Thermodynamic Studies of Lectin−Carbohydrate Interactions by Isothermal Titration Calorimetry

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I. Introduction

Lectins are a large group of carbohydrate binding proteins which are widely found in nature including plants, animals, and lower organisms.[1] Animal lec-

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Tarun Kanti Dam studied various fundamental aspects of lectins during his doctoral research and received his Ph.D. degree from the University of Calcutta, India, under the supervision of Professor Amalesh Choudhury. He was introduced to invertebrate lectins by Dr. Manju Sarkar of the Indian Institute of Chemical Biology. Subsequently, he extended his study to plant lectins in Professor Avadhesha Surolia's laboratory at the Indian Institute of Science, Bangalore. In 1996, he joined the Albert Einstein College of Medicine, New York, as a postdoctoral fellow to work with Professor Fred Brewer, where he focused on structural and functional glycobiology. Using a well-defined plant lectin model system, he systematically studied several important thermodynamic aspects of carbohydrate binding by lectins. He was given the Young Scientist Award at the Biocalorimetry 2001 conference for his contributions in the field of microcalorimetry. Dr. Dam joined the faculty of the Albert Einstein College of Medicine in 2000, as an instructor. His primary research interest is the thermodynamic and structural basis of ligand recognition and binding.

Fred Brewer did his graduate studies with Dan Santi leading to a Ph.D. degree in Chemistry from the University of California, Santa Barbara, in 1971. He did postdoctoral research on the application of ^{13}C NMR to lectin–carbohydrate interactions with Arthur Grollman and Don Marcus at the Albert Einstein College of Medicine and Himan Sternlicht at Bell Laboratories, and he then joined the Albert Einstein College of Medicine faculty in 1974, where he is currently Professor of Molecular Pharmacology, and Microbiology and Immunology. Professor Brewer is on the Editorial Boards of the *Journal of Biological Chemistry* and *Glycobiology* and an Associate Editor of *Trends in Glycosiences and Glycotechnology*. He has been on the Awards Committee of the Society for Glycobiology and is past Chairman of the Division of Carbohydrate Chemistry of the American Chemical Society. Professor Brewer's interests include carbohydrate enzymology, immunochemistry, and lectin biochemistry, thermodynamics, and structural biology.

tins have been shown to be involved in diverse biological processes such as trafficking and clearance of glycoproteins, adhesion, immune defense, malignancy, and apoptosis.[2] Lectins, especially those from plants which are easily obtained in large quantities, have also been widely used to isolate carbohydrate receptors from cells by affinity chromatography and as tools for investigating the structural and functional aspects of carbohydrates in biological systems.

The biological activities of lectins are due to their carbohydrate binding properties,[2] although some lectins possess non-carbohydrate binding sites. Hence, it is important to elucidate the carbohydrate binding properties of a lectin including its thermodynamic binding parameters in order to understand its biological functions. Hemagglutination and precipitation inhibition techniques have been used to determine the relative affinity and specificity of lectins for carbohydrates.[3] However, these techniques do not provide thermodynamic data about such interactions. Other methods such as equilibrium dialysis, spectrophotometry, fluorimetry, nuclear magnetic resonance, and surface plasmon resonance have provided important insights into carbohydrate−lectin interactions but have limitations in providing thermodynamic binding data. Isothermal titration calorimetry (ITC), on the other hand, is able to directly determine the thermodynamic binding parameters of lectin−carbohydrate interactions and has found growing use over the past decade.

There are many review articles in the literature on the thermodynamics of biomolecular interactions.[4−13] However, a comprehensive review of thermodynamic data in the literature for lectin−carbohydrate interactions over the past decade has not been done to our knowledge. This article presents such a review including studies of plant and animal lectins excluding selectins. ITC-derived thermodynamic data with selectins appear to be absent in the literature.

Calorimetry is one of the oldest techniques in experimental science, and calorimeters have evolved for several hundred years[14] giving rise to the modern versions. For the past several decades, basically three different types of microcalorimeters, batch, flow, and titration calorimeters, have been used. Introduction of microelectronics and advancements in instrument designing has radically improved the sensitivity of the instruments since the mid-1980s, broadening the scope of the technique. In 1990 an improved version of the titration calorimeter was introduced,[15,16] which has been further improved during the past 10 years. On the basis of its application, calorimetry is divided in two classes. Differential scanning calorimetry (DSC) includes studies of the thermal unfolding and phase transitions of biomolecules. Isothermal titration calorimetry (ITC), on the other hand, is primarily employed to investigate the thermodynamics of ligand−macromolecule interactions. The impact and importance of ITC is evident from the surge of publications dealing with biological systems including protein−carbohydrate interactions.

ITC directly estimates the binding thermodynamics through measurement of the energetics of molecular interactions at constant temperature. A series of data points representing the amount of heat released (exothermic) or absorbed (endothermic) per mole of injectant (usually the ligand) after each injection is plotted as a function of the molar ratio $[L_T]/[M_T]$ of the total ligand concentration, $[L_T]$, and the total macromolecule concentration, $[M_T]$, to generate the

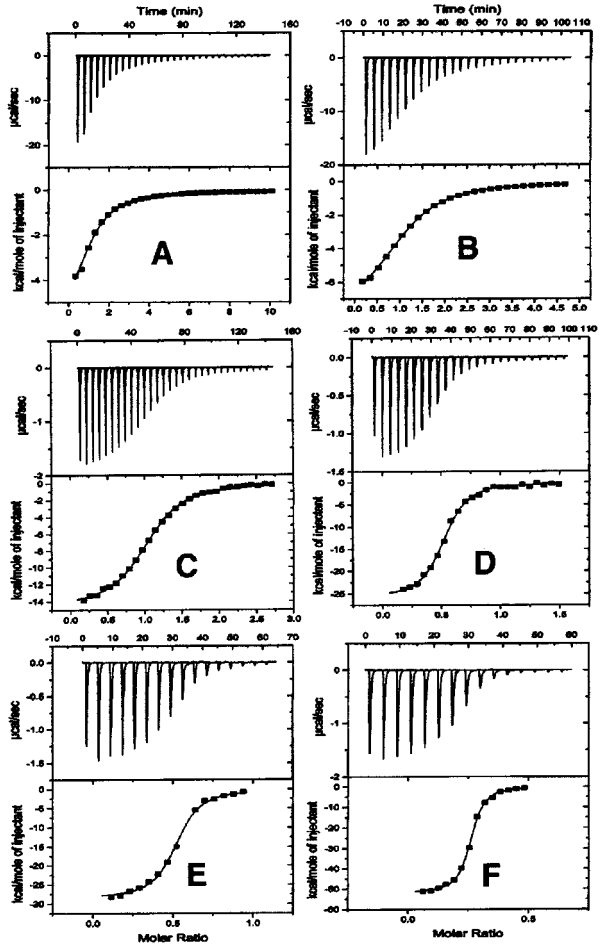


Figure 1. ITC profiles (A−F) of ConA with various carbohydrate ligands possessing different affinities: **(A)** 470 μM ConA with 37 mM isomaltose ($K_a = 5.1 \times 10^3$ M^{-1}); (B) 350 μM ConA with 20 mM MeαMan ($K_a = 1.2 \times 10^4$ M^{-1}); (C) 30 μM ConA with 880 μM trimannoside ($K_a = 4.0 \times 10^5$ M^{-1}); (D) 20 μM ConA with 400 μM multivalent sugar 1 ($K_a = 2.4 \times 10^6$ M^{-1}); (E) 18 μM ConA with 350 μM multivalent sugar 2 ($K_a = 4.5 \times 10^6$ M^{-1}); (F) 19 μM ConA with 235 μM multivalent sugar 3 ($K_a = 1.3 \times 10^7$ M^{-1}). Top of each profile shows data obtained from automatic injections, 4 mL each, of the ligands; bottom of each profile presents the integrated curve showing experimental points and the best fit (T. K. Dam, R. Roy, and C. F. Brewer, unpublished data).

binding isotherm (Figure 1). The thermodynamic binding parameters are then determined by nonlinear least-squares analysis of the binding isotherm. The adjustable parameters in these fits are $\Delta H°$, the change in enthalpy (kcal/mol) upon binding, K_a, the association constant (M^{-1}), and n, the number of binding sites per monomer of the (macro)molecule in the cell. From the equation

$$\Delta G° = -RT \ln K_a \quad (1)$$

$\Delta G°$, the free energy of binding (kcal/mol), can be calculated. From the Gibbs Free energy equation

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

$T\Delta S°$, the entropy of binding (kcal/mol) at the experimental temperature, can be determined.

Assessment of the temperature dependence of the enthalpy and entropy changes allows evaluation of the changes in heat capacity ($\Delta C_p°$)

$$\Delta H° (T_1) = \Delta H° (T_0) + \Delta C_p° (T_1 - T_0) \quad (3)$$

$$\Delta S° (T_1) = \Delta S° (T_0) + \Delta C_p° (T_1 - T_0)/T_0 \quad (4)$$

where T_1 and T_0 denote different temperatures (over a narrow range).[12] Changes in the solvent-accessible surface area upon binding primarily contribute to a finite (nonzero) ΔC_p value.[7,17] The number of binding site(s), n, per molecule of the protein is described as follows

$$q = nV\Delta H° [ML_n] \quad (5)$$

where q is the heat absorbed or evolved, n is the number of binding site(s) per molecule of the receptor, V is the cell volume, $\Delta H°$ is the binding enthalpy per mole of ligand, and [ML] is the concentration of the bound ligand.[6]

The superscript zero in the thermodynamic parameters $\Delta G°$, $\Delta H°$, $T\Delta S°$, and $\Delta C_p°$ indicates that the parameters have been measured when all species are present in their standard states (temperature, pressure, composition, and concentration) or that they have been corrected to standard states after measurements under different conditions.[12] The thermodynamic data presented in this review were collected under varied experimental conditions and were not corrected to standard states. Hence, throughout the review ΔG, ΔH, $T\Delta S$, and ΔC_p are presented without the superscript zero.

Importantly, ITC is the only technique that allows simultaneous determination of the thermodynamic binding parameters K_a, ΔG, ΔH, $T\Delta S$, and n in a single experiment[16] and ΔC_p from temperature-dependent measurements. From these binding data, important information about ligand recognition by a macromolecule can be obtained.

II. Animal Lectins

Among an increasing variety of animal lectins being described, two major groups, the calcium-independent S-type lectins or galectins[18] and the calcium-dependent C-type lectins,[19] have been the subject of ITC thermodynamic binding studies. This review will cover work performed with these two groups of animal lectins.

A. Galectins

The galectins are soluble β-galactoside-specific proteins, and their expression and distribution appear to be developmentally regulated.[20] They are a highly conserved family of lectins defined by common consensus sequences and structures.[21−23] Twelve members of the family have currently been identified in mammals and designated as galectin-1 through galectin-12.[24] The structures of the mammalian galectins can be identified as prototype (including galectin-1, -2, -5, -7, and -10) which exist as monomers or homodimers consisting of one carbohydrate

recognition domain (CRD), chimera type (galectin-3) that contains a nonlectin N-terminal collagen-like repeat segment connected to the C-terminal CRD, and tandem repeat type (including galectin-4, -6, -8, and -9) composed of two CRD domains in a single polypeptide chain.[25] Galectin-1 is involved in inflammation, development, mRNA splicing, differentiation, cell adhesion, and apoptosis of activated T cells.[21,23,26] Galectin-3 has also been shown to exhibit roles in regulating inflammation, cell growth, and cell adhesion (cf. ref 21). Galectin-3, unlike galectin-1, possesses anti-apoptotic effects in a variety of cells, and its expression has been shown to correlate with the metastatic potentials of certain cancers.[27] The preferential binding of galectins to *N*-acetyllactosamine (Galβ1,4GlcNAc) or LacNAc suggests that the functions of these lectins are mediated through their binding to proteins containing LacNAc residues or related structures. Studies of the carbohydrate binding specificities, employing ITC and other techniques, of the galectins have largely been restricted to galectin-1 and -3.[28−34]

Binding inhibition studies have suggested that 4-OH and 6-OH of the galactopyranosyl ring and 3-OH of the glucopyranoside ring in lactose and lactosaminoglycans are primarily responsible for interactions with galectin-1 and -3.[29,35,36] The crystal structure of the bovine spleen galectin (galectin-1) complexed with LacNAc at 1.9 Å resolution[37] confirmed the above interactions with possible hydrogen bonding of the 4-OH (of Gal) with His44 and Arg48, 6-OH (of Gal) with Asn61, and 3-OH of the *N*-acetylglucopyranoside ring with Arg48, Glu71, and Arg73. Additional possible hydrogen-bonding interactions involving water were predicted between His52, Asp54, and Arg73 and the nitrogen of the *N*-acetyl group on the C-2 of the glucopyranoside ring. Although two galectin monomer units combine to form a dimer with a topology similar to that of the legume lectins, the ligand binding site is topologically different from that of the legume lectins and consists of a unique set of salt bridges.[37]

1. Galectin-1 from Sheep Spleen

ITC studies of the binding of galectin-1 from sheep spleen to carbohydrates were reported by Ramkumar et al.[32] A comparison of the binding data obtained with several mono- and disaccharides showed that 4-methylumbelliferyl α-D-galactopyranoside (Mumb-α-Gal) and LacNAc possessed the highest association constant among the mono- and disaccharides tested, respectively. The affinity of Gal was low and associated with a low ΔH of ca. −0.7 kcal/mol and relatively large unfavorable entropy. Introduction of a methyl group in the α or β configuration (methyl α- and β-galactopyranoside) did not improve binding, but the presence of a 4-methylumbelliferyl group in the α-configuration (Mumb-α-Gal) significantly increased the affinity (∼50-fold). This increase for Mumb-α-Gal was largely due to a favorable change in the binding entropy. The authors concluded that the methylumbelliferyl group in the α-configuration interacted with a nonpolar site adjacent to the Gal binding site and that binding was probably accompanied with the release of highly structured water molecules.[32] The methylumbelliferyl group in the β-configuration was a poor ligand. The values of ΔH for lactose, LacNAc, and thiodigalactoside were −8.1, −9.3, and −12.2 kcal/mol, respectively, more than the ΔH of methyl β-galactopyranoside. This observation indicated that the reducing end hexapyranosyl groups of these disaccharides were accommodated in a subsite of the protein in addition to the Gal binding site. The affinity of LacNAc was greater than lactose, presumably due to additional van der Waals interactions between the acetamido group at of the GlcNAc residue of LacNAc and the side chains of Arg73 and Glu71.[37]

The X-ray crystal structures of galectin-1 complexed with lactose[38] and LacNAc[37] clearly show that OH groups of Gal are involved in a larger number of contacts with the lectin compared to that of Glc at the reducing end. Moreover, the Gal residue occupies the primary binding site. The authors[32] suggested that water molecules played a major role in the energetics of lactose binding. Different energetics for Gal binding relative to the other sugars tested were cited as the reason for the deviations of Gal, methyl α- and β-galactopyranoside from the enthalpy−entropy compensation plot. It is also possible that the low affinities of these monosaccharides precluded accurate ITC data due to low *c* values[16] under the conditions of the experiments.

2. Galectin-1 from Bovine Spleen

Schwarz et al.[34] used a series of disaccharides with a nonreducing Gal moiety and various substituents at the subterminal position [(Galβ1,4Glc), (Galβ1,-4Man), (Galβ1,3Ara), (Galβ1S1βGal), (Galβ1,4Glcβ-OMe), (Galβ1,4Fruc), (Galβ1,3GlcNAc)] to study the thermodynamic basis of carbohydrate binding by galectin-1 from bovine spleen. The binding data obtained allowed insight into the role of the reducing sugar in their binding energetics. The authors derived energy-minimized structures from the crystal structure of the Galβ1,4GlcNAc-galectin complex[37] (Figure 2) with suitable substitutions on the glucopyranoside moiety, e.g., with OH replacing the NHAc group on C-2 for the starting structure of Galβ1,4Glc. They attempted to correlate the number of atoms on the disaccharide within 4 Å of the atoms of the amino acid residues at the galectin binding site to the binding enthalpy, i.e., the number of close contacts, on the assumption that the greater the number of contacts, the more negative the binding enthalpy. They also compared the experimental binding enthalpies to those calculated from changes in the solvent-accessible surface area of the galectin binding site upon binding of the disaccharide.[39] The thermodynamic binding quantities for Galβ1,4GlcβOMe and 2′-O-methyllactose (MeO-2Galβ1,4Glc) were determined to evaluate the role of a particular OH group in the binding thermodynamics.

