_t/n[P]_t + 1/nK_b[P]_t)^2 - 4[L]_t/n[P]_t]^{1/2}\}/2 \quad (1a)$$

where $n$ is the stoichiometry of binding and $V$ is the cell volume. The heat released per $i$th reaction, $\Delta Q_i$, is given by the following equation of Yang et al. (*26*):

$$\Delta Q_i = Q_i + dV_i/2V\,[Q_i + Q_{i-1}] - Q_{i-1} \quad (1b)$$

where $dV_i$ is the volume of titrant added to the solution. A least square fit of $\Delta Q_i$ to eq 1b obtained by each run of titration calorimetry gives values for $\Delta H^\circ_b$ and $K_b$. Since WBA I is a homodimeric protein with one binding site per subunit, an identical site model utilizing a site concentration $[P]_t$ = 2× concentration of the lectin dimer of 29 kDa subunits (*16*) was the simplest binding model found to provide the best fit to the ITC data. Values for $\Delta S^\circ_b$ were obtained from the basic equation of thermodynamics:

$$\Delta G^\circ_b = \Delta H^\circ_b - T\Delta S^\circ_b \quad (2)$$

where

$$\Delta G^\circ_b = -nRT \ln K_b \quad (3)$$

and $n$ is the number of moles, $T$ is the absolute temperature, and $R$ = 8.315 J mol$^{-1}$ K$^{-1}$.

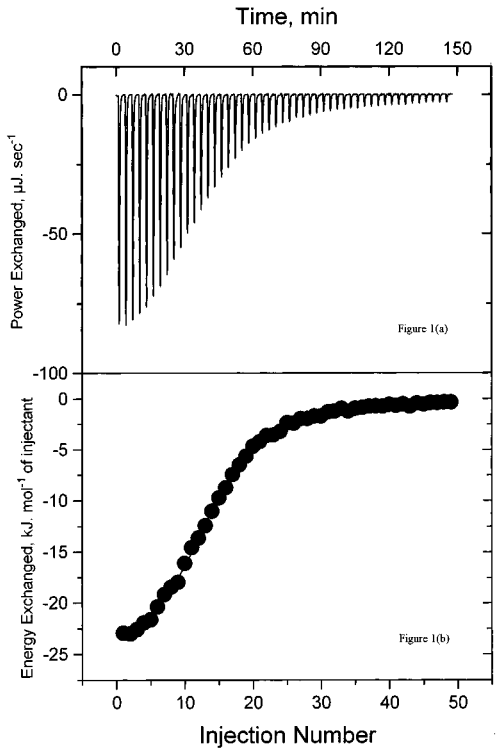


FIGURE 1: Calorimetric titration of 2MeOGal solution into WBA I solution. (a) Titration of 5.0-μL aliquots of 20 mM 2MeOGal with 1.05 mM WBA I at 6.5 °C in 20 mM phosphate buffer at pH 7.2 containing 150 mM NaCl. (b) Fit of the incremental heat for the titration in panel a by injection number to eqs 1a and 1b in the text.

## RESULTS

A representative titration curve for the binding of 2-methoxygalactose to WBA I at 6.5 °C along with the incremental heats of interaction to the injection number is shown in Figure 1a. The results exhibit a monotonic decrease in the exothermic heat of binding with each successive injection until saturation is achieved. Consequently as shown in Figure 1b, the incremental heats per mole of added ligand follow closely the injection number as defined by eq 1b. The thermodynamic parameters, $\Delta G^\circ_b$, $\Delta H^\circ_b$, and $T\Delta S^\circ_b$ for the binding of a variety of deoxy-, fluorodeoxy-, and methoxygalactopyranoside sugars in the temperature range of 6−25 °C are presented in Table 1. Each ligand binding to WBA I displayed the same stoichiometry of 1 (±0.05) per subunit (29 kDa) of WBA I. The binding enthalpies for various

Table 1: Thermodynamic Quantities for Binding of Galactopyranoside Derivatives to WBA I

| sugar | [sugar] (mM) | [lectin][a] (mM) | $T$ (K) | $K_b$ ($M^{-1}$) | $-\Delta G°_b$ (kJ/mol$^{-1}$) | $-\Delta H°_b$ (kJ mol$^{-1}$) | $-\Delta S°_b$ (J mol$^{-1}$ K$^{-1}$) | $-T\Delta S°_b$ (kJ mol$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| D-Gal | 26.0 | 1.44 | 281.7 | 2460 (±89) | 18.29 | 26.00 (±0.22) | 27 | 7.71 |
| | 24.0 | 1.36 | 286.4 | 2062 (±104) | 18.17 | 27.61 (±0.21) | 33 | 9.43 |
| | 20.0 | 1.28 | 298.3 | 1457 (±110) | 18.07 | 26.45 (±0.24) | 28 | 8.33 |
| | 25.0 | 1.24 | 304.2 | 1008 (±94) | 17.49 | 28.19 (±0.24) | 35 | 10.71 |
| 1HGal | 25.0 | 1.26 | 281.9 | 2196 (±123) | 18.03 | 24.71 (±0.26) | 24 | 6.68 |
| | 24.0 | 1.20 | 286.2 | 1573 (±106) | 17.52 | 25.04 (±0.21) | 26 | 7.52 |
| | 25.0 | 1.24 | 297.8 | 992 (±91) | 17.08 | 25.14 (±0.28) | 27 | 8.06 |
| | 26.0 | 1.20 | 298.1 | 956 (±86) | 17.01 | 24.88 (±0.29) | 26 | 7.87 |
| 2HGal | 76.0 | 4.95 | 283.0 | 306 (±26) | 13.47 | 16.36 (±0.34) | 10 | 2.89 |
| | 82.0 | 4.98 | 286.6 | 260 (±22) | 13.25 | 17.02 (±0.39) | 13 | 3.77 |
| | 80.0 | 4.98 | 295.0 | 248 (±20) | 13.52 | 17.21 (±0.44) | 13 | 3.69 |
| | 82.0 | 4.96 | 298.2 | 224 (±18) | 13.42 | 16.74 (±0.48) | 11 | 3.32 |
| 3HGal | 24.0 | 1.94 | 298.2 | NB[c] | NB | NB | NB | NB |
| 4HGal | 26.0 | 1.93 | 298.2 | NB | NB | NB | NB | NB |
| D-Fuc | 84.0 | 5.01 | 282.9 | 482 (±36) | 14.53 | 20.88 (±0.26) | 22 | 6.35 |
| | 72.0 | 4.95 | 288.2 | 388 (±32) | 14.28 | 20.58 (±0.28) | 22 | 6.30 |
| | 80.0 | 4.98 | 298.1 | 342 (±27) | 14.46 | 20.72 (±0.27) | 21 | 6.26 |
| | 84.0 | 4.98 | 304.6 | 214 (±23) | 13.59 | 18.14 (±0.35) | 15 | 4.55 |
| α1FGal | 20.0 | 1.65 | 281.8 | 2510 (±116) | 18.34 | 27.11 (±0.20) | 31 | 8.77 |
| | 24.0 | 1.40 | 298.2 | 1480 (±104) | 18.10 | 27.64 (±0.20) | 32 | 9.54 |
| 2FGal | 20.0 | 1.28 | 282.0 | 1584 (±86) | 17.27 | 24.11 (±0.14) | 24 | 6.84 |
| | 18.5 | 1.26 | 288.4 | 1423 (±88) | 17.41 | 25.34 (±0.16) | 28 | 7.93 |
| | 22.4 | 1.28 | 298.1 | 1168 (±76) | 17.51 | 27.31 (±0.13) | 33 | 9.80 |
| | 20.6 | 1.25 | 304.7 | 985 (±89) | 17.46 | 27.36 (±0.17) | 33 | 9.90 |
| 3FGal | 27.0 | 1.95 | 298.2 | NB | NB | NB | NB | NB |
| 4FGal | 26.0 | 1.26 | 298.2 | NB | NB | NB | NB | NB |
| 6FGal | 20.0 | 1.28 | 281.8 | 2023 (±46) | 17.83 | 25.61 (±0.17) | 28 | 7.78 |
| | 24.0 | 1.24 | 286.2 | 1847 (±58) | 17.90 | 25.99 (±0.14) | 28 | 8.09 |
| | 22.0 | 1.38 | 296.7 | 1044 (±114) | 17.15 | 25.74 (±0.15) | 29 | 8.59 |
| | 25.0 | 1.24 | 298.2 | 978 (±88) | 17.07 | 24.83 (±0.12) | 26 | 7.76 |
| αMeOGal[b] | | | 298.2 | 6600 | 21.80 | 23.50 | 6 | |
| βMeOGal[b] | | | 298.2 | 1000 | 17.10 | 19.70 | 9 | |
| 2MeOGal | 20.0 | 1.05 | 279.7 | 10 694 (±59) | 21.57 | 29.11 (±0.11) | 27 | 7.54 |
| | 9.6 | 1.22 | 285.6 | 9433 (±181) | 21.73 | 30.62 (±0.14) | 31 | 8.89 |
| | 25.0 | 1.28 | 291.2 | 8304 (±262) | 21.85 | 30.81 (±0.11) | 31 | 8.96 |
| | 26.0 | 1.24 | 298.1 | 7032 (±257) | 21.95 | 31.04 (±0.13) | 30 | 9.09 |
| 3MeOGal | | | ND[d] | ND | ND | ND | ND | ND |
| 4MeOGal | | | ND | ND | ND | ND | ND | ND |
| 6MeOGal | 25.0 | 1.64 | 298.1 | NB | NB | NB | NB | NB |
| L-Ara | 25.0 | 1.85 | 298.1 | NB | NB | NB | NB | NB |