In the X-ray crystallographic structure of galectin-1 complexed with LacNAc,[37] the glucopyranoside moiety of the disaccharide exhibits possible hydrogen-bonding interactions between the acetyl group on C-2 and the side chains of Arg73 and Asp54 and the

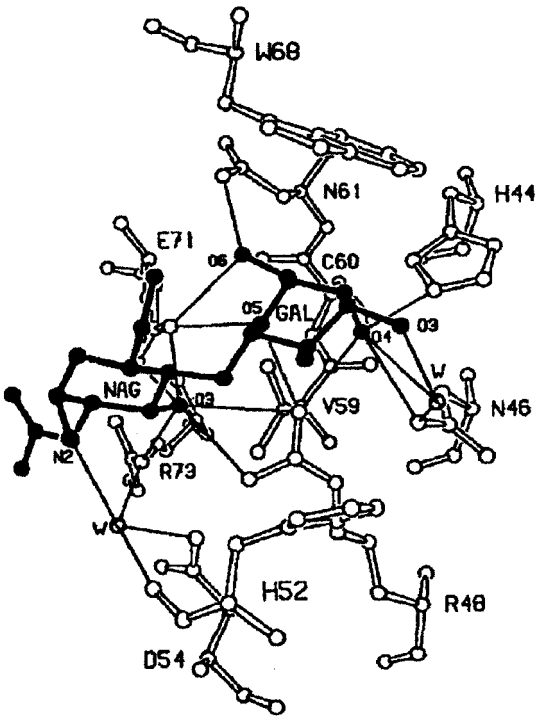


Figure 2. Schematic representation of the bovine spleen galectin-1 carbohydrate binding site complexed with *N*-acetyllactosamine. Bonds between carbohydrate atoms are solid, and those between protein atoms are open. Hydrogen bonds between protein and sugar atoms are shown in thin lines.[37] (Reprinted with permission from ref 37. Copyright 1994 National Academy of Sciences U.S.A.)

carbonyl of His52 mediated by a water molecule and between the C-3 OH group and Arg73, Arg48, and Glu71. Since modification of the reducing Glc moiety alters the binding thermodynamics of the disaccharide for galectin-1, this must involve changes in the interactions between the amino acid residues at the binding site and groups on C-2 and C-3 of the glucopyranoside. In relating changes in the binding thermodynamics to the molecular interactions between the substituted glucopyranoside moiety and galectin, the binding enthalpies were compared since enthalpy−entropy compensation minimizes changes in ΔG and changes in ΔH more accurately reflect changes in the carbohydrate−protein interactions of the complex. Comparison of the binding enthalpies to the structural interactions observed in the crystal complex were made on the assumption that the disaccharides being compared had the same solvation energy in water. With the exception of the MeO-2Galβ1,4Glc derivative, differences in the solvation energy of the disaccharide were argued to arise from differences in the solvation energies of the substituted glucopyranoside moieties since the galactopyranoside moiety remained the same in all disaccharide structures.

The ΔH values of Galβ1,4Man (−8.6 kcal/mol), Galβ1,3Ara (−9.6 kcal/mol), and Galβ1S1βGal (−10 kcal/mol) were almost 2−4 kcal/mol more negative than the binding enthalpies of Galβ1,4Glc (−6.1 kcal/mol) and Galβ1,3GlcNAc (−6.1 kcal/mol), while the other disaccharides tested possessed ΔH values close to −7 kcal/mol.[34] Since the structures of the disaccharides differ in their reducing end regions, any differences in the binding enthalpies were argued to reflect the number and type of contacts formed between the substituted glucopyranoside moiety and the galectin binding site. No correlation was found between the number of close contacts and the binding enthalpies (Table 1). The agreement between the enthalpies calculated from the change in the solvent-accessible surface area at the galectin binding site and experimentally determined enthalpies in this table was poorer than that found when the same parameters were used in systems involving protein−peptide and protein folding interactions.[40,41] It was clear that the calculations were able to model some general characteristics of the interactions between the disaccharides and the galectin binding site but failed to incorporate more detailed characteristics that lead to tighter binding for some of the sugars. It appears that a more complete model should include, in addition to the averaged terms represented by the buried surface area, terms that reflect the type of interactions such as the strength of the hydrogen bonds and polar/nonpolar interactions.

Table 1. Comparison[a] of Experimental to Calculated Binding Enthalpies for Binding of the Disaccharides to Galectin[34]

disaccharide	no. of close contacts at distances <4.0 Å	exp $-\Delta H$ (kcal/mol)	calcd $-\Delta H^b$ (kcal/mol)
Galβ1,3GlcNAc	5	5.9	6.6
Galβ1,4Glc	7	6.1	5.9
Galβ1,4Fruc	11	7.2	5.9
Galβ1,4GlcNAc	6	7.4	6.6
Galβ1,4Man	7	8.6	5.8
Galβ1,3Ara	7	9.6	5.6
Galb1S1bGal	7	10.0	6.0

[a] Reprinted with permission from ref 34. Copyright 1998 American Chemical Society. [b] Calculated using differences in the solvent-accessible surface area as described in Luque et al. (1996).[39]

The correlation of the binding enthalpy with the calculated binding enthalpy of the energy-minimized conformations for most of the disaccharides tested showed that the method based on changes in the solvent-accessible surface area could predict the binding enthalpies from the energy-minimized conformation of the complex derived from just one of the known crystal structures of the complex, the LacNAc-galectin-1 complex.[37] Nevertheless, the approach did not appear as unequivocal because the calculated and experimentally determined ΔH of some of the disaccharides did not show any agreement.

3. Galectin-1 from CHO Cells

Galectin-1 from CHO cells and its C2S mutants were expressed as recombinant proteins in *E. coli*.[42] Biosynthetic studies reveal that the lectin is secreted by CHO cells and is found both at the cell surface, where it is bound to surface glycoconjugates, and in the media in free form.[43] C2S-Gal-1 was constructed as a stable form of the lectin,[42] since it was demonstrated to be more stable than the native lectin in solutions lacking reducing agents,[42] thus providing evidence that Cys-2 is responsible for the observed instability of the lectin. Another monomeric mutant

of the galectin (N-Gal-1), possessing the C2S mutation as well as three other amino acid changes in the N-terminal region, was expressed in *E. coli* and shown to have similar binding activity.

The K_a values, as determined from ITC studies,[33] of galectin-1, C2S-Gal-1, and N-Gal-1 for LacNAc were nearly the same: 6.2×10^3, 2.9×10^3, and 8.7×10^3 M^{-1}, respectively. These results agreed well with equilibrium dialysis experiments. The data were also consistent with the observation that Gal-1 and C2S-Gal-1 bind to immobilized laminin with essentially equal avidities.[42] Importantly, the ΔH and $T\Delta S$ values of the three forms of the lectin binding to LacNAc were substantially different. For the native galectin, ΔH was -6.6 kcal/mol, while for C2S-Gal-1 and N-Gal-1 the ΔH values were -2.8 and -0.6 kcal/mol, respectively. Furthermore, K_a values of the native galectin and C2S-Gal-1 for dithiogalactoside were also nearly the same: 2.9×10^3 and 2.5×10^3 M^{-1}, respectively. However, ΔH for the native lectin was -3.8 kcal/mol, while ΔH for the C2S mutant was -2.7 kcal/mol. Thus, the differences in ΔH (and $T\Delta S$) values for galectin-1, C2S-Gal-1, and N-Gal-1 were due to intrinsic differences in the proteins and not to the individual saccharides.

The position of Cys-2 in the X-ray structures of bovine spleen Gal-1 as well as human galectin-2[38] is approximately 20 Å from the carbohydrate binding sites of the lectins. Thus, the differences in ΔH values for binding LacNAc and dithiogalactoside to galectin-1 (-6.6 and -3.8 kcal/mol, respectively) as compared to C2S-Gal-1 (-3.8 and -2.6 kcal/mol, respectively) were due to long-range effects and not to contact residues in the binding site. Changes in the N-terminal region of N-Gal-1 (residues 2, 4, 5, and 6) were likewise far removed from the carbohydrate binding site of the lectin. The same must be true for the differences in the ΔH value (-0.6 kcal/mol) of N-Gal-1 binding LacNAc as compared to the other two lectins. Similar long-range changes in ΔH (and $T\Delta S$) without changes in ΔG have been observed in mutants of glucoamylase from *Aspergillus niger*.[44] It would thus be important to determine the molecular mechanisms that gave rise to the differences in ΔH between native galectin-1 and the C2S mutant in order to understand the thermodynamic binding parameters of the lectin. It is interesting to note that the ΔH and $T\Delta S$ values for galectin-1 and its mutants, C2S-Gal-1 and N-Gal-1, binding to LacNAc fell on the same line in an enthalpy−entropy plot. Such a plot is reported in all forms of weak association processes,[45] although the meaning of this plot is not fully understood. The compensatory behavior is mainly attributed to solvent reorganization upon ligand binding and to the changes in rotational degrees of freedom of the ligand. Data (enthalpy and entropy) obtained under identical experimental conditions with a group of ligands are expected to show a linear relationship if they follow a similar binding mechanism. If the binding is dominated by enthalpy, the slope value would be greater than unity, whereas a slope value smaller than unity suggests that the binding is dominated by entropy. The correlation coefficient often demonstrates the quality of ITC-derived K_a and ΔH values. Some of the reported plots have been shown in the subsequent sections.

The crystal structure of galectin-1 from bovine spleen complexed with LacNAc, determined at 1.9 Å resolution,[37] showed that the 4- and 6-hydroxyl groups and ring oxygen atom of the Gal residue and the 3-hydroxyls of the GlcNAc residue of LacNAc were directly involved in hydrogen binding to the protein (Figure 2). These interactions were likely to be conserved in the galectin-1 from CHO cells, since its primary structure was 95% identical to bovine galectin-1, and to contribute to the observed thermodynamic parameters. The mutants C2S-Gal-1 and N-Gal-1 possessed greater contributions of $T\Delta S$ to binding of the disaccharides than the native galectin.

4. Galectin-1 from Chicken Liver

Galectin-1 from chicken liver also possessed a weak affinity for Gal[46] that could be increased by introducing an apolar aglycone.[47] ITC data for several disaccharides binding to the galectin showed that substitution of the reducing residue of lactose with Gal (as in thiodigalactoside) or GlcNAc (as in LacNAc and Galβ1,3GlcNAc) increased their association constants. While ΔH of thiodigalactoside was 1.8 kcal/mol more negative than that of lactose, the ΔH values of LacNAc and Galβ1,3GlcNAc were reduced by ~2 kcal/mol from that of lactose. The affinities of LacNAc and Galβ1,3GlcNAc increased due to favorable relative entropic contributions, but a relative enthalpic gain contributed to the higher affinity of thiodigalactoside.[47] The chicken lectin-LacNAc complex was modeled by Varela et al.[48]

5. Human Galectin-3

Previous studies[35,49] have shown the importance of an axial configuration of the hydroxyl group at the C4 position of the sugar in the binding interaction with galectin-3. This, in turn, suggests that the combining site specifically accommodates a terminal nonreducing Gal. The crystal structures of the CRD of galectin-3 complexed with lactose and LacNAc demonstrated that Glu165, Arg186, Glu184, Arg162, Asn160, Asn174, Arg144, and His158 of galectin-3 made contacts with lactose and LacNAc (Figure 3).[50] ITC studies with a selected panel (Figure 4) of carbohydrates by Bachhawat-Sikder et al.[51] provide further insights into the carbohydrate recognition properties of human galectin-3. Their data complement the reported relative inhibition values obtained with hapten inhibition assays.[35,49] The K_a and ΔH values of lactose were 1160 M^{-1} and -4.8 kmol/mol, respectively. Addition of a Fucα1,2 at the nonreducing end of lactose (2′FL) or substitution of the reducing end Glc with a sulfur-linked Gal as in thiodigalactoside (TDG) marginally increased the affinity as a result of favorable changes in enthalpies for the two oligosaccharides.[51] The C4−OH of the fucosyl residue of 2′FL may form a water-mediated hydrogen bond with Glu165 according to modeling studies.[52] The authors[51] suggest that the second Gal residue of thiodigalactoside is hydrogen bonded with Arg162 and Glu184 of galectin-3. These additional possible hydrogen bonds may contribute to the greater negative enthalpies of these two ligands.

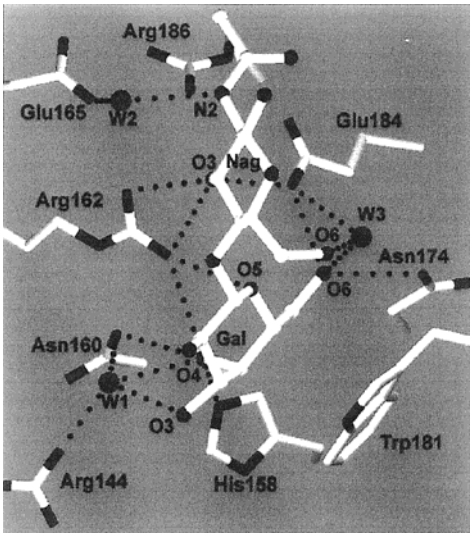


Figure 3. Human galectin-3 carbohydrate binding site. Residues interacting with the bound LacNAc moiety through direct and water-mediated hydrogen bonds or through van der Waals contacts are shown. Water molecules are labeled W1−W3. Potential hydrogen bonds are shown as dotted lines.[50] (Reprinted with permission from ref 50. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

LacNAc possesses 7-fold higher affinity than lactose for galectin-3 and a ΔH that is −3.3 kcal/mol of more favorable than that of lactose.[51] These results appear to be due to the acetamide group of GlcNAc that makes favorable contacts with Glu165 of the protein via a water molecule, in addition to the hydrogen bonds established by the Arg162 and Glu184 with O3 of GlcNAc.[52] For Galβ1,3GlcNAc (LNB), modeling suggests that the acetamido group is oriented away from Glu165 due to the β1,3 linkage, reducing the value of K_a and ΔH as compared to LacNAc.[52]

The presence of additional subsites for galectin-3 binding site was suggested from the binding data of LNTet that exhibited a 16-fold higher affinity relative to lactose at 8 °C, with a favorable enthalpy change (ca. −14.4 kcal/mol).[51] The K_a for LNhex was 30-fold greater than that of lactose, but ΔH for LNhex was less than that of LNTet (ca. −10 kcal/mol). The increased affinity of LNhex primarily resulted from favorable entropic effects. There was no indication of oligomerization of galectin-3 upon LNhex binding as the interaction was found to be monovalent.

The affinities of human blood group oligosaccharides, B-tri, A-tri, and A-tetra for galectin-3 were 19-, 24-, and 37-fold higher, respectively, than lactose at 280 K. A comparison of the ΔH values of A-tetra (ΔH = −30.1 kcal/mol) and 2′ FL (ΔH = −7.2 kcal/mol) emphasizes the energetic contribution of the α1,3-linked terminal GalNAc of the tetrasaccharide in the binding process.[51] The higher affinity and ΔH of A-tetra was argued to be due to the additional hydrogen bonds as observed in docking experiments (Figure 5).[52] An increase in the binding enthalpy of A-tri and A-tetra confirmed the observation that the combining site of galectin-3 is extended. Moreover, the extended site interaction was found to be quite specific because sugars such as lacto-*N*-triose were very poor ligands. On the basis of their results, the authors endorsed the view of Knibbs et al.[49] that the CRD of galectin-3 is comprised of four subsites.

Sugar	Structure
Lac	Galβ1-4Glc
2'FL	Fucα1-2Galβ1-4Glc
TDG	Galβ1-S-1βGal
LNB	Galβ1-3GlcNAc
LacNAc	Galβ1-4GlcNAc
LNTet	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
LNhex	Galβ1-4GlcNAcβ1 6 Galβ1 4Glc 3 Galβ1-3GlcNAcβ1
B-Tri	Galα1-3Galβ1-4GlcNAc
A-Tri	GalNAcα1-3Gal 2 Fucα1
A-tetra	GalNAcα1-3Galβ1-4Glc 2 Fucα1

Figure 4. Carbohydrate ligands used for the binding study of galectin-3.[51] (Reprinted with permission from ref 51. Copyright 2001 FEBS Society.)

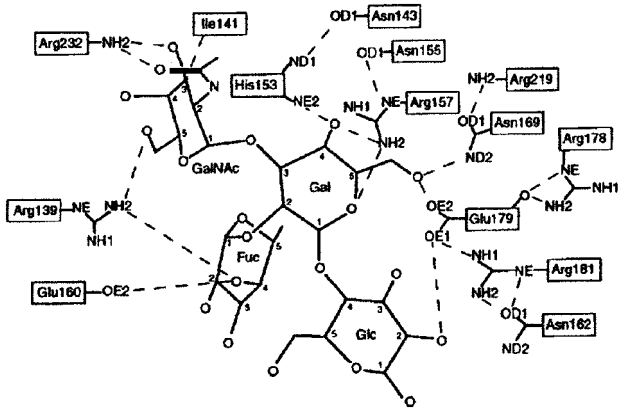


Figure 5. Outlines of potential ligand binding sites in hamster galectin-3 CRD mutants. The complete binding pocket for mutant-RR complexed with GalNAcα1,3-[Fucα1,2]-Galβ1,4Glc.[52] (Reprinted with permission from ref 52. Copyright 1998 Oxford University Press.)