[a] Site concentration. [b] From ref 19. [c] NB, nonbinding. [d] ND, not determined.

derivatives are essentially independent of temperature. 3- and 4-deoxy and fluorodeoxy derivatives were completely inactive; hence, the binding of 3- and 4-methoxy derivatives were not determined. L-Arabinose and 6-methoxygalactose were inactive whereas 6-deoxygalactose and 6-fluorodeoxygalactose were poorer ligands as compared to galactose.

The changes in binding enthalpies and entropies are compensatory in nature (Figure 2) and because $\Delta G°_b$ is a function of difference between these two parameters, i.e., $\Delta H°_b$ and $\Delta S°_b$, $\Delta G°_b$ values are not being compared for the ligand. Hence the differences between $\Delta H°_b$ and $\Delta S°_b$ for various derivatives and the values of galactose are presented in Table 2.

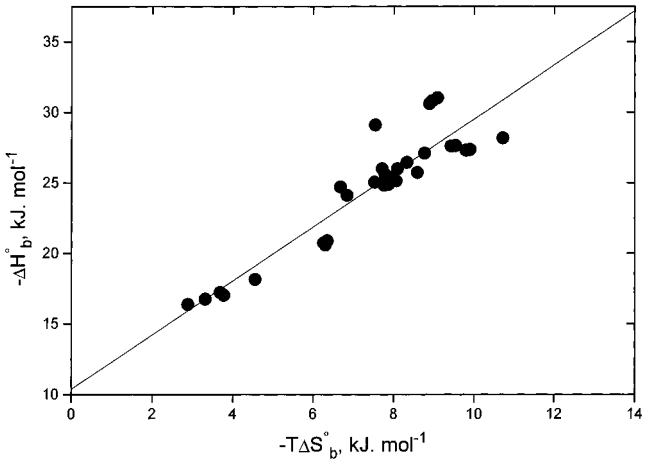


FIGURE 2: Plot of $-T\Delta S°_b$ as a function of $-\Delta H°_b$ for the binding of D-galactopyranoside derivatives to WBA I. The plot shows a linear relationship with a slope of 1.95 with a correlation coefficient to 0.91.

## DISCUSSION

*Energetics of Hydrogen-Bonding in Aqueous Solution.* Thermodynamic binding parameters for the interactions between the galactose derivatives and the amino acid residues at the binding site are associated with the structural differences between the galactose derivatives and the effects of dehydration of both the derivative and the lectin binding site. Replacement of an OH group by a substituent, such as H or F, or a relatively bulkier one such as a methyl group on a six-membered ring is expected to have little effect on its conformation in aqueous solution (*27*−*29*).

The ability of water to compete for hydrogen-bonding sites on both proteins and ligands makes the molecular interactions in aqueous solution a complex process (*30*, *31*). The hydrogen bonding between a single donor site of a lectin (L-H) with an acceptor site of sugar (A-S) can therefore be

Table 2: Summary of Differences in Thermodynamic Parameters between D-Galactopyranoside Derivatives at 298.1−298.3 K

| sugar | $-\Delta\Delta H°_b$ (kJ mol$^{-1}$) | $-\Delta\Delta S°_b$ (J mol$^{-1}$ K$^{-1}$) | sugar | $-\Delta\Delta H°_b$ (kJ mol$^{-1}$) | $-\Delta\Delta S°_b$ (J mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|---|
| D-Gal | 0 | 0 | 4FGal | NB | NB |
| 1HGal | −1.57 | −2 | 6FGal | −1.62 | −2 |
| 2HGal | −9.71 | −17 | αMeOGal | −2.95 | −22 |
| 3HGal | NB[a] | NB | βMeOGal | −6.75 | −19 |
| 4HGal | NB | NB | 2MeOGal | 4.59 | 2 |
| D-Fuc | −5.73 | −7 | 3MeOGal | ND[b] | ND |
| α1FGal | 1.11 | 4 | 4MeOGal | ND | ND |
| 2FGal | 0.86 | 5 | 6MeOGal | NB | NB |
| 3FGal | NB | NB | L-Ara | NB | NB |

[a] NB, nonbinding. [b] ND, not determined.

| Lectin | Loop A | Loop B | Loop C | Loop D |
|---|---|---|---|---|
| **WBA I** | QPFP--RPHPA**D**GLVF | GE-GG**G**YFG | VEFDT**FRN**--TWDP | GFSAATG**D**PSGKQRNATETHDILSW |
| **EcorL** | QPYT--RPLPA**D**GLVF | AQ-GY**G**YLG | VEFDT**FSN**--PWDP | GLSGATG**A**----QRDAAETHDVYSW |
| **SBA** | APDT---KRLA**D**GLAF | QT-HA**G**YLG | VEFDT**FRN**--SWDP | GFSAATG**L**DIP-----GESHDVLSW |
| **PNA** | KD--IKDYDPA**D**GIIF | GSIGG**G**TLG | VEFDT**YSN**SEYNDP | GFSA-SG**S**L-----GGRQIHLIRSW |
| **GS IV** | KNYG---APTA**D**GLAF | KDY-G**G**FLG | VEFDT**WIN**KDWNDP | GFSAGVG**Y**--------DEVTYILSW |
| | ** * | * | ** ** | * * |

FIGURE 3: Carbohydrate binding site loops of WBA I compared with those of four Gal/GalNAc-specific legume lectins whose crystal structures are known. The residues that are highly conserved are indicated by asterisk (*), and the key binding site residues are shown in bold. The alignment was obtained by structure-based manual adjustments of the corresponding regions.

expressed as

$$\text{L-H}\cdots\text{OH}_2 + \text{HOH}\cdots\text{A-S} \rightarrow [\text{L-H}\cdots\text{A-S}] + \text{HOH}\cdots\text{OH}_2 \quad (4)$$

Although the multiplicity and the bonding roles for a given interaction may vary, the numbers and types of hydrogen bonds would be conserved. The efficiency of a given binding process would thus be determined by the enthalpic contribution arising from the differences in hydrogen-bond geometries and the increase in entropy associated with the release of water from the binding site of the lectin and the sugar into bulk solvent.