B. C-Type Lectins

The C-type lectins are a family of extracellular carbohydrate recognition proteins characterized by

a common sequence motif of 115−130 amino acid residues. This domain, the carbohydrate recognition domain (CRD), usually shows specific, weak (with dissociation constants in the millimolar range), and calcium-dependent binding to a variety of monosaccharides.[53] C-type lectin CRDs are found as building blocks in a variety of multidomain proteins involved in organizing the extracellular matrix, in endocytosis, in the primary immune system, and in interactions of blood cells.[54] In the current Pfam database of protein families,[55] 389 C-type lectin sequences have been identified in a wide range of animals (in nematodes, molluscs, arthropods, echinoderms, tunicates, and in a large number of vertebrates). The importance of the C-type lectins is also reflected by the fact that they represent the seventh most common protein domain identified in the *Caenorhabditis elegans* genome.[56] C-type lectin domains adopt a typical fold: one-half of the molecule consists of a long two-stranded β-sheet and two α-helices, while the second half contains the calcium and carbohydrate binding site(s) and is mostly formed of nonrepetitive loop structures. This fold is conserved in all the examples of known C-type lectin structures, including the human and rat serum Man-binding proteins (MBP-A),[57,58] and rat liver Man-binding protein (MBP-C).[59]

Insight into the mechanism of carbohydrate binding in C-type lectins was first obtained from the crystal structure of MBP-A complexed with an oligomannose asparaginyl−oligosaccharide.[60] Later, the structures of MBP-C complexed with different monosaccharides and a mutant of MBP-A that bound Gal were solved by X-ray crystallography.[59,61] The binding site of the C-type lectins is quite exposed and is located on the surface of the loop region, with the 3- and 4-hydroxyl groups of the carbohydrate coordinating to a bound calcium ion. Additional hydrogen bonds are formed between the sugar and the protein side chains involved in binding this calcium ion, with van der Waals contacts further stabilizing the bound sugar. In all structures of Gal-specific lectins, including the Gal-binding mutant of MBP-A, additional hydrophobic stacking between an aromatic protein side chain and the apolar side of the Gal was observed.[62]

1. C-Type Lectin from the Tunicate Polyandrocarpa misakiensis (TC14)

The binding affinity of the tunicate lectin for monosaccharides was weak, which was consistent with that observed for most of the C-type lectin family. Recombinant TC14 showed the expected selectivity for a Gal-specific C-type lectin, and the results were also in agreement with binding studies performed on the native protein.[63] ITC experiments by Poget et al.[64] for TC14 showed the highest affinities for Gal (K_d = 0.44 mM, ΔH = −18.6 kcal/mol) and Fuc (K_d = 0.33 mM, ΔH = −6.1 kcal/mol), with no detectable binding for Man or Glc and only very weak binding for GalNAc (K_d = 2.0 mM, ΔH = −3.6 kcal/mol). Two disaccharides, 6-β-D-galactopyranosyl-D-galactopyranose (K_d = 0.38 mM, ΔH = −11.2 kcal/mol) and 4-α-D-galactopyranosyl-D-galactopyranose

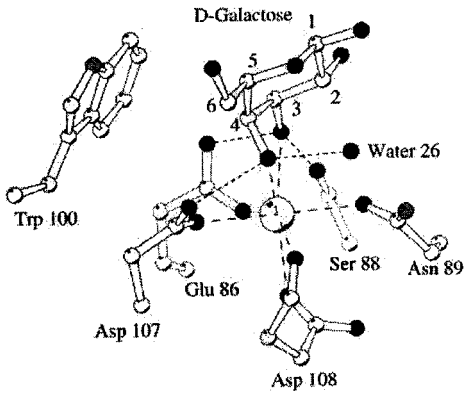


Figure 6. Galactose binding in TC14 (tunicate C-type lectin). Hydrogen bonds are shown in broken lines.[64] Ca^{2+} is shown as a larger sphere. (Reprinted with permission from ref 64. Copyright 1999 Academic Press.)

(K_d = 0.58 mM, ΔH = −9.8 kcal/mol), showed similar affinities to that of Gal, although the ΔH value of Gal was much higher than the disaccharides.

The sugar binding in TC14 occurs predominantly through a bound Ca^{2+} ion (Figure 6). The authors concluded that the ITC data indicated only one Ca^{2+}-binding site per domain, and in the TC14 structure, the sugar-binding calcium site was likely to be the only metal site.[64] The X-ray crystal structure of TC14 in the same study demonstrated that the protein ligands for the calcium ion are the side chain oxygen atoms of Glu86, Asn89, Asp107, and Asp108 as well as the main-chain carbonyl oxygen of Asp108. To form pentagonal bipyramidal coordination of the bound Ca^{2+} ion, the last two coordination sites are occupied by the 3- and 4-hydroxyl oxygen atoms of Gal. Hydrogen bonds between protein side chains and the Gal hydroxyl groups were formed between the 3-OH and Glu86 and Ser88 as well as between the 4-OH and Asp107. Solvent molecules played a role in the structure of the complex by mediating indirect protein−carbohydrate interactions that were bridged by a single water atom. Such interactions could be found between the 2-hydroxyl group and Ser88 and between the 5-hydroxyl group and Asn89. The Gal 6-hydroxyl group was linked to the side chains of the three residues Asp52, Gln98, and Arg115 through a single water molecule. Additional stabilization of the complex was achieved through hydrophobic stacking of the side chain of Trp100 to the apolar side of Gal. The importance of this hydrophobic interaction was also reflected in the slightly higher binding affinity of TC14 for Fuc than for Gal. The less polar sugar could form a stronger hydrophobic interaction with the side chain of Trp100.[64] The ΔH (−18.7 kcal/mol) of Gal was unusually high compared to the value of any other monosaccharide in the literature. The crystal structure did not provide an explanation for this higher value.

2. Mannose Binding Proteins (MBPs)

The collectins are a group of C-type lectins which share a common arrangement of structural domains: a cysteine-rich domain at the amino terminus is followed by a collagenous domain, an oligomerization domain, and a COOH-terminal carbohydrate

recognition domain (CRD).[59,65] Mannose binding proteins (MBPs), often mentioned as mannose binding lectin (MBL), belong to the collectin family. In rat, MBPs are found in serum (MBP-A) and in liver (MBP-C). MBP-A plays crucial roles in antibody-independent carbohydrate-mediated host defense against pathogens. Although the function of MBP-C is not clearly known, it is suggested that it is probably involved in host defense, cell−cell interactions, or glycoprotein trafficking.[59]

Both MBP-A and MBP-C bind to a number of monosaccharides containing equatorial hydroxyl groups at the 3- and 4-positions such as Man, GlcNAc, and Fuc.[66] Man binds at a conserved Ca^{2+} site, designated site 2, through vicinal, equatorial 3- and 4-OH groups that form coordination bonds with the Ca^{2+} and hydrogen bonds with amino acid side chains that also serve as Ca^{2+} site 2 ligands (Figure 7). Sequence alignment, mutagenesis, and crystallographic studies suggest that this binding site is well-conserved among C-type lectins and that the sequence of amino acids around this site determines binding specificity.[59]

Despite the conserved nature of the monosaccharide binding site, MBP-A and MBP-C possess different affinities for some oligosaccharides and multivalent ligands including neoglycoproteins. MBP-C binds the Man3GlcNAc2 core structure of *N*-glycosides, whereas MBP-A does not.[67] MBP-C CRD consistently bound a series of Man-containing divalent ligands with a 10−20-fold higher affinity than monovalent Man derivatives, while MBP-A CRD did not show such affinity enhancements.[68] On the other hand, MBP-A binds GlcNAc−BSA much more tightly than MBP-C, even though the two proteins bind comparably to free GlcNAc.[67]

The interactions of MBP-A and MBP-C CRD with MeO-Man (anomer not indicated), MeO-GlcNAc (anomer not indicated), NAcYD(G-ah-Man)$_2$, and NAcYD-(G-ah-GlcNAc)$_2$ were measured by ITC by Quesenberry et al.[69] Except for NacYD(G-ah-Man)$_2$ binding to the MBP-C CRD, all other titrations were done with higher ligand concentration to generate measurable amounts of heat. Due to the much lower solubility of NAcYD(G-ah-GlcNAc)$_2$, titrations could not be performed with this ligand at higher concentration.

The K_D values obtained calorimetrically agreed reasonably well with the I_{50} values of the inhibition assay.[69] The ΔG (−3.8 kcal/mol) values of MeO-Man and MeO-GlcNAc were identical for MBP-A. Similar values of ΔG for the interaction of MBP-C with these two monosaccharides were also observed. Their binding enthalpies for both lectins were comparable (−4.7 to −5.1 kcal/mol). In contrast, the affinity of NAcYD-(G-ah-Man)$_2$ for MBP-C CRD ($\Delta G = -5.9$ kcal/mol) was higher than its affinity for MBP-A ($\Delta G = -3.8$ kcal/mol). The enhancement was derived mainly from ΔH, since the ΔH (−7.4 kcal/mol) value obtained for the divalent ligand was approximately 1.5 times greater than the ΔH (−5.1 kcal/mol) of the monovalent ligand (Me O-Man).

Fitting of the ITC data using the number of binding sites per monomer (N) set at 1 or 2 showed that $N =$ 1 fitted the data as well as $N = 2$ for binding of Me O-GlcNAc to MBP-A and MBP-C CRD and the binding of Me O-Man to MBP-A CRD. However, the binding of Me O-Man to MBP-C CRD fitted better for $N = 2$ than for $N = 1$. The fit with $N = 1$ was excellent for the binding of NacYD(G-ah-Man)$_2$ to MBP-C CRD. The authors suggested that the enhanced binding affinity of NAcYD(G-ah-Man)$_2$ to MBP-C CRD involved binding of one molecule of

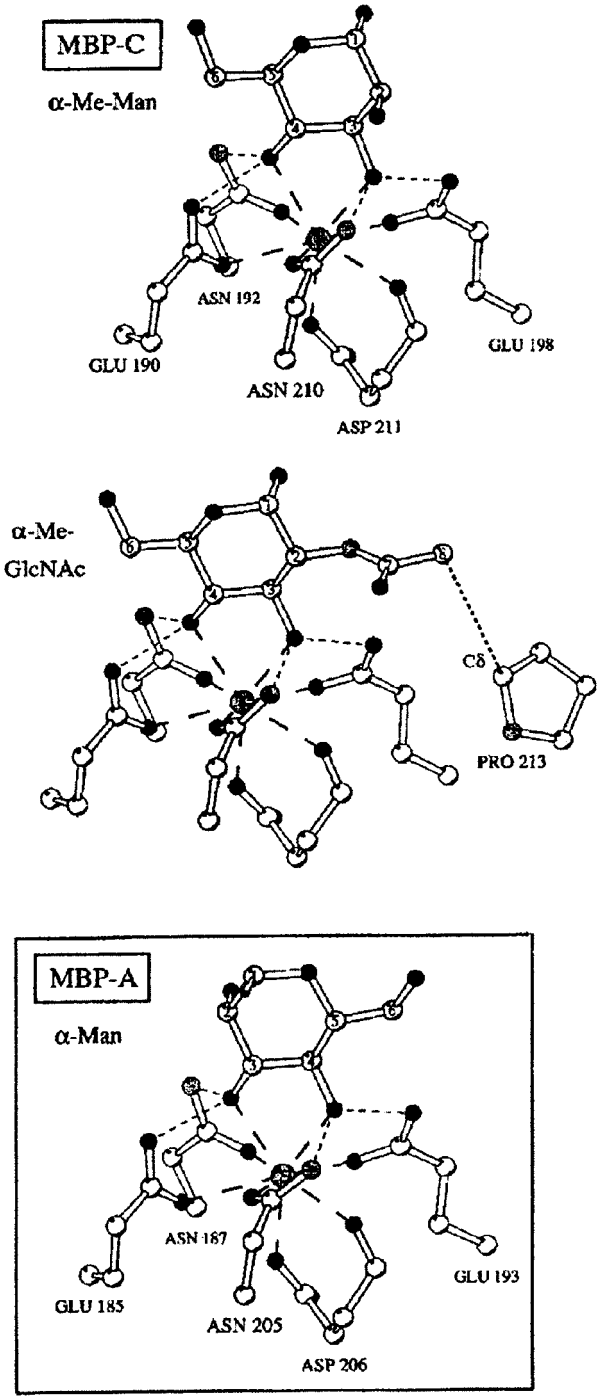


Figure 7. Structures of monosaccharides bound to MBP-A and MBP-C. Ca^{2+} is shown as a larger gray sphere. Carbon atoms of the bound sugars are numbered. Long-dashed lines denote coordination bonds with Ca^{2+}, medium-dashed lines denote hydrogen bonds, and short-dashed lines denote van der Waals' contacts.[59] (Reprinted with permission from ref 59. Copyright 1996 American Society for Biochemistry and Molecular Biology.)

ligand at two sites within the same monomer.

The forces involved in the binding of a Man residue appear to be similar for MBP-A and MBP-C as determined by X-ray crystallography.[59] A network of hydrogen bonds and coordination bonds that connect the 3-OH and 4-OH of Man with amino acid residues and a calcium ion in the binding site apparently generate a significant portion of the total binding force (Figure 7).

Since the cloned MBP fragments each should possess a unique and uniform primary structure and the binding mechanisms were essentially the same for both the MBPs, the most plausible explanation for the enhanced affinity of NacYD(G-ah-Man)$_2$ was the presence of two sugar-binding sites on MBP-C monomer: both sites could bind Man, but only one of the sites accommodated GlcNAc (Figure 49). Simultaneous occupation of the two sites by Man-containing ligand enhanced the binding affinity.

A secondary binding site for methyl α-mannopyranoside which was only observable in the presence of a very high concentration of the ligand was reported for MBP-C CRD by Ng et al.[59] At the second site only a single amino acid side chain (Lys 130) made hydrogen bonds with 6-OH, 2-OH, and ring-O of the sugar ring. The involvement of the axial 2-OH of Man in the binding process suggested perhaps GlcNAc, lacking this axial OH group, might not bind at the second site. This site was about 25 Å away from the first site, and since the divalent Man-containing ligand with enhanced affinity for MBP-C had two Man residues separated by 25 Å or longer, one molecule of the divalent ligands should be able to bind both sites simultaneously. The binding of two terminal Man residues simultaneously at the two Man-binding sites in a single CRD of MBP-C would explain the preferential binding by MBP-C CRD of certain high Man-type oligosaccharide structures.

The existence of secondary binding sites has been observed in related C-type CRDs.[70] The authors observed that the MBP CRDs might contain a vestigial secondary Man binding site which degenerated into a less functional site in MBP-A than in MBP-C. Although MBP-A CRD barely showed any cluster effect toward small synthetic di- and trivalent ligands, it produced large cluster effects of similar magnitude as MBP-C CRD toward neoglycoproteins, such as BSA derivatives containing multiple residues of Man, GlcNAc, or Fuc.[66,68] Undoubtedly such affinity enhancement was generated by the clustering of three monomeric units of MBP CRDs.

III. Mannose Binding Plant Lectins

A. Legume Lectins

1. Concanavalin A

Concanavalin A (ConA), isolated from the seeds of the Jack bean *Canavalia ensiformis*, is one of the most widely utilized lectins in biology. It is also a member of the Diocleinae subtribe, which is a family of Man binding proteins with comparable properties. ConA is well-known for its several uses, such as, probing the dynamics and structures of normal and tumor cell membranes,[71,72] establishing glycosylation mutants in transformed cells,[73] and yielding preparations of polysaccharides, glycopeptides, and glycoproteins from lectin affinity columns.[74] Early studies showed that ConA recognized α-glucopyranoside and α-mannopyranoside residues with free 3-, 4-, and 6-hydroxyl groups.[3] The lectin is reported to be a tetramer above pH 7 and a dimer below pH 6. Studies have shown that it exists in a dimer−tetramer equilibrium which, in addition to pH, is also influenced by salt concentration (Dam and Brewer, unpublished data). Each monomer of ConA possesses one saccharide binding site as well as a transition metal ion site (S1) and a Ca^{2+} site (S2).[3,75] Sanders et al.[76] studied the binding of D-glucopyranose, D-mannopyranose, MeαMan, and MeαGlc to Cd^{2+}-, Co^{2+}-, and Ni^{2+}-substituted ConA by ITC. The results show that substitution of the Mn^{2+} ion at S1 with Cd^{2+}, Co^{2+}, and Ni^{2+} has, in most case, little effect on the thermodynamics of carbohydrate binding to the lectin in solution.The ability of ConA to bind with high affinity to certain N-linked carbohydrates has made it the subject of a number of studies to determine its fine carbohydrate binding specificity.

Early studies by Goldstein and co-workers showed the presence of two classes of linear oligosaccharides which differed in their affinities for ConA. The first class possesses affinities similar to monosaccharide binding and includes α(1,3), α(1,4), and α(1,6) oligosaccharides with nonreducing terminal Glc or Man residues.[77] The second class shows higher affinities and includes the α(1,2) oligomannosides.[78,79] The 5- and 20-fold enhanced affinities of the α(1,2) di- and trimannosyl oligosaccharides with respect to MeαMan, prompted speculation that ConA possessed an extended binding site that accommodated these oligosaccharides.[80] Solvent proton nuclear magnetic relaxation dispersion (NMRD) studies[81,82] suggested that the enhanced affinities of the α(1,2) oligomannosides were primarily due to their increased probability of binding because of the presence of multiple Man residues with free 3-, 4-, and 6-hydroxyl groups in each molecule.[81] These findings were supported by rapid flow kinetic analysis of the binding of fluorescent labeled α(1,2)-mannosyl oligosaccharides to the protein.[83]

Studies of the binding of a series of oligomannose and bisected hybrid glycopeptides and complex type oligosaccharides which possess higher affinities (∼50-fold or greater) than MeαMan showed that their NMRD profiles were different from those of simple mono- and oligosaccharides.[84−87] These studies observed that the "core" trisaccharide (Figure 8) possessed nearly 100-fold higher affinity than MeαMan and gave an NMRD profile similar to those of the larger N-linked carbohydrates.[84,87] These results suggested that the trimannosyl moiety in N-linked carbohydrates was responsible for their high-affinity binding to ConA. These conclusions were supported by structure−activity studies of Kasai and co-workers[88] and NMR studies of the binding of methyl trimannoside by Carver et al.[89]

A number of ITC studies of the thermodynamics of binding of mono- and oligosaccharides and glyco-

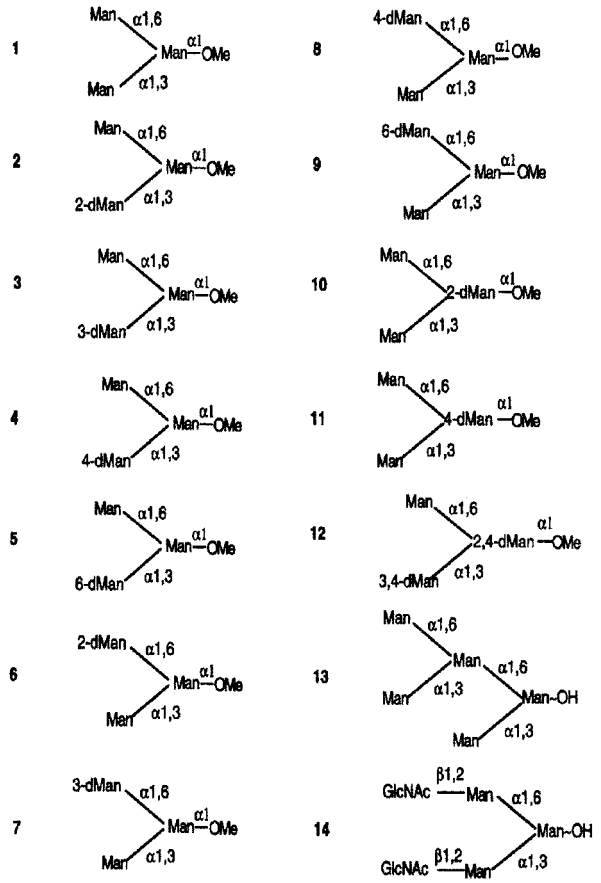


Figure 8. Structures of core trimannoside **1**, deoxy analogues **2**−**12**, Man 5 oligomannose carbohydrate **13**, and biantennary complex carbohydrate **14**. Man, GlcNAc, 2-d-Man, 3-dMan, 4-dMan, and 6-dMan represent mannose, *N*-acetylglucosamine, 2-deoxymannose, 3-deoxymannose, 4-deoxymannose, and 6-deoxymannose residues, respectively. (Reprinted with permission from ref 107. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

peptides to ConA have since been reported.[90−94]

ITC Studies of the Binding of the Core Trimannoside to ConA. Brewer and co-workers[92] reported an ITC study of the binding of a series of mono- and oligosaccharides to ConA including the "core" trimannoside. The K_a (4.9×10^5 M^{-1}) and ΔH (−14.4 kcal/mol) values for ConA binding the trimannoside (**1**) (Figure 8) are much greater than those of MeαMan ($K_a = 8.2 \times 10^3$ M^{-1}; $\Delta H = -8.2$ kcal/mol) and constituent disaccharides, Manα(1,3)Man and Manα(1,6)Man, which represent the two arms of the 3,6-trimannoside. These results provided direct evidence that ConA possessed an extended binding site that recognized the two nonreducing Man residues of the trimannoside and that these extended binding interactions were, in part, responsible for the high affinity of the trimannoside.[92] Similar ITC data were obtained at pH 7.2 and pH 5.2. Williams et al.[90] reported $\Delta H = -9.8$ kcal/mol and $\Delta G = -7.2$ kcal/mol for binding of trimannoside to ConA at pH 5.2. Swaminathan et al.[95] determined the ΔH for ConA−trimannoside interaction at pH 5.2, which was similar to that reported by Mandal et al.[92]

Binding of Complex-Type Oligosaccharide to ConA. A biantennary complex pentasaccharide (**14**) (Figure 8) possessing a terminal β(1,2)GlcNAc residue on each arm of the core trimannoside was shown by ITC measurements to possess an affinity nearly 4-fold greater than that of the core trimannoside.[92] However, ΔH of the complex oligosaccharide was −10.6 kcal/mol as compared to −14.4 kcal/mol for the trimannoside. These results indicated that the increase in affinity of the longer chain complex oligosaccharide was due to entropic effects and not increases in enthalpy of binding. The disaccharide, GlcNAcβ(1,2)Man, which constitutes the two branched chains of the oligosaccharide also showed a relatively low ΔH but a favorable $T\Delta S$ contribution to the binding free energy. ITC data for α(1,2) dimannoside (Manα(1,2)Man) and α(1,2) trimannoside (Manα(1,2)-Manα(1,2)Man) binding to ConA both showed enhanced entropic contributions to binding.[92] These results were consistent with a sliding mechanisms between Man residues of the oligosaccharides at the primary "monosaccharide" binding site of the lectin for their enhanced affinities, similar to that proposed from earlier NMRD studies.[81] Indeed, subsequent X-ray crystallographic data supported these findings. The presence of terminal α(1,2) Man residues in large oligomannose glycopeptides may explain some of the higher affinity of these molecules for ConA relative to shorter chain analogues.

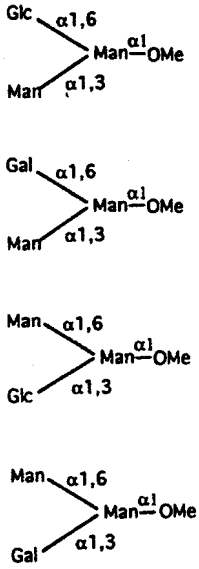


Figure 9. Glucose (Glc) and galactose (Gal) substituted trimannoside (Man is mannose). (Reprinted with permission from ref 93. Copyright 1994 American Chemical Society.)

Nature of the Extended Binding Site of ConA for the Core Trimannoside. Binding of a variety of synthetic analogues (Figure 9) of the methyl α-anomer of the core trisaccharide to ConA was investigated by ITC binding studies. Data for analogues possessing an α-glucosyl or α-galactosyl residue substituted at either the α(1,6) or α(1,3) position of the trimannoside indicated that the α(1,6) residue of the parent trimannoside occupied the so-called "monosaccharide site" and the α(1,3) residue to a weaker secondary site.[93] A complete set of deoxy analogues of the α(1,3)Man residue of the trimannoside was also synthesized and examined by ITC.[93] The ITC data demonstrated that only the 3-deoxy

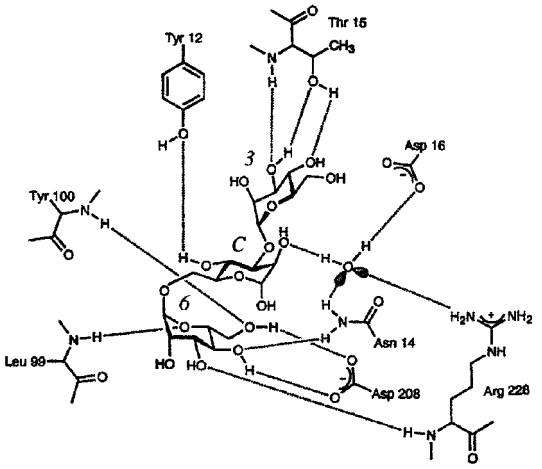


Figure 10. View of the X-ray crystal structure of trimannoside **1** (no anomeric methoxy group) bound to ConA. The trimannoside is shown with the central Man indicated by ***C***, the α(1,6) Man by ***6*** and the α(1,3)Man by ***3***.[96] (Reprinted with permission from ref 96. Copyright 1996 American Society for Biochemistry and Molecular Biology.)

analogue of the trimannoside on the α(1,3)Man arm (**3**) (Figure 8) bound with ∼10-fold lower affinity and 3.4 kcal mol^{-1} lower enthalpy than the parent trimannoside (**1**). This suggested that the 3-hydroxyl of the α(1,3)Man arm makes specific hydrogen bonds with the protein at a secondary binding site. The enthalpy of binding of the 3-deoxy analogue (**3**) (−11 kcal mol^{-1}) was still, however, higher than that of MeαMan (−8.4 kcal mol^{-1}), which indicated another site of contact between the trimannoside and ConA, most likely the central Man residue. Thus, ConA was predicted to have an extended binding site which included a high-affinity site that recognized the 3-, 4-, and 6-hydroxyl groups of the α(1,6)Man residue of the trimannoside, a lower affinity that bound the 3-hydroxyl of the α(1,3)Man residue, and a third site which appeared to involve the "core" Man residue.[93]

The X-ray crystal structure of ConA complexed with the core trimannoside[96] was subsequently published after the above studies and supported most of the ITC findings. The results demonstrate that ITC can provide important structural information about ligand−receptor complexes in solution as well as thermodynamic data. Further ITC studies using a complete set of deoxy analogues (Figure 8) as well as di- and trideoxy analogues of the trimannoside[94] showed excellent agreement with the X-ray data.[96]

Detailed Comparison of the ITC Data with the X-ray Crystal Structure of Trimannoside Complexed to ConA. A view of the H-bonding interactions between the hydroxyl groups of the trimannoside and the binding site of ConA, derived from the X-ray data, is shown in Figure 10.[96] The data show that the 3-, 4-, and 6-hydroxyl groups of the α(1,6) Man residue of the trimannoside binds in the same manner as MeαMan in its crystalline complex with ConA.[97] These results agree with the ITC data for the α(1,6)3-deoxy (**7**), α(1,6)4-deoxy (**8**), and α(1,6)6-deoxy (**9**) trimannoside analogues.[94] All these analogues showed reduced K_a and Δ*H* values compared to trimannoside. The X-ray data also show binding of the 3-OH of the α(1,3) Man residue to the N−H and side chain O of Thr 15 and the 4-OH of the α(1,3) Man residue to the side chain −OH of Thr 15 (Figure 10). 2-OH and 4-OH of the central Man residue were also found hydrogen bonded to the lectin. These observations were also in agreement with the thermodynamic data. The K_a and Δ*H* values for the α(1,3)3-deoxy (**3**), α(1,3)4-deoxy (**4**), "core" 2-deoxy (**10**), and "core" 4-deoxy (**11**) analogues were all less than the parent trimannoside.[94]

Nonlinearity of the ΔΔ*H* and ΔΔ*G* Values of the Individual Hydroxyl Groups of 1. Thermodynamic data indicated that the ΔΔ*H* values for the monodeoxy analogues were nonlinear.[94] For example, the combined ΔΔ*H* values for the 3-OH and 4-OH of the α(1,3) Man residue of trimannoside obtained from **3** and **4**, respectively, and the 2-OH and 4-OH of the central Man residue obtained from **10** and **11**, respectively, was ca. −8.8 kcal/mol. This could be compared to the difference in Δ*H* between **1** and MeαMan of −6.2 kcal/mol, which reflects binding of the α(1,3) Man and the central Man residues of **1**. Furthermore, the sum of the ΔΔ*H* values for the 3-, 4-, and 6-OH of the α(1,6) Man residue (**7**, **8,** and **9**), the 3- and 4-OH of the α(1,3) Man residue (**3** and **4**), and the 2- and 4-OH of the central Man residue (**10** and **11**) was −17.5 kcal/mol, which was greater than the Δ*H* for **1** of −14.4 kcal/mol. Thus, the sum of the ΔΔ*H* values for the hydroxyl groups of **1** obtained from the monodeoxy analogues did not correspond to the measured Δ*H* of **1**. In all of the above cases, the sum of the ΔΔ*H* values for specific hydroxyl groups on certain Man residues of **1** obtained from the corresponding monodeoxy analogues was greater than the measured Δ*H* for that residue(s). This nonlinear relationship in ΔΔ*H* was also present in the di- and trideoxy analogues. The same nonlinearity was also present in the ΔΔ*G* values of the monodeoxy analogues.

The ΔΔ*H* and ΔΔ*G* values for each monodeoxy analogue of **1** also did not scale with the number of H-bonds at each position as determined from X-ray crystallography. The ΔΔ*H* values for the monodeoxy analogues are not proportional to the number or type of H-bonds involved at specific hydroxyl groups of **1**. This is of particular interest since it has been suggested that the free energy associated with elimination of a H-bond between an uncharged donor/acceptor pair is 0.5−1.5 kcal/mol and between a neutral−charged pair 3.5−4.5 kcal/mol. The data, however, indicate no such relationship in the free energy difference (ΔΔ*G*) of monodeoxy analogues that represent the loss of one or more H-bonds such as **7** versus **8** and **9**.

The presence of nonlinear relationships in the ΔΔ*H* and ΔΔ*G* values for the deoxy analogues indicates other contributions to these terms such as solvent and protein effects. Thus, the magnitude of the ΔΔ*H* and ΔΔ*G* values represent not only the loss of the H-bond(s) involved, but also differences in the solvent and protein contributions to binding of **1** and the deoxy analogues. Contribution of solvent to the Δ*H* of sugar by lectin has been shown experimentally and presented in a different section (section V) of this review. Thus, ITC measurements of the binding of

deoxy analogues of a substrate to a macromolecule do not provide direct measurements of the free energy and enthalpy of the H-bonding involved.

Interaction of ConA with a Conformationally Constrained Trisaccharide. Navarre et al.[98] studied the binding of the trisaccharide, GlcNAcβ(1,2)-Manα(1,3)ManαOMe, as well as a conformationally constrained cyclic analogue to ConA by ITC. Binding of the cyclic analogue was associated with a more favorable entropic effect compared to the linear form. However, the enthalpy of binding of the cyclic analogue was less favorable than that of the linear trisaccharide; therefore, no improvement in the affinity of the former was observed. On the other hand, a study with an anti-carbohydrate monoclonal antibody[99] showed that the introduction of conformational constraints in the carbohydrate ligand had little effect on the overall thermodynamics of binding. The study of Navarre et al.[98] points to one important limitation of ligand design: attempts to overcome certain thermodynamic barriers (entropy effects, in this case) through rational design of the ligand may not always give rise to high-affinity binding because of other factors such as steric effects, etc.

2. ITC Studies of the Lectin from Dioclea grandiflora (DGL)

The seed lectin from *Dioclea grandiflora* (DGL) is a Man/Glc binding protein obtained from North East Brazil and a member of a group of lectins from the subtribe Diocleinae. DGL is devoid of covalently linked carbohydrate and is reported to be a tetramer with a molecular mass of 100 KDa.[100] As with other legume lectins, DGL requires Ca^{2+} and a transition metal ion for its binding activity. The lectin possesses a high degree of sequence homology with the jack bean lectin, ConA, another member of the Diocleinae subtribe, differing in 52 out of 237 residues.[101] Six of the seven residues that have been implicated as ligands for the Ca^{2+} and transition metal ion sites are conserved, and the amino acid residues surrounding the carbohydrate binding site of ConA are also conserved in DGL.[101]

DGL, like ConA, binds with high affinity to the "core" trimannoside, but their affinities for a complex carbohydrate **14** (Figure 8) are different.[102] Furthermore, both ConA and DGL possess different biological activities such as histamine release from rat peritoneal mast cells.[103] It is shown in the subsequent section of this review that this biological property is correlated to the affinity of the lectins for the complex carbohydrate. The thermodynamic study revealed the molecular basis of trimannoside and complex carbohydrate binding by DGL highlighting some important similarities and differences in carbohydrate recognition by homologous lectins, namely, ConA and DGL.

DGL Binding to Trimannoside. DGL binds to the core trimannoside with a ΔH of -16.2 kcal/mol and a K_a of 1.2×10^6 M^{-1}. The ΔH was -8.0 kcal/mol greater and K_a 270-fold higher for **1** than that of MeαMan. These results also suggest that DGL, like ConA, possesses an extended binding site for the trimannoside. The trend of increased K_a and ΔH values for the trimannoside relative to MeαMan is similar to that reported by Chervenak and Toone.[104]

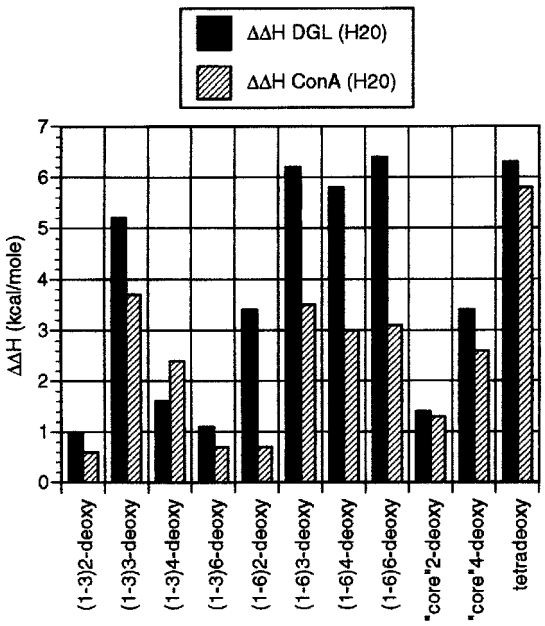


Figure 11. $\Delta\Delta H$ values of DGL and ConA binding to deoxy trimannoside **1** analogues. The solid bars are the DGL data and the hatched bars the ConA data.[105] (Reprinted with permission from ref 105. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

Study with a complete set of monodeoxy analogues of the core trimannoside indicate that DGL recognizes the 2-, 3-, 4-, and 6-hydroxyl groups of the α-(1,6) Man residue, the 3- and 4-hydroxyl groups of the α(1,3) Man residue, and the 2- and 4-hydroxyl groups of the central Man residue of the trimannoside. These assignments were in total agreement with the X-ray crystal structure of DGL complexed with trimannoside.[105] DGL and ConA recognize the same set of hydroxyl groups on trimannoside (Figures 10 and 38) but there exist certain important differences.