The binding of a lectin with a sugar analogue in which a potential hydrogen-bonding group is removed may or may not lead to the loss of full energy of the hydrogen bond as in such a case hydrogen bond between the sugar analogue and water would not exist as shown in eq 5:

$$\text{L-H}\cdots\text{OH}_2 + \text{HOH} + \text{S} \rightarrow [\text{L-H S}] + \text{HOH}\cdots\text{OH}_2 \quad (5)$$

The energetic difference between two systems described by eqs 4 and 5 will therefore equal the difference in strengths of hydrogen bonds formed by the sugar with the lectin and with water. The deoxygenation (OH→H) of galactose would abolish its hydrogen bonding at the corresponding position leaving space insufficient to accommodate a water molecule and provides the case depicted in eq 5. The fluorination (OH→F), on the other hand, is more complex since the fluorine atom could participate in hydrogen bonding only as an acceptor giving rise to two situations:

$$\text{L-H}\cdots\text{OH}_2 + \text{HOH}\cdots\text{F-S} \rightarrow [\text{L-H}\cdots\text{F-S}] + \text{HOH}\cdots\text{OH}_2 \quad (6)$$

$$\text{L-A}\cdots\text{OH}_2 + \text{HOH}\cdots\text{F-S} \rightarrow [\text{L-A F-S}] + \text{HOH}\cdots\text{OH}_2 \quad (7)$$

At the position where hydrogen bond is donated by the lectin, the fluorodeoxy derivatives of the sugar would show affinity comparable to the unsubstituted ligand as shown in eq 6. Should the lectin group be an acceptor (eq 7), there would be a net loss of a hydrogen bond leading to poor binding affinity, which might be lower than the corresponding deoxy derivative since fluorine can still hydrogen bond as an acceptor with water.

*Molecular Basis of Recognition by Gal/GalNAc Binding Lectins*. The crystal structure of four Gal/GalNAc binding lectins are known. These are lectins from *Erythrina corallodendron*, EcorL(*31*); *Arachis hypogea*, PNA(*32*,*33*); *Griffonia simplicifolia*, GS IV(*34*); and *Glycine max*, SBA(*35*). The carbohydrate binding site of these lectins is mainly constructed by residues from four sequentially apart regions that are called loops A−D (*36*), represented in Figure 3. In all the four known structures, the conserved aspartic acid from loop A recognizes the equatorial C3-OH and axial C4-OH of galactose. The C3-OH makes additional hydrogen bonds with the backbone NH of Gly in loop B and the Asn in the loop C, whereas the C4-OH makes another hydrogen bond with loop D (Table 3). The conformationally flexible C6-OH is oriented such that it interacts with a polar side chain from either loop D (glutaminyl in EcorL and aspartyl in SBA) or loop A (aspartyl in PNA).

In Gal/GalNAc-specific lectins, considerable variation is observed in the sequence, size, and nature of loops C and D. The primary interactions of galactose orient the pyranose ring such that the residues from loop C interact with C2-OH and those from loop D interact with C1-OH and C6-OH. The nature of interaction and driving force of binding of sugars is, therefore, determined by the orientation of residues constituting loops C and D, which along with associated water establish the energetics of sugar binding. GalNAc binding, in general, involves polar interactions with C6-OH and both polar and nonpolar interactions at the C2-acetamido group. The preference at the C1 position varies among GalNAc binding lectins.