Comparison of Thermodynamic Data for DGL and ConA Binding to Deoxy Analogues of Trimannoside Based on X-ray Structural Data. The α(1,3)4-deoxy analogue (**4**) shows a loss in $-\Delta H$ ($\Delta\Delta H$) of 2.4 kcal/mol and a ~4-fold reduction in K_a for ConA binding, but a loss in $\Delta\Delta H$ of 1.6 kcal/mol and a 2-fold reduction in K_a for DGL binding, indicating some difference in the mode of binding at this position.

ITC data for deoxy analogues **7**, **8,** and **9** of the α-(1,6)-arm of **1** indicate binding of the 3-, 4-, and 6-OH groups to DGL, as observed in ConA. However, unlike ConA, the data for deoxy analogue **6** indicate binding of the 2-OH to DGL. The crystal structure of the DGL complex with **1**, however, shows no direct protein–carbohydrate binding interactions at this site.[105] The reduction in binding indicated by the thermodynamic data for the α(1,6)Man 2-deoxy analogue appears to reflect indirect binding to the protein of the 2-hydroxyl at this position in **1** via a water molecule in the binding site. Although the overall pattern of $\Delta\Delta H$ data for DGL is similar to that for ConA, the magnitude of the $\Delta\Delta H$ data for certain analogues of the α(1,6)-arm of **1** is different (Figure 11). Thus, the 3-, 4-, and 6-deoxy α(1,6) Man analogues of **1** possess $\Delta\Delta H$ values that are nearly twice as great for DGL (~6.1 kcal/mol) as for ConA (~3.2 kcal/mol). It is clear

that the two protein complexes are nearly identical, both in terms of the residues involved in binding to **1** as well as in terms of the number of hydrogen bonds and their distances to **1**. Therefore, differences in the $\Delta\Delta H$ values of the two lectins for the 3-, 4-, and 6-deoxy α(1,6) Man analogues of **1** are not due to differences in the direct lectin−carbohydrate hydrogen-bonding interactions. Furthermore, the ∼2.9 kcal/mol difference in the average $\Delta\Delta H$ values for the 3-, 4-, and 6-deoxy α(1,6) analogues binding to DGL (6.1 kcal/mol) and to ConA (3.1 kcal/mol) is nearly the same as the ∼2.7 kcal/mol difference in $\Delta\Delta H$ (with regard to trimannoside) values for the 2-deoxy α(1,6) analogue (**6**) binding to DGL (3.4 kcal mol^{-1}) versus to ConA (0.7 kcal/mol) (Figure 11). This suggests a common mechanism underlying the differences in the thermodynamics of binding of all four α(1,6) deoxy analogues to the two lectins. In this regard, it is interesting that the average $\Delta\Delta H$ values for the 3-, 4-, and 6-deoxy α(1,6) analogues are nearly the same magnitude for each lectin. The relatively constant $\Delta\Delta H$ values of the 3-, 4-, and 6-deoxy α(1,6) analogues for each lectin occur despite the different number and type of hydrogen bonds at each respective position in the parent trisaccharide. This further suggests a common thermodynamic mechanism of binding of the 3-, 4-, and 6-deoxy α(1,6) analogues to each lectin.

There are four amino acid differences found within a more extended area surrounding the ligand (**1**), which are residue 21 (Asn in DGL, Ser in ConA), residue 168 (Asn in DGL, Ser in ConA), residue 205 (Glu in DGL, His in ConA), and residue 226 (Gly in DGL, Thr in ConA). These residues are indirectly involved in ligand binding. They interact with a network of hydrogen-bonded ordered water molecules, which in turn, directly interact with **1** (Figure 38).

The largest deviation in ordered water molecule organization appears near the shift in residues 222−227. In ConA, the side chain of Thr 226 is oriented to make direct hydrogen-bond interactions with the ordered water molecule network, and the smaller side chain of Ser 168 accommodates a water between itself and Thr 226. In DGL, residue 226 is a Gly and ordered water molecules fill the space of the missing side chain. Importantly, this network of hydrogen-bonded ordered water molecules directly interacts with the hydroxyl oxygens at positions 2 and 3 of the α(1,6)-arm of **1**, and thermodynamic data indicate that the strength and specificity of DGL and ConA binding differ at these positions in **1**.[106] Thus, the differences observed in the $\Delta\Delta H$ values for DGL and ConA binding to the 2-, 3-, 4-, and 6-deoxy α(1,6) Man analogues of **1** may be due to altered structural water molecules in this region of the binding sites of the lectins.

Alternatively, DGL and ConA may undergo different conformational transitions upon binding **1** and the deoxy analogues, which may contribute to the observed differences in the $\Delta\Delta H$ values of the analogues. In any case, it is clear that differences in the $\Delta\Delta H$ values of the 2-, 3-, 4-, and 6-deoxy α(1,6) Man analogues of **1** binding to DGL and ConA are not due to direct protein−ligand interactions.

Binding of Tetradeoxy Analog. The K_a and ΔH values obtained with α(1,3)3,4-deoxy, "core" 2,4-deoxy analogue **12** (Figure 8) for both ConA and DGL are almost comparable with those of MeαMan.[106] These results are consistent with the fact that tetradeoxy analogue is functionally equivalent to MeαMan as it is devoid of all participating hydroxyl groups on α-(1,3)Man and "core" Man residues.

Nonlinearity of the $\Delta\Delta H$ and $\Delta\Delta G$ Values of the Individual Hydroxyl Groups of Trimannoside. Nonlinearity and a lack of scaling of $\Delta\Delta H$ and $\Delta\Delta G$ values with the number and nature of hydrogen bonds were observed with the deoxy trimannosides binding to DGL as noted with ConA.[106]

Different Thermodynamics of Binding of a Biantennary Complex Carbohydrate to DGL and ConA and Its Structural Basis. Hemagglutination inhibition experiments and affinity column chromatography have shown that DGL binds the biantennary complex carbohydrate **14** (Figure 8) much more poorly than ConA.[102] The differential specificity of ConA and DGL for **14** has also been shown to be present in other members of the Diocleinae subtribe as mentioned in a separate section of this review. Importantly, the ability of these nine Diocleinae lectins to induce histamine release from rat peritoneal mast cells[103] was shown to correlate with the relative affinities of the lectins for **14**.[107]

The ITC-derived K_a value of **14** for DGL is 4.7×10^4 M^{-1} as compared to a K_a of 1.2×10^6 M^{-1} for ConA. The ΔH values of the two lectins for **14** are very different, with $\Delta H = -10.6$ kcal/mol for ConA and $\Delta H = -4.6$ kcal/mol for DGL. These results indicate that although both lectins share high affinities and specificities for trimannoside (**1**), they possess very different affinities and specificities for **14**.

The X-ray crystal structure of ConA complexed with **14** reveals that the β(1,2)-GlcNAc residue on the α(1,6)-arm of the pentasaccharide fits into an extended groove of ConA and makes hydrogen-bond contacts on both sides of the sugar ring.[108] The specific interactions of the β(1,2)-GlcNAc residue on the α(1,6)-arm have been shown with Thr 226 and Ser 168 of ConA (Figure 13). Superposition of the X-ray crystal structure of DGL bound to trimannoside onto that of ConA complexed with 14 reveals that proper contacts between DGL and the pentasaccharide are prevented due to key amino acid differences at residue 226 (Thr in ConA, Gly in DGL) and at residue 168 (Ser in ConA, Asn in DGL) and the shift in the backbone of residues 222− 227 (Figure 12).

Superposition of the structure of trimannoside in DGL with the structure of complex carbohydrate **14** bound to ConA[108] has shown that the core trimannoside moiety of **14** bound to ConA deviates by less than 0.5 Å from the position of **1** bound to DGL, indicating similar binding of the trimannoside moiety in both complexes. However, the β(1,2)-GlcNAc residue on the α(1,6)-arm of **14** modeled into DGL reveals contacts different from those observed in the ConA complex. In DGL, the side chain is missing from residue 226 and therefore no hydrogen bond can be formed to the 3-hydroxyl of the β(1,2)-GlcNAc resi-

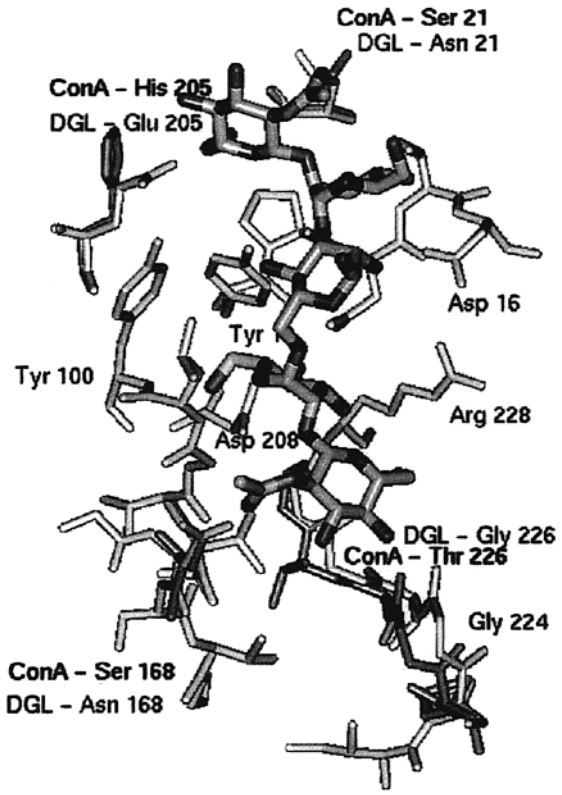


Figure 12. Modeling of **14** binding site of ConA and DGL.[105] (Reprinted with permission from ref 105. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

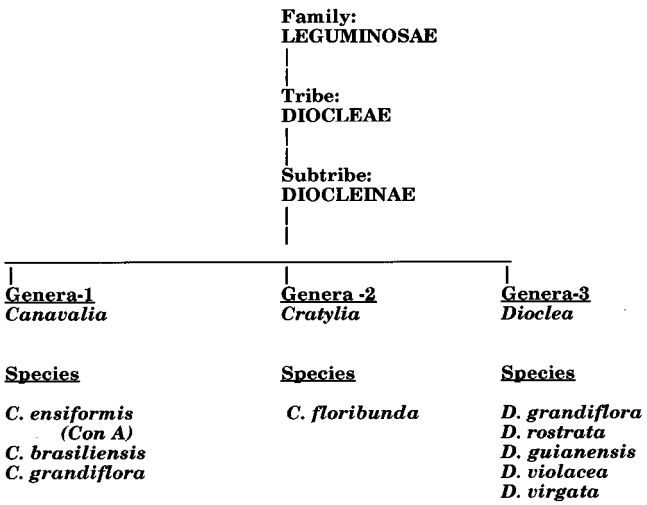
due. In addition, the backbone carbonyl oxygen of Gly 224 is too far to make a proper hydrogen bond with the 4-hydroxyl of the β(1,2)-GlcNAc residue, and the side chain of Asn 168 is too large to accommodate a proper hydrogen bond to the 7-hydroxyl of the β(1,2)-GlcNAc residue.[105] These differences in the interactions of the β(1,2)-GlcNAc residue on the α(1,6)-arm of **14** in DGL appear to explain the 30-fold lower affinity as well as the lower ΔH value of the lectin relative to ConA. The observed interactions of β(1,2)-GlcNAc residue on the α(1,3)-arm of **14** in DGL suggest little interference in binding of this region of the complex.

The contact differences for the β(1,2)-GlcNAc residue on the α(1,6)-arm of **14** in DGL and ConA also explain why DGL fails to bind the disaccharide GlcNAcβ(1,2)Man while ConA binds well to the disaccharide.[102]

3. Diocleinae Lectins: An Extended Family of Mannnose Binding Lectins

ConA and DGL belong to the subtribe Diocleinae. Seven other lectins from the same subtribe were used for a detailed thermodynamic study. The lectins were isolated from *Canavalia brasiliensis, Canavalia bonariensis, Cratylia floribunda, Dioclea rostrata, Dioclea virgata, Dioclea violacea, and Dioclea guianensis* (Scheme 1). Despite their phylogenetic proximity and apparently conserved sequences, the above Diocleinae lectins possess different biological activities such as histamine release from rat peritoneal mast cells,[103] lymphocyte proliferation and interferon γ production,[109] peritoneal macrophage stimulation and inflammatory reaction,[110] as well as induction of paw edema and peritoneal cell immigration in rats.[111] Hemagglutination and thermodynamic studies revealed important similarities and differences in the carbohydrate binding properties of these lectins. Differential carbohydrate specificities were found to be correlated with the biological activity of the Diocleinae lectins.

Scheme 1[a]



[a] Reprinted with permission from ref 114. Copyright 2000 American Society for Biochemistry and Molecular Biology.

Thermodynamics of MeαMan Binding to the Diocleinae Lectins. The thermodynamic binding parameters of seven Diocleinae lectins to MeαMan determined by ITC measurements[107] showed minor differences. *C. brasiliensis* displays the highest K_a value (1.3×10^4 M^{-1}) with *D. rostrata* possessing the lowest K_a value (1.7×10^3 M^{-1}). *C. brasiliensis, D. guianensis, D. violacea,* and *D. virgata* had ΔH values between -5.8 and -4.9 kcal/mol, while *C. bonariensis, C. floribunda, D. rostrata,* ConA, and DGL possessed $-\Delta H$ values between -6.9 and -8.9 kcal/mol. However, the relative K_a values of the lectins for binding MeαMan did not correlate with their respective $-\Delta H$ values, indicating compensating entropic factors.

Trimannoside Shows Extended Site Interactions with Diocleinae Lectins. ITC data indicated that all seven new Diocleinae lectins showed enhanced K_a and $-\Delta H$ values for trimannoside relative to MeαMan. The $-\Delta H$ values for all seven lectins binding to trimannoside are -5 to -7 kcal/mol greater than that for MeαMan, similar to the differences observed for ConA and DGL. These data strongly suggest similar extended binding sites for all nine Diocleinae lectins including ConA and DGL.[107]

Binding of Man5 Oligomannose Carbohydrate. Hemagglutination inhibition data showed that the Diocleinae lectins bind Man5 oligosaccharide **13** with almost the same inhibitory potency as **1**. This indicates that the trimannoside moiety on the α(1,6)-arm is the primary epitope for interaction, as observed for ConA and DGL.[102] ITC data confirmed these results.[107]

Differential Binding of Biantennary Complex Oligosaccharide 14. As discussed above, the affinity

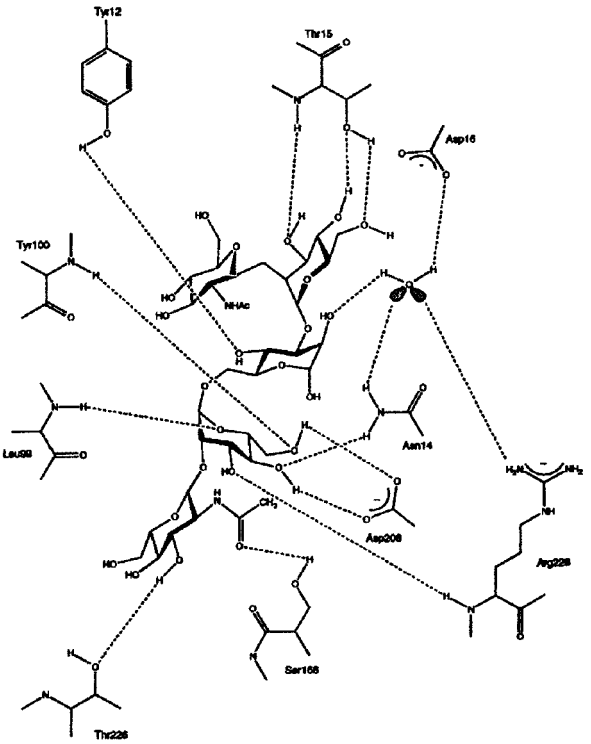


Figure 13. Schematic representation of the hydrogen bonds between ConA and the pentasaccharide **14**.[108] (Reprinted with permission from ref 108. Copyright 1998 Oxford University Press.)

of DGL for biantennary complex oligosaccharide **14** has been shown to be weak compared to that of ConA, and a structural explanation for this difference is provided by Rozwarski et al.[105] All of the Diocleinae lectins tested showed distinct correlated binding affinities toward **14** and its constituent disaccharide, GlcNAcβ(1,2)Man. Hemagglutination inhibition results indicated that **14** had much higher inhibition potencies with *C. brasiliensis*, *D. guianensis*, and *D. virgata* as compared to the other new Diocleinae lectins. This parallels the binding activities of the lectins toward GlcNAcβ(1,2)Man. ITC data showed order of magnitude greater K_a values of *C. brasiliensis*, *D. guianensis*, and *D. virgata* for **14** relative to the other four lectins. Among the nine Diocleinae lectins, ConA showed the highest K_a value for **14** while DGL showed the lowest K_a.[107] The relative K_a values for all nine lectins binding to **14** (along with trimannoside) with respect to MeαMan are shown in Figure 14. *C. brasiliensis*, *D. guianensis*, and *D. virgata* possessed greater $-\Delta H$ values for **14** of the seven lectins, while the $-\Delta H$ values for the other four lectins were comparatively lower.

An enthalpy−entropy compensation plot of the data for **14** yielded different slopes for the above two groups of the Diocleinae lectins (Figure 15). The lectins from *C. brasiliensis*, *D. guianensis*, *D. virgata*, and ConA fell on a line with a slope of 1.44 (correlation coefficient 0.85), while a slope of 0.85 was found for the lectins from *C. bonariensis*, *C. floribunda*, *D. rostrata*, *D. violacea*, and DGL (correlation coefficient 0.98). By comparison, a similar plot of the lectins binding to **1** shows a single line with a slope of 1.21 (correlation coefficient 0.97) (Figure 15). These results indicate different energetic mechanisms of

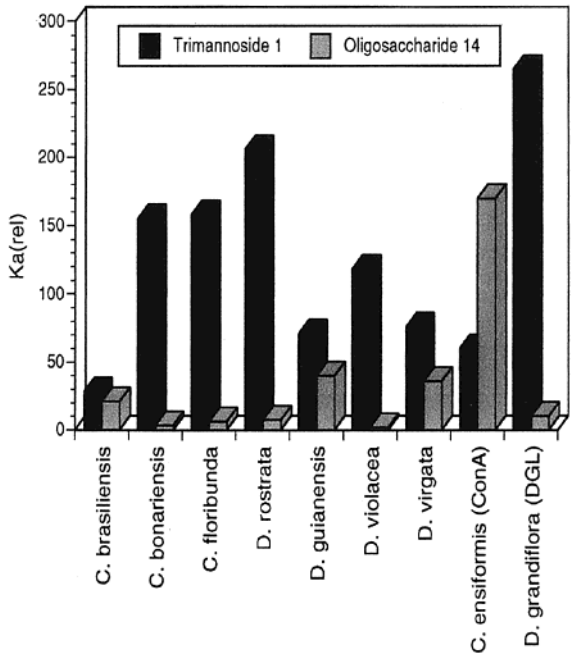


Figure 14. Plot of the ratio of K_a values of the nine Diocleinae lectins for trimannoside **1** and complex carbohydrate **14**, relative to MeαMan, derived from the ITC data.[107] (Reprinted with permission from ref 107. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

binding of the four relatively high-affinity lectins for **14**, as compared to the five lower affinity lectins. Thus, although all nine Diocleinae lectins show conserved high-affinity binding for **1**, four of the lectins show relatively high affinities for **14** with the other five lectins showing relatively low affinities. Therefore, binding discrimination among this group of lectins occurs toward biantennary complex carbohydrates.

Histamine Release Activities of the Diocleinae Lectins are Correlated with Relative Affinities for 14. ConA has long been known for its ability to induce histamine release from cells.[112,113] Recently, Gomes and co-workers[103] investigated the histamine release properties from rat peritoneal mast cells of several other lectins from the same subtribe. At the level of 10 μg/mL lectin concentration, ConA, *C. brasiliensis*, *D. guianensis*, and *D. virgata* induced a higher level of histamine release from rat peritoneal mast cells, whereas *D. grandiflora*, *C. bonariensis*, *C. floribunda*, *D. rostrata*, and *D. violacea* displayed lower abilities for induction. A significant correlation between the histamine releasing properties of these lectins and their affinity constants for **14** is presented in Figure 16, which shows that the K_a values of the Diocleinae lectins for **14** and the amount of histamine released by the lectins at 10 μg/mL are correlated. The strong histamine inducing lectins ConA, *C. brasiliensis*, *D. guianensis*, and *D. virgata* exhibit relatively high affinities (K_a) for **14**. On the other hand, the remaining relatively inactive lectins possess lower affinities for the complex carbohydrate. It appears, therefore, that induction of histamine release from rat peritoneal mast cells by ConA, *C. brasiliensis*, *D. guianensis*, and *D. virgata* involves binding of the lectins to a biantennary complex

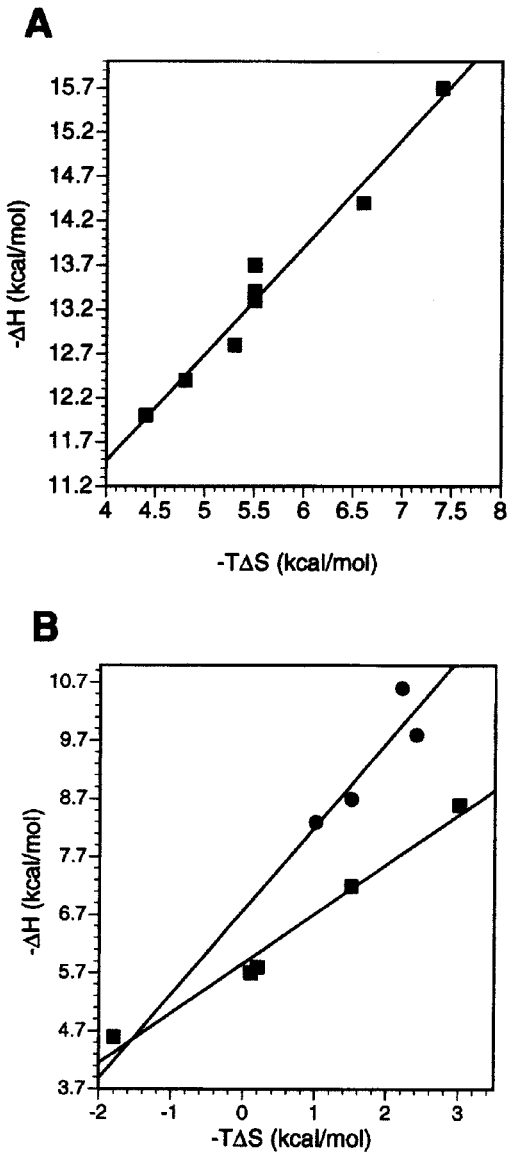


Figure 15. Enthalpy−entropy compensation plots for the binding of the Diocleinae lectins with (A) trimannoside **1** (slope = 1.21) and (B) biantennary complex carbohydrate **14** at 27 °C (300 K). In B, closed circles (●) represent the values for ConA, *C. brasiliensis, D. guianensis,* and *D. virgata* (slope = 1.4) while the closed squares (■) represent the values for *D. grandiflora, C. bonariensis, C. floribunda, D. rostrata,* and *D. violacea* (slope = 0.85).[107] (Reprinted with permission from ref 107. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

carbohydrate and/or structurally homologous epitope present on the cell surface.

ITC Studies of Trimannoside Binding by Diocleinae Lectins. To determine which hydroxyl groups of the trimannoside are involved in binding to the Diocleinae lectins, hemagglutination inhibition experiments were performed using different deoxy analogues of the trimannoside (Figure 8) The results indicated the involvement of the 3-, 4-, and 6-hydroxyls of the α(1,6) Man, the 3- and 4-hydroxyls of the α(1,3) Man, and the 2- and 4-hydroxyls of the central Man of trimannoside in binding. There was also indication of possible participation of the 2-hydroxyl of the α(1,6)-arm, as observed for a few of the lectins. The tetradeoxy analogue (**12**) showed very

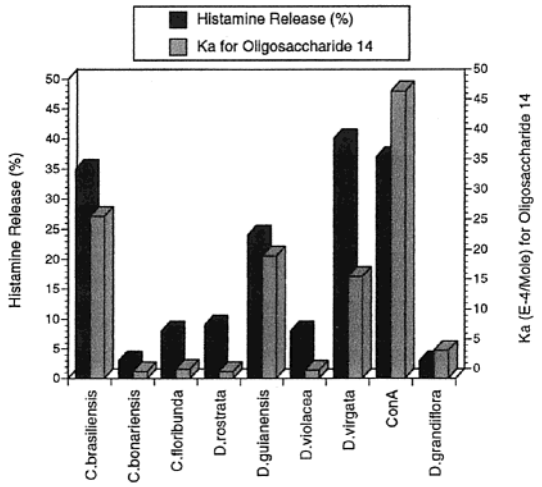


Figure 16. Graph showing K_a values (gray bars) of the seven Diocleinae lectins for complex carbohydrate **14** and their histamine-releasing properties (black bars).[107] (Reprinted with permission from ref 107. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

little inhibition potency relative to **1** and is comparable to that of MeαMan. Hemagglutination data showed a similar pattern of inhibition by the analogues for the seven Diocleinae lectins as observed for ConA and DGL. These results indicate highly conserved binding sites for **1** in all nine Diocleinae lectins.

Subsequent ITC studies confirmed this pattern of binding. Additionally, it provided further insight into the finer aspect of trimannoside recognition.[114]

Interaction of a (1,6) Man Residue of Trimannoside with the Diocleinae Lectins. The ITC-derived K_a and ΔH values of **7**, **8,** and **9** are significantly lower than that of **1** for all seven lectins which support the involvement of the 3-, 4-, and 6-hydroxyl groups of the α(1,6) Man of **1**, but not the 2-hydroxyl, in binding to all seven Diocleinae lectins.[114] The results also suggest that the α(1,6) Man residue of **1** occupies the so-called "monosaccharide binding site" in all of the lectins.[3]

Interaction of a (1,3) Man Residue of Trimannoside with the Diocleinae Lectins. Deoxy analogues **2**, **3**, **4,** and **5** were used to determined the involvement of the 2-, 3-, 4-, and 6-hydroxyl groups of α(1,3) Man of **1**, respectively, in binding to the seven Diocleinae lectins.[114] Only analogue **3** exhibited a loss in K_a and ΔH relative to **1**. These findings thus suggest that the 3-hydroxyl of the α(1,3) Man of **1** binds to the seven Diocleinae lectins.

Importantly, the X-ray crystal structures of ConA and DGL complexed with the core trimannoside[96,105] show that the 3-hydroxyl of the α(1,3) Man of **1** is involved in H-bonds with conserved contact residues of two lectins. However, the X-ray data for both trimannoside complexes also indicate the involvement of the 4-hydroxyl of the α(1,3) Man of **1** in H-bonds to the ConA and DGL (Figure 38). ITC data for binding of ConA[94] to **4** shows a 5-fold reduction in K_a value and a loss in $\Delta\Delta H$ of 2.1 kcal/mol relative to **1**. ITC data for DGL binding to **4** shows a 2-fold reduction in K_a and a loss in $\Delta\Delta H$ of 1.6 kcal/mol relative to **1**.[106] Thus, although the X-ray crystal

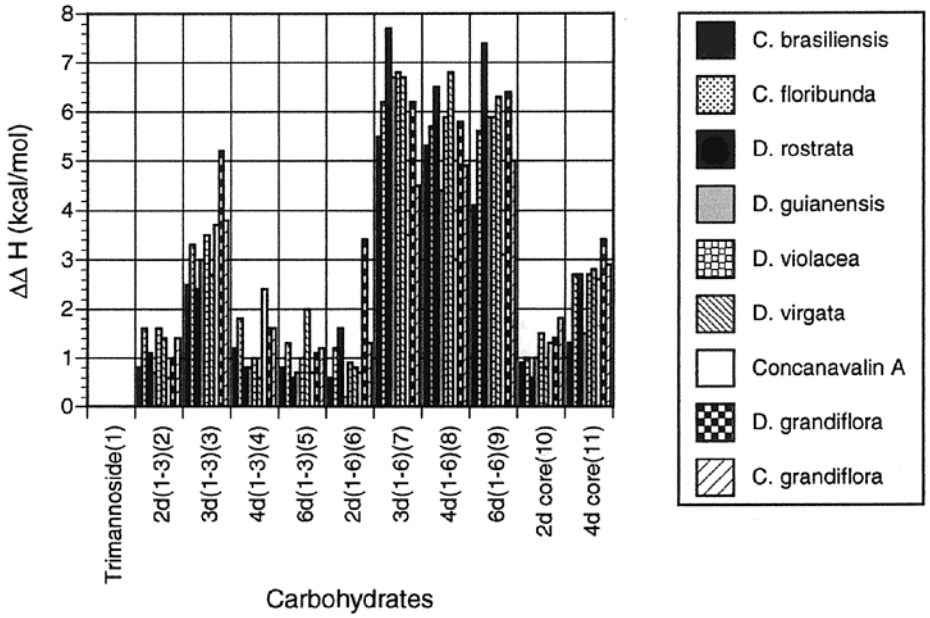


Figure 17. Bar graph showing $\Delta\Delta H$ values of the seven Diocleinae lectins as well as ConA and DGL for deoxy analogues **2**−**11**.[114] (Reprinted with permission from ref 114. Copyright 2000 American Society for Biochemistry and Molecular Biology.)

structures show the involvement of the 4-hydroxyl of the α(1,3) Man of **1** with both lectins, the thermodynamic data for **4** suggests weaker interactions of the 4-hydroxyl with the two proteins.

The thermodynamic data for **4** with the seven Diocleinae lectins also do not support strong interactions of the 4-hydroxyl group of the α(1,3) Man of **1** with the Diocleinae lectins.

Interactions of the "Core" Man Residue of Trimannoside with the Diocleinae Lectins. The X-ray crystal structures of the trimannoside complexes of ConA[96] and DGL[105] showed evidence for the involvement of the 2- and 4-hydroxyl groups of the core Man of trimannoside. Unlike the 4-hydroxyl group, the 2-hydroxyl group does not have any direct H-bond with the lectin, the contact is essentially water mediated (Figure 38). ITC measurements showed $\Delta\Delta H$ values of 2.3 and 3.4 kcal/mol, respectively, for ConA and DGL binding to **11**.[94,106] However, the $\Delta\Delta H$ values for ConA and DGL binding to **10** were 1.0 and 1.4 kcal/mol, respectively.[94,106]

A similar pattern of $\Delta\Delta H$ values is observed with the seven Diocleinae lectins, with larger $\Delta\Delta H$ values for **11** as compared to **10**. Only two lectins (*C. grandiflora* and *D. violacea*) have $\Delta\Delta H$ values for **10** large enough to be considered as evidence for H-bonding of the respective hydroxyl group of **1** to the lectins. The presence or absence of H-bonding of the 2-hydroxyl group of the core Man of **1** to the remaining five Diocleinae lectins will have to await X-ray crystallographic analysis of their trimannoside complexes. Thus, the absence of $\Delta\Delta H$ values greater than 1.0 kcal/mol for certain deoxy analogues such as **10** with five of the Diocleinae lectins may be taken as evidence that such H-bonds are either energetically very weak or absent in the corresponding solution complexes of **1** with the lectin.

Differences in the Magnitude of the Thermodynamic Binding Parameters of the Diocleinae Lectins Despite Structural Similarities and Conserved Binding. Thermodynamic data in combination with the available structural information clearly show that all nine Diocleinae lectins interact with the same set of hydroxyl groups of the trimannoside. The X-ray crystal structures of ConA[96] and DGL[105] complexed with the core trimannoside show conserved contact residues for both proteins (Figure 10). This fact is also reflected in Figure 17. The lectins from *D. guianensis*, *C. floribunda*, and *C. brasiliensis* also have these same contact residues for **1**, as shown in their primary sequence data (Figure 18).[115] However, the ITC data in Figure 17 also show a range of $\Delta\Delta H$ values for certain deoxy analogues which have corresponding hydroxyl groups involved in binding to the nine Diocleinae lectins.[114] These include $\Delta\Delta H$ values ranging from 6.5 to 7.7 kcal/mol for **7**, **8,** and **9** binding to *D. rostrata* to the much lower values of ∼3 kcal/mol for ConA.[94] Thus, there is a wide variation in the $\Delta\Delta H$ values of the nine Diocleinae lectins in Figure 17. This is true for not only the same deoxy analogue with different lectins, but also for different deoxy analogues that have corresponding hydroxyl groups that bind to the same lectin. For example, analogues **3**, **7,** and **11** possess different $\Delta\Delta H$ values in binding to ConA and DGL, respectively, even though their respective hydroxyl groups of **1** show hydrogen bonds to both lectins (Figures 10 and 38). Another example comes from the comparison of ConA and *C. brasiliensis.* The X-ray crystal structure of the lectin from *C. brasiliensis* shows only two amino acid changes relative to ConA.[116] Gly-58 and Gly-70 in *C. brasiliensis* are replaced by Asp and Ala, respectively, in ConA. Neither of the residues are near the carbohydrate binding sites in both lectins, and only small changes in the quaternary structures of the two lectins were noted. However, these two amino acid changes result in significant differences in the $\Delta\Delta H$ values of both lectins binding to analogues **7**, **8,** and **9** (∼5 kcal/mol for *C. brasiliensis* versus ∼3.0 kcal/mol for ConA).

	10	* * * * *		40
CA	ADTIVAVELD	TYPNTDIGDP	SYPH IGIDIK	SVRSKKTAKW
CB	ADTIVAVELD	TYPNTDIGDP	SYPH IGIDIK	SVRSKKTAKW
CF	ADTIVAVELD	TYPNTDIGDP	NYQHIGINIK	S IRSKATT RW
DGU	ADTIVAVELD	SYPNTDIGDP	S YPHIGIDIK	S IRSKSTARW
DGL	ADTIVAVELD	SYPNTDIGDP	NYPHIGIDIK	S IRSKSTARW
	50			80
CA	NMQNGKVGTA	HIIYNSVDKR	LSAVVSYPNA	DSATVSYDVD
CB	NMQNGKVGTA	HIIYNSVGKR	LSAVVSYPNG	DSATVSYDVD
CF	NVQ DGKVGTA	HISYNSVAKR	LSA IVSYPGG	SSATVSYDVD
DGU	NMQTGKVGTA	HISYNSVAKR	LSAVVSYTGS	SSTTVSYDVD
DGL	NMQTGKVGTV	HISYNSVAKR	LSAVVSYSGS	SSTTVSYDVD
	90	**		120
CA	LDNVLPEWVR	VGLSASTGLY	KETNTILSWS	FTSKLKSNST
CB	LDNVLPEWVR	VGLSASTGLY	KETNTILSWS	FTSKLKSNST
CFL	LNNILPEWVR	VGLSASTGLY	KETNTILSWS	FTSKLKTNST
DGU	LNNVLPEWVR	VGLSATTGLY	KETNTILSWS	FTSKLKTNSI
DGL	LNNVLPEWVR	VGLSATTGLY	KETNTILSWS	FTSKLKTNSI
	130			160
CA	HETNALHFMF	NQFSKDQKDL	ILQGDATTGT	DGNLELTRVS
CB	HETNALHFMF	NQFSKDQKDL	ILQGDATTGT	DGNLELTRVS
CFL	ADAQSLHF TF	NQFSQNPKDL	ILQGDASTDS	DGNLQLTRVS
DGU	ADANSLHF SF	NQFSQNPKDL	ILQGDATTDS	DGNLELTKVS
DGL	ADE NSLHF SF	HKFSQNPKDL	ILQGDAFTDS	DGNLELTKVS
	170			200
CA	SNGSPQGSSV	GRALFYAPVH	IW ESSAVVAS	FEATFTFLIK
CB	SNGSPQGSSV	GRALFYAPVH	IW ESSAVVAS	FEATFTFLIK
CFL	- NGSPQSNSV	GRALYYAPVH	VWDKSAVVAS	FDATFTFLIK
DGU	SSGDPQGSSV	GRALFYAPVH	IWEKSAVVAS	FDATFTFLIK
DGL	SSGDPQGNSV	GRALFYAPVH	IWEKSAVVAS	FDATFTFLIK
	* 210		*	237
CA	SPDSHPADGI	AFFISNID SS	IPS GSTGRLL	GLFPDAN
CB	SPDSHPADGI	AFFISNID SS	IPS GSTGRLL	GLFPDAN
CFL	STDSDI ADGI	AWFIANTDSS	IPHGSGGRLL	GLFPDAN
DGU	SPDRDPADGI	TFFIANTDTS	IPS GSGGRLL	GLFPDAN
DGL	SPDREPADGI	TFFIANTDTS	IPS GSGGRLL	GLFPDAN

Figure 18. Primary sequences of ConA (CA) and the lectin from *D. grandiflora* (DGL)[105] and the lectins from *C. brasiliensis* (CB),[116] *C. floribunda* (CF), and *D. guianensis* (DG).[115] The asterisks indicate conserved contact residues (12−16, 99, 100, 208, and 228) of the lectins with the core trimannoside.[114] (Reprinted with permission from ref 114. Copyright 2000 American Society for Biochemistry and Molecular Biology.)

In addition, although the K_a values of the two lectins for **1** are similar (3.7×10^5 M^{-1} for *C. brasiliensis* and 4.9×10^5 M^{-1} for ConA), the lectin from *C. brasiliensis* possesses a ΔH of −12.4 kcal/mol for **1** while ConA possesses a ΔH of −14.4 kcal/mol for **1**.[106] It is also interesting that ConA and the *C. brasiliensis* lectin are reported to have different lectin-induced nitric oxide production in murine peritoneal cells in vitro.[117] Differences in the binding thermodynamics in a homologous group of lectins where the binding residues are conserved indicate the indirect but important roles of the nonconserved residues away from the carbohydrate binding site. Subtle changes in the hydration of the lectins or subtle differences in their conformation due to the minor alteration of amino acid residues away from the carbohydrate binding sites may be responsible for their thermodynamic binding differences.[114] Effects of single-site mutations on conformational features of lectins have been shown by Siebert et al.[118]

Similar changes in the thermodynamics of binding of lectins with amino acid substitutions away from the carbohydrate binding site have been reported. For example, galectin-1 from Chinese hamster ovary cells was reported to undergo significant changes in ΔH and $T\Delta S$ but not ΔG in binding to LacNAc when single or multiple mutations were introduced in the N-terminal region of the protein.[33] ΔH for the parent galectin-1 binding to LacNAc is −6.6 kcal/mol, while a Cys-2 to Ser-2 mutant possesses a ΔH of −2.8 kcal/mol for LacNAc. Both lectins possess essentially the same K_a value for the disaccharide. Interestingly, a monomeric 4-substituted mutant with amino acid substitutions at the 2, 4, 5, and 6 positions also shows essentially no change in K_a but possesses a ΔH of −0.6 kcal/mol, thus making binding a largely entropy driven process. Importantly, all of these mutations were ∼20 Å from the carbohydrate binding site of the galectin.

B. Monocot Mannose Binding Lectins

Several Man-binding lectins have been reported from various monocot families such as Amaryllidaceae, Alliaceae, Araceae, Orchidaceae, Iridaceae, and Liliaceae.[119] These structurally and evolutionarily related lectins, which constitute the monocot lectin superfamily, exhibit strict specificity for Man, unlike other reported Glc/Man-specific dicotyledonous legume lectins as well as the C-type Man binding animal lectins. The specificity is so well-defined for Man that they do not bind to its epimer, Glc. Lectins from three species (*Galanthus nivalis, Allium sativum*, and *Narcissus pseudonarcissus*) of this superfamily have been investigated for their sugar binding properties by ITC.

1. Galanthus nivalis Agglutinin (GNA)

Precipitation and hapten inhibition studies along with affinity chromatography showed that oligosaccharides with terminal Manα(1,3)Man residues and glycopeptides with the same disaccharide units were the most preferable ligands for GNA.[120] ITC studies by Chervenak and Toone[104] confirmed the hapten inhibition data[121] that GNA did not recognize α-glucosides and β-glycosides. The disaccharides maltose and isomaltose did not bind to the lectin. The affinity of Man and MeαMan was very weak as determined by inhibition assays, and their affinities were too low to be determined by ITC. The authors estimated a binding constant of MeαMan to GNA as less than 100 M^{-1}. The association constants of Manα(1,3)Man, Manα(1,6)Man, and trimannoside were 3300, 1200, and 3000 M^{-1}, respectively. The ΔH values of these three ligands were (−3.1, −2.1, and −3.9 kcal/mol, respectively) less negative than the respective values of free energy (−4.8, −4.2, and −4.8 kcal/mol) that indicated favorable entropic effects in the binding. In most examples of carbohydrate−lectin interactions, binding enthalpy is generally more negative than the free energy. Binding of two other disaccharides, Manα(1,2)Man and Manα(1,4)Man, was not detectable by ITC. On the basis of these results, the authors proposed an extended binding site of GNA which was composed of distinct sites for the α(1,3)- and α(1,6)-arms.[104]

The crystal structure of GNA with Manα(1,3)Man shows that axial C2−OH from both Man residues are hydrogen bonded with the lectin (Figure 21). The disaccharide could bind to GNA with either the reducing or nonreducing Man bound in the specificity pocket depending on which site was selected. This binding feature differs from many other lectins which

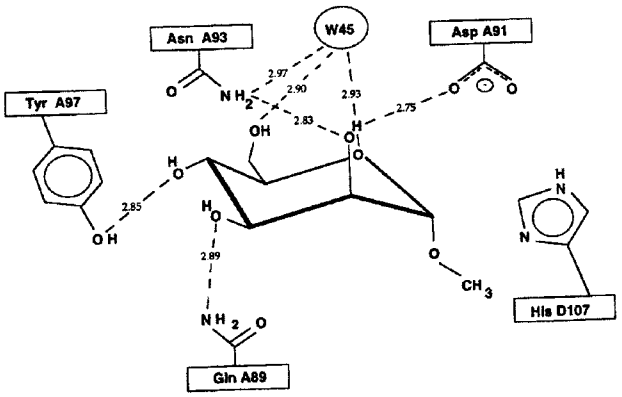


Figure 19. Schematic illustration of the binding site of GNA showing all hydrogen-bond contacts that stabilize MeαMan. Dashed lines represent hydrogen bonds. Residues labeled 'A' or 'D' belong to subunits A and D, respectively, and 'W' designates bound water.[219] (Reprinted with permission from ref 219. Copyright 1995 Nature Publishing Group.)

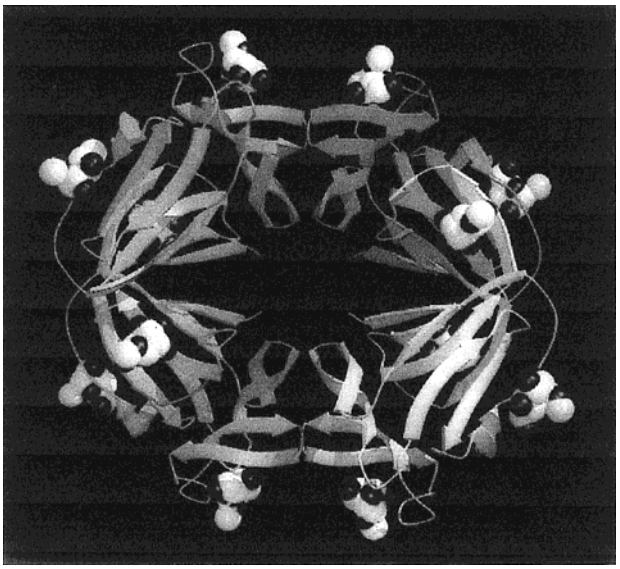


Figure 20. GNA tetramer substituted by MeαMan at all 12 binding sites. The crystallographically-independent monomers are distinguished by different shades.[122] (Reprinted with permission from ref 122. Copyright 1996 Academic Press.)

bind the terminal nonreducing sugar in the specific (primary) binding pocket.[122] Structures of GNA complexed with trimannoside[123] and Manα(1,3)Man[122] showed differential hydrogen bonding of these two saccharides with the lectin (Figures 21 and 22). Compared to Manα(1,3)Man, the trimannoside made more hydrogen bonds and van der Waals contacts with the lectin. These differential contacts were not reflected in the energetics of binding as ITC studies showed that these two ligands had similar association constants and binding enthalpies. A lack of correlation between the number of hydrogen bonds and binding energetics was also found when the binding of Manα(1,3)Man was compared with the binding of Man. Although Man showed more contacts with the lectin (Figure 19) than did Manα(1,3)Man, the affinity of the former was severalfold lower than the disaccharide.[104] This once again reflects the

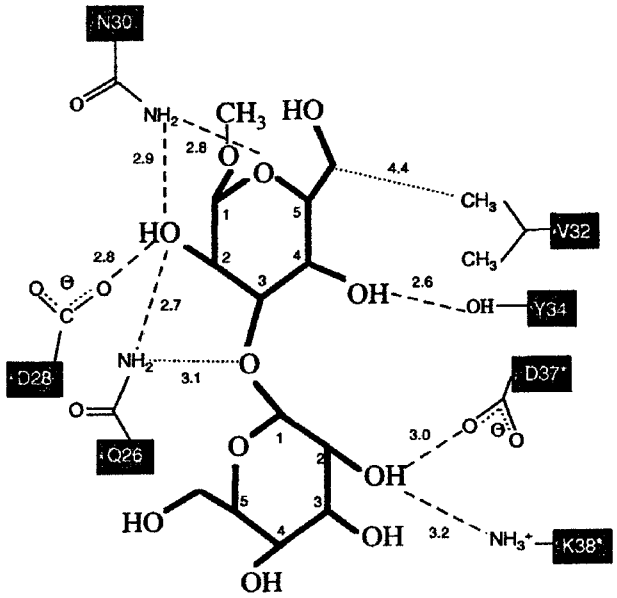


Figure 21. Schematic illustration of the binding interactions between bound Manα(1,3)ManaOMe and site 3 of GNA. Residues D37* and K38* below to the dimer-related subunit. H-Bond contacts are indicated by broken lines and van der Waals contacts by dotted lines.[122] (Reprinted with permission from ref 122. Copyright 1996 Academic Press.)

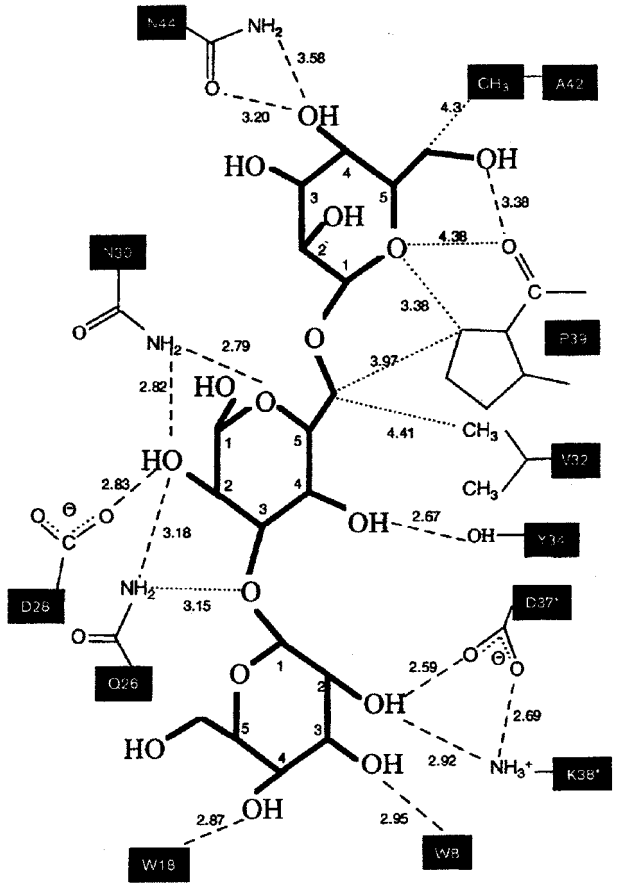


Figure 22. Schematic illustration of all ligand−protein contacts observed for trimannoside bound at the extended CRD3 binding site of GNA. Dashed lines indicate hydrogen bonds and dotted lines van der Waals contacts.[123] (Reprinted with permission from ref 123. Copyright 1996 Elsevier Science.)

nonlinear relationship between binding thermodynamics and number of hydrogen bonds.

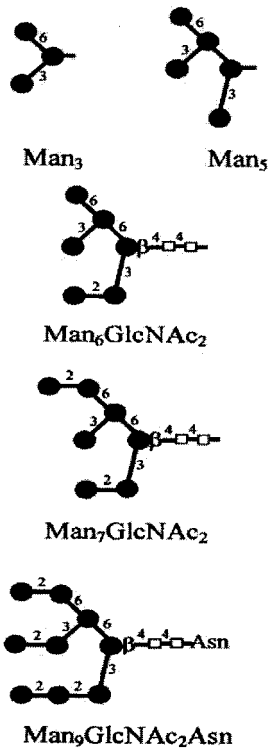


Figure 23. Structures of mannooligosaccharides used for the binding study of ASA by Bachhawat et al.[126] (Reprinted with permission from ref 126. Copyright 2001 American Society for Biochemistry and Molecular Biology.)

2. Allium sativum Agglutinin (ASA)

Among the disaccharides that were tested, Manα-(1,3)Man was the best inhibitor of ASA[124,125] but oligosaccharides and glycoproteins containing α(1,2)-linked Man residues were found to be the most potent ligands of ASA.[125] Using ITC and surface plasmon resonance (SPR), Bachhawat et al.[126] further characterized the carbohydrate binding properties of ASA. The binding constants of trimannoside and Man5 (Figure 23), as determined by ITC, were 144 and 162 M^{-1}, respectively. The K_a of trimannoside for ASA was even less than that of GNA. The values of ΔH for these two ligands were found to be −4.8 and −5.8 kcal/mol, respectively. Unlike the data obtained with GNA, binding enthalpies of all the ligands for ASA were more negative than the respective free energy (ΔG). Extension of Man5 with α(1,2)-linked mannosyl residues, as in $Man_9GlcNAc_2Asn$, resulted in a huge enhancement in the association constant (91×10^4 M^{-1}) and binding enthalpy (−10.3 kcal/mol). The ITC-derived association constants agreed well with those determined by surface plasmon resonance studies. The primary mannose binding site of ASA is shown in Figure 25.

Addition of α(1,2)-linked Man residues on the α-(1,6)- and α(1,3)-arms of Man5, as in $Man_7GlcNAc_2$ (Figure 23), increased the affinity by almost 500 times (determined by SPR). On the other hand, extension of the α(1,3)-arm of Man5 alone, as in $Man_6GlcNAc_2$, showed an adverse effect in binding. This highlighted the crucial role of α(1,2)-linked Man residues on the α(1,6)-arm played in the stronger interaction with ASA. The affinity of $Man_9GlcNAc_2$-Asn, where all the arms were extended with α(1,2)-linked Man residues, was even better than Man_7-$GlcNAc_2$, which suggested that α(1,2)-linked Man residues on other arms were recognized by ASA only after the binding of the α(1,2)-linked Man residue on α(1,6)-arm.