Table 3: Putative Hydrogen Bonds between Monosaccharides and WBA I Derived from Homology with Different Gal-/GalNAc-Binding Legume Lectins

| sugar residue | EcorL | PNA | GS IV | SBA | WBA I[a] |
|---|---|---|---|---|---|
| O2 | | $H_2O$·NH Gly$^{104}$ | $H_2O$·O$\delta$2 Asp$^{137}$ | | |
| | | $H_2O$·O$\epsilon$2 Glu$^{129}$ | | | |
| O3 | O$\delta$2 Asp$^{89}$ | O$\delta$2 Asp$^{83}$ | O$\delta$2 Asp$^{89}$ | O$\delta$2 Asp$^{88}$ | O$\delta$2 Asp$^{87}$ |
| | N$\epsilon$2 Asn$^{133}$ | N$\delta$2 Asn$^{127}$ | N$\delta$2 Asn$^{135}$ | N$\delta$2 Asn$^{130}$ | N$\epsilon$2 Asn$^{128}$ |
| | NH Gly$^{107}$ | NH Gly$^{104}$ | NH Gly$^{107}$ | CO Ala$^{105}$ | NH Gly$^{105}$ |
| | | | | O$\delta$1 Asp$^{88}$ | |
| O4 | O$\delta$1 Asp$^{89}$ | O$\delta$1 Asp$^{83}$ | O$\delta$1 Asp$^{89}$ | O$\delta$1 Asp$^{88}$ | O$\delta$1 Asp$^{87}$ |
| | NH Ala$^{218}$ | O$\gamma$ Ser$^{211}$ | | NH Leu$^{214}$ | O$\delta$/CO Asp$^{212}$ |
| O5 | | O$\gamma$ Ser$^{211}$ | | | |
| O6 | N$\epsilon$2 Gln$^{219}$ | O$\delta$1 Asp$^{80}$ | | O$\delta$1 Asp$^{215}$ | ? Gln$^{217}$ |
| | $H_2O$·CO Leu$^{86}$ | | | C$\delta$1 Ile$^{216}$ | ? His$^{84}$ |
| TOTAL | 7 | 9 | 5 | 8 | 7 |

[a] ? indicates as to which of the two residues, viz., $H_{84}^{A}$ or $Q_{217}^{D}$, donating a hydrogen bond to the C6-OH group of galactose is not yet identifiable.

*Consequences of Hydroxyl Substitution by Deoxy, Fluorodeoxy, and Methoxy Groups on Binding*. The average $\Delta H°_b$ and $\Delta S°_b$ values in Table 1 show that the binding enthalpies for the most part are invariant with regard to temperature as observed earlier (*17*−*19*, *37*−*39*). Differences in the enthalpies and entropies between the derivatives and galactose presented in Table 2 show the importance of single group substitutions on the binding thermodynamics of carbohydrates to lectins. Replacement of the OH group by F and H on C3 and on C4 apparently eliminates binding of the galactose derivatives to the lectins. These results are consistent with C3-OH and C4-OH acting as hydrogen bond donors to Asp89 in EcorL and Asp83 in PNA and with the fact that the strongest hydrogen bonds in protein−carbohydrate complexes are those where a carbohydrate OH serves as the donor. The requirement of an acidic residue for a hydrogen bond acceptor in WBA I is perhaps fulfilled by the corresponding aspartic acid residue, Asp87. Furthermore, a situation equivalent to other Gal/GalNAc-specific lectins would imply the presence of bifurcated and trifurcated hydrogen bonds with the C4-OH and C3-OH, respectively. Since all other residues have to be hydrogen bond donors, these interactions are most probably between the conserved Gly105 NH group of the main chain and the Asn128 residues with C3-OH and Asp212 with C4-OH. Overall, the requirement of a polar environment in the vicinity of C3 and C4 hydroxyl seems necessary for interaction of WBA I with galactose derivatives (Figure 4).

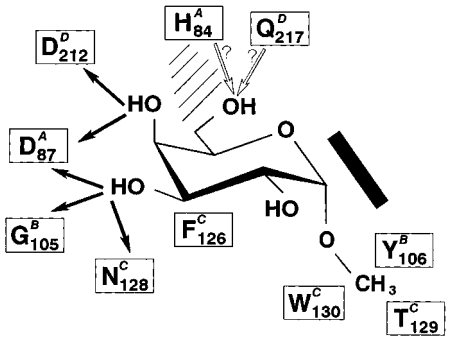


FIGURE 4: Schematic representation of the combining site of WBA I complexed with methyl-α-D-galactopyranoside. The residues of WBA I participating in sugar binding are enclosed in rectangular boxes and are denoted by one-letter abbreviation, subscripts refer to their positions in the sequence of the protein, while superscripts refer to the loop that it belongs to. $F_{126}^{C}$ is involved in stacking interactions with the hydrophobic face ($\beta$-face) of the sugar ring. This residue is identical to $F_{131}^{C}$ of EcorL implicated in stacking interactions. Key to figure: locus where steric hindrance occurs (■); van der Waals interaction (/////); hydrogen-bond donating sugar hydroxyl group (←); hydrogen-bond accepting sugar hydroxyl group (⟸). Question mark indicates that which of the two residues, viz., $H_{84}^{A}$ or $Q_{217}^{D}$, donating a hydrogen bond to the C6-OH group of galactose is not yet identifiable.

Because of the lack of additional structural information on this system together with the fact that the C2-OH of galactose in EcorL−lactose complex is not involved in hydrogen bonding, the amino acid residue(s) in WBA I participating in hydrogen bond formation at this locus remains unidentified. The affinity for the C2 derivatives of galactose decreases in the order GalNAc > 2MeOGal > 2FGal ≅ Gal > 2HGal, which suggests that both polar and nonpolar residues surround the C2 locus of galactose. This is consistent with the observed high affinity of WBA I toward GalNAc, where the acetamido group at C2 position is probably stabilized by both nonpolar interactions with the methyl group and polar interactions with the NH group or the carbonyl group of the acetamido substituent. The comparison of the WBA I sequence with that of EcorL, PNA, and SBA suggests that the amphipathic pocket is probably formed by residues Tyr106, Thr129, and Trp130. Both Thr and Trp could mediate interaction through a bound water molecule, which probably occupies the space in which the C2-acetamido group can be accommodated. Interactions through such a water molecule could be conserved in binding of 2FGal and lost in binding to 2HGal, which explains the extremely poor binding of the latter to WBA I. Binding of 2MeOGal, on the other hand, would involve strong hydrophobic interactions that being entropically-driven are accompanied by the displacement of the bound water molecule(s). Unlike the interaction of ConA, pea, and lentil lectins with glucose, the equatorially oriented C2-OH group of galactose interacts tenaciously with the corresponding loci in WBA I by both the nonpolar and the hydrogen-bonding interactions. Had that not been the case, the C2 fluorodeoxy derivative should have displayed considerably diminished binding activity if the interaction at C2 position was exclusively nonpolar in nature.

Apparently, substitution of the OH group on C1 by the bulkier MeO group as well as removal of the OH group does not alter the binding enthalpy ($\Delta H°_b$) between the derivative and the lectin binding site, indicating the lack of hydrogen bond formation between the C1 hydroxyl group and WBA I. Nearly identical $\Delta H°_b$ and $\Delta G°_b$ values for the binding of 1HGal and 1FGal with that of galactose suggests that the anomeric hydroxyl group of galactose bound to WBA I is not hydrogen bonded to any of the residues in the combining site of the lectin. Despite the lack of change in $\Delta H°_b$, there is an increase in the binding affinity ($\Delta G°_b$) for the αMeOGal

binding to WBA I. The alkyl group of αMeOGal promotes local structuring of the water molecules in aqueous solutions. Such a hydration shell interaction for αMeOGal (*40*), which is lost upon removal of the derivative from water through binding to the lectin, is perhaps responsible for its stronger affinity as compared to galactose. The preferences of WBA I for αMeOGal over galactose is 6. The stronger binding interaction of WBA I for αMeOGal is perhaps due to a nonpolar interaction between the hydrophobic pocket and its methyl group (Figure 4).