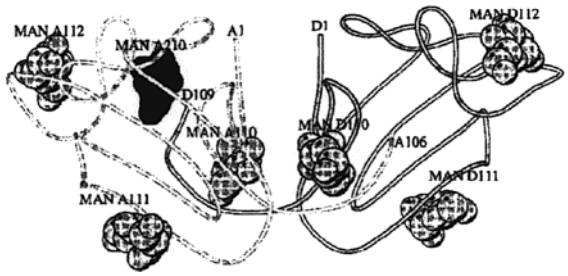


Figure 24. Location of the mannose-binding sites in the AD dimer of ASA. Mannose is shown as CPK (grey) objects and labeled as 110, 111, and 112 in both the subunits. The additional mannose in subunit A is labeled as 210 and is shown in black. Mannose 110 refers to the primary binding site in each subunit.[220] (Reprinted with permission from ref 220. Copyright 1999 Academic Press.)

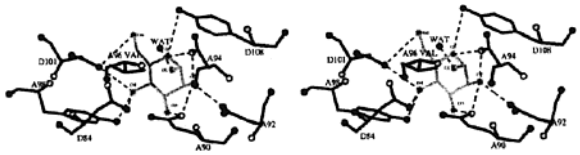


Figure 25. Stereoview of the primary mannose-binding site in subunit A of ASA. Hydrogen bonds are indicated by broken lines. Interacting residues from subunits A and D are labeled appropriately. The mannose molecule is shown in gray.[220] (Reprinted with permission from ref 220. Copyright 1999 Academic Press.)

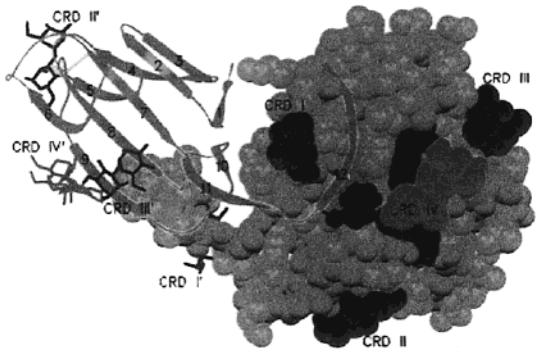


Figure 26. Illustration of the dimer interface of NPL showing molecule A in a ribbon model and molecule D in a space-filling model (light gray). Molecule A demonstrates the triangular *β*-prism motif with its 12th strand fitting in a cleft of molecule D. The three main CRDs (I−III) are occupied by α(1,3) mannobioses (black). CRDIV containing an additional α(1,3) mannobiose.[128] (Reprinted with permission from ref 128. Copyright 1999 Academic Press.)

3. Narcissus pseudonarcissus Lectin (NPL)

The affinities of Manα(1,2)Man and Manα(1,3)Man for NPL determined by inhibition studies were found to be similar, and they were more potent ligands than MeαMan.[127] Among the oligosaccharides tested, the best inhibitor was α(1,6)-linked trimannoside. Binding of bovine fetuin and viral glycoproteins by NPL confirmed that the lectin efficiently recognized oligomannose, complex, and hybrid glycans.[128−130] ITC studies showed that Manα(1,3)Man binding to NPL

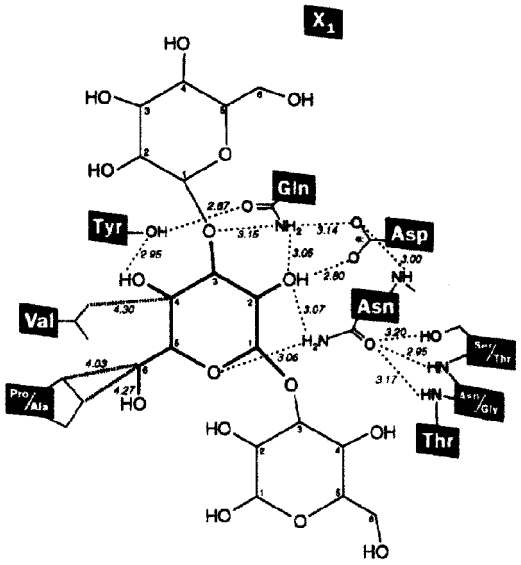


Figure 27. Schematic illustration of the two binding modes of Man(α1,3)Man by NPL, superimposed, showing the mannose residue in the main binding pocket in bold lines. Given distances for H-bond contacts (thin broken lines) and van der Waals contacts (bold broken lines) are average values of all three main CRDs. Residue X_1 of the minor pocket stands for Ser in CRDII and Glu in CRDIII.[128] (Reprinted with permission from ref 128. Copyright 1999 Academic Press.)

is an exothermic process ($\Delta H = -4$ kcal/mol) with an association constant of 500 M^{-1}.[128] In NPL, the specific binding pocket could bind either the reducing or the nonreducing Man residue of Manα(1,3)Man (Figure 27).

ITC studies with GNA, ASA, and NPL demonstrated that the affinities of these lectins for mono- and disaccharides were remarkably low compared to other Man binding plant lectins. This suggests that larger multivalent oligosaccharides with defined structures are the natural ligands of the lectins of this superfamily. This view is supported by the fact that an increasing number of Man residues in the oligosaccharide with appropriate linkage were necessary for the enhancement of affinity and specificity in ASA.[126] One of the most distinct features of the monocot Man binding lectin super family is their higher number of carbohydrate binding sites. In contrast to two or four binding sites per molecule found in most other reported lectins, GNA, ASA, and NPL contain 12, 7, and 16 potential binding sites, respectively, per lectin molecule (Figures 20, 24, and 26). Availability of such a large number of sites allows a large number of multivalent interactions to occur.

C. Other Lectins

1. Artocarpin

Artocarpin, a Man-specific nonglycosylated lectin isolated from jack fruit (*Artocarpus integrifolia*) seeds, is a homotetrameric protein (M_r 65 000) with one binding site per subunit. Artocarpin is of considerable interest because of its potent mitogenic effect on B-cells.

The order of binding affinity of artocarpin, as determined by ITC,[131] is as follows: trimannoside (**1** of Figure 8) > Manα(1,3)Man > $GlcNAc_2$ Man_3 (**14** of Figure 8) > MeαMan > Man > Manα(1,6)Man > Manα(1,2)Man > MeαGlc > Glc. The ΔH values for the interaction of Manα(1,3)Man, Manα(1,6)Man, and MeαMan are similar and ~5 kcal/mol lower than that of trimannoside (**1**). This indicates that while Manα(1,3)Man and Manα(1,6)Man interact with the lectin exclusively through their nonreducing end monosaccharide with the subsites specific for the α-(1,3)- and α(1,6)-arms, the trimannoside interacts with the lectin simultaneously through all three of its mannopyranosyl residues. From further ITC studies[132] with trimannoside and its monodeoxy as well as Glc and Gal analogues (Figure 8), the authors conclude that 2-, 3-, 4-, and 6-hydroxyl groups of the α(1,3) Man and α(1,6) Man residues and the 2- and 4-OH groups of the central Man residue are involved in binding. α(1,3) Man is the primary contributor to the binding affinity, unlike other Man/Glc binding lectins which exhibit a preference for α(1,6) Man. The free energy and enthalpy contributions to binding of individual hydroxyl groups of the trimannoside estimated from the corresponding monodeoxy analogues show nonlinearity, suggesting differential contributions of the solvent and protein to the thermodynamics of binding of the analogues.

2. Banana Lectin

The banana lectin (*Musa acuminata*) is composed of four identical subunits of 15 kDa. ITC studies with several mono- and oligosaccharides[133,134] showed the following association constants: 3.33×10^2 M^{-1} (MeαMan), 3.65×10^2 M^{-1} [Galα(1,3)ManαOMe], 3.72×10^2 M^{-1} [Meα(1,3)Man], 5.1×10^2 M^{-1} [MeβFruc*p*], 5.43×10^2 M^{-1} [Glcα(1,2)Glc], and 8.3×10^2 M^{-1} [Glcβ(1,3)Glc].

IV. Galactose Binding Plant Lectins

A. Soybean Agglutinin

The soybean agglutinin (SBA) from *Glycine max* is a tetrameric GalNAc/Gal-specific glycoprotein of M_r 120 kDa.[135] Each SBA subunit contains one sugar binding site as well as one Mn^{2+} and one Ca^{2+} site as observed in all legume lectins.[3] SBA is known to be mitogenic toward lymphocytes[136] and to localize carbohydrate receptors on the surface of normal and transformed cells.[137]

ITC experiments[33] confirmed that SBA possesses 20- and 50-fold higher affinity for GalNAc and MeβGalNAc, respectively, compared to MeβGal, as previously determined by other techniques.[138,139] Comparison of the methyl β-anomers of the two monosaccharides showed that MeβGalNAc possessed a ΔH of −13.9 kcal/mol compared to −10.6 kcal/mol for MeβGal. The larger $-\Delta H$ for MeβGalNAc is consistent with greater binding of the acetamido group of MeβGalNAc relative to the hydroxyl group at C-2 in MeβGal. The X-ray crystal structure of SBA cross-linked with a biantennary pentasaccharide possessing terminal LacNAc residues suggested that replacement of the bound Gal moiety in LacNAc by GalNAc would allow the *N*-acetyl group of the latter

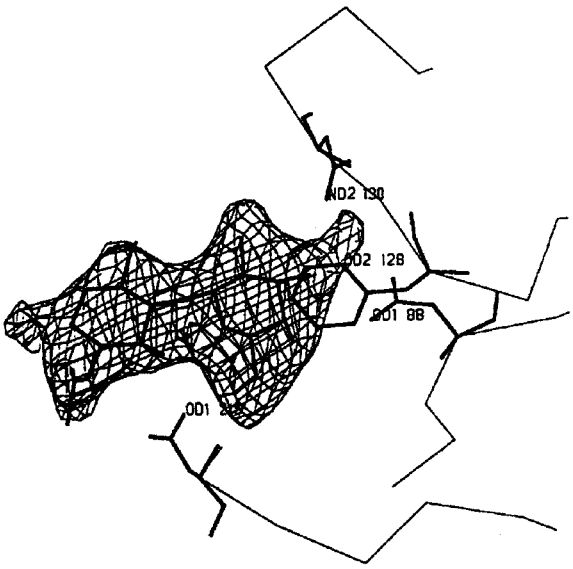


Figure 28. Four amino acid residues of SBA, namely, Asp 215, Asp 88, Asn 130, and Phe 128, were found in contact with the carbohydrate ligand, (β-LacNAc)$_2$Gal-β-R, where R is $-O(CH_2)_5COOCH_3$. (Reprinted with permission from ref 140. Copyright 1995 American Chemical Society.)

to form a hydrogen bond with the side chain of Asp 88.[140] Interestingly, the affinity of MeβGalNAc for SBA was 3-fold greater than GalNAc, and MeβGalNAc possessed 4.4 kcal/mol more negative ΔH, indicating either the involvement of the β-methyl group of MeβGalNAc in binding or the negative effects of a free anomeric hydroxyl group of GalNAc. Binding of GalNAc was entropically more favorable ($T\Delta S = -4.0$ kcal/mol) than that of MeβGalNAc ($T\Delta S = -7.9$ kcal/mol).

The ΔH values of lactose (-5.5 kcal/mol), LacNAc (-8.2 kcal/mol), and MeβLacNAc (-7.5 kcal/mol) were less negative than that of MeβGal (-10.6 kcal/mol), even though the affinities of the disaccharides were nearly the same as the monosaccharide. The presence of the acetamido group in LacNAc and MeβLacNAc increased their ΔH values relative to lactose, indicating the involvement of the acetamido group in binding. The X-ray crystal structure of SBA (Figure 28) shows binding of the acetamido nitrogen of the GlcNAc residue of LacNAc via a hydrogen bond to Asp 215 of the lectin.[140] The X-ray crystallographic data also showed binding of the 3-, 4-, and 6-hydroxyl groups of the terminal Gal residue of LacNAc to SBA, consistent with the dominant role of the Gal moiety of LacNAc in binding to the lectin.

B. Lectins from Erythrina spp.

Lectins from *Erythrina corallodendron* (ECorL), *Erythrina cristagalli* (ECL), and *Erythrina indica* (EIL) are dimeric proteins that possess comparable physicochemical and carbohydrate binding properties.[141,142] The molecular masses of ECL and ECorL are 56 and 68 kDa for EIL. All three are Gal-specific lectins with one carbohydrate binding site per monomer. LacNAc among simple oligosaccharides binds to all three lectins with the highest affinity.[142]

Gupta et al.[33] reported ITC data indicating ECorL and ECL possess similar thermodynamic binding data for the saccharides tested. For example, ECorL bound MeβGal with a ΔH of -4.4 kcal/mol and a $K_a = 0.43 \times 10^3$ M^{-1}, while ECL bound to the monosaccharide with a ΔH of -4.7 kcal/mol and a $K_a = 0.88 \times 10^3$ M^{-1}. Both ECorL and ECL possessed approximately 3−4-fold higher affinities and slightly greater $-\Delta H$ values for MeβGalNAc as compared to MeβGal, suggesting binding of the acetamido group of the former. The thermodynamic binding data for GalNAc and MeβGalNAc were similar for both lectins, which indicated that the β-anomeric methyl group of MeβGalNAc was not involved in binding. ECorL and ECL possessed slightly higher affinities (3−4-fold, respectively) and somewhat greater $-\Delta H$ values ($\Delta\Delta H$ of -1.3 to -2.3 kcal/mol, respectively) for lactose as compared to MeβGal. The affinity constants of ECorL and EIL for LacNAc were greater (6- to 10-fold, respectively) than for MeβGal, and LacNAc possesses an even greater $-\Delta H$ ($\Delta\Delta H = -6.9$ and -6.2 kcal/mol, respectively) than the monosaccharide. Thus, the data suggest extended site binding interactions of the two lectins with both the Gal and GlcNAc residues of LacNAc. The thermodynamic values for LacNAc and MeβLacNAc are similar, indicating little sensitivity to binding of the β-anomeric methyl group of the latter.

The X-ray crystal structure of lactose bound to ECorL has been determined at 2 Å resolution.[143] The structure shows that the combining site of ECorL is a shallow depression on the protein surface that binds to the 3-, 4-, and 6-hydroxyls of the Gal moiety of lactose. An open space in the binding site was observed close to the 2-OH of the bound Gal residue which could accommodate the acetamido group of GalNAc, as suggested by the thermodynamic data. The crystal structure also showed that the Glc residue of lactose resided mostly outside the binding pocket and was poorly defined in the electron density map, indicating its flexibility in the bound complex and lack of strong interactions. This was in agreement with the relatively small differences in the thermodynamic data for MeβGal and lactose. However, the thermodynamic data also indicated that the GlcNAc moiety of LacNAc contributes to binding.

An ITC study of the binding of mono- and oligosaccharides to ECorL was reported by Surolia and coworkers.[144] Their findings are in good agreement with those obtained by Gupta et al.,[33] except for a larger $-\Delta H$ value for lactose. The Surolia laboratory[144] also reported that the highest affinity of ECorL was for Me α-*N*-dansylgalactosaminide. Most of the carbohydrates that bind to ECorL were observed to be enthalpically driven with the exception of Fuc, Me α-*N*-dansylgalactosaminide, and Gal. 2′-Fucosyllactose was observed to possess enhanced affinity relative to lactose, which was associated with a more favorable $T\Delta S$ contribution. The entropically driven binding of Me α-*N*-dansylgalactosaminide to ECorL was suggested to be due to enhanced nonpolar contacts between the aromatic dansyl moiety at C2 of the ligand and Trp-135 in the binding site of ECorL. The nonpolar interaction of Me α-*N*-dansylgalactosaminide with ECorL was suggested to be also evident by its change in negative heat capacity. The

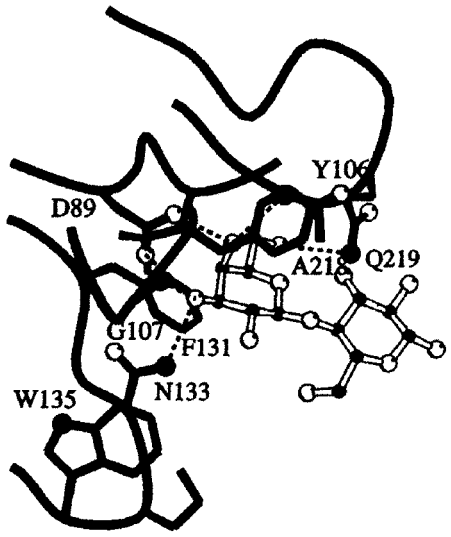


Figure 29. Combining site of ECorL -lactose complex. Hydrogen bonds between the galactose and the side chain of D89, N133, and Q219 and of the main chain amides of G107 and A218 displayed on the basis of the structure by Shaanan et al.[143] (Reprinted with permission from ref 144. Copyright 1996 American Society for Biochemistry and Molecular Biology.)

$-\Delta H$ values for GalNAc, MeαGal, and MeβGal were greater than that of Gal.[144] A similar increase in ΔH for ConA was observed when the C1 hydroxyls of Man and Glc were converted to the methyl α-anomers.[91] In this case, the α-anomers of Man and Glc are known to bind to ConA with much higher affinities than the β-anomers.[3]

From X-ray crystallographic studies,[145] the structural basis