Significant reduction in the enthalpy occurs for 6HGal binding to WBA I, whereas the 6MeOGal does not exhibit any affinity. This is in contrast to 6FGal and galactose, both of which display comparable binding energy, indicating the presence of favorable polar interactions with a hydrogen-bond donor in the vicinity; binding of C6 derivatives follows the order: Gal > 6FGal > D-Fuc ≫ 6MeOGa ≅ L-Ara. Thus, a polar residue in either loop A or loop D appears to favor binding of a hydrogen-bonding group at C6 of galactose. Structural comparison with other GalNAc binding legume lectins suggests that the probable residue could be His84 from loop A or more likely with groups from a residue from loop D viz. Gln217 (*31*, *36*). Steric hindrance by the bulky methoxy group of 6MeOGal with the corresponding loci in WBA I is apparently responsible for its poor binding. In contrast to the locus corresponding to the C2 substituent of galactose, which can accommodate a variety of groups, the region abutting the C6 substituent is most complementary toward a hydroxymethyl group. A larger substituent such as a methoxy group at C6 sterically compromises the binding while the replacement of C5 hydroxymethyl group by hydrogen as in L-arabinose diminishes the binding propensity significantly. Thus van der Waal's interaction between the hydroxymethyl group of the ligand and the corresponding loci in the lectin play an important role in binding. The most likely residue from WBA I participating in such an interaction appears to be His84 from loop A, which occupies an analogous position to Leu86 of EcorL. Involvement of His84 is also supported by chemical modification studies with diethyl pyrocarbonate, which abrogates the binding of WBA I to galactose (Chittoor P. Swaminathan, Vivek Sharma, and Avadhesha Surolia, unpublished data).

Site-specific mutagenesis studies of Fersht et al. (*5*) have indicated that stronger hydrogen bonds are formed between charged and neutral atoms than those between two neutral atoms. An examination of the structure of EcorL−lactose complex indicates that the equatorial hydroxyl group at C3 and axial hydroxyl group at C4 of galactose are involved in donating hydrogen bonds to atoms emanating from charged residues. Since the recently solved structure of peanut lectin shows identical residues at analogous position to that of EcorL, it is reasonable to assume that similar contacts also operate in WBA I−galactose complex (*33*). According to these expectations, the greatest loss in binding enthalpy should have occurred with 3- and 4-deoxy derivatives of galactose as observed experimentally. Such a correlation appears to be qualitatively consistent for the binding of monosaccharides in contrast to the binding of oligosaccharides where additional factors may preclude a direct correlation between the $\Delta H$ values and the strength of the hydrogen bonds (*41*).

The enthalpically driven nature of the binding reaction between the galactose derivatives and the WBA I is maintained although there are variable $\Delta S°_b$ contributions to $\Delta G°_b$. Enthalpy and entropy compensation is also observed as shown in Figure 3 so that changes in the enthalpy are linearly compensated by changes in the entropy as discussed earlier (*37*, *42*, *43*). Observation of enthalpy−entropy compensation is not surprising as thermodynamic studies on several lectins, both from legumes and other sources, strongly emphasize the importance of water as a mediator in protein−carbohydrate recognition (*39*, *44*−*47*); other factors such as subtle shifts in protein conformational states cannot be altogether ignored at the moment (*48*).

Changes in the thermodynamic parameters caused by substitutions of the OH groups on the pyranoside ring of galactose can be explained qualitatively in terms of the solution properties of the derivative. This implies that there is very little variation in the overall structure of the WBA I complex following substitution of the OH groups. Determination of the contributions of the binding enthalpy and entropy changes to the binding affinities for the derivatives is important in developing a quantitative model for the binding interactions (*37*).

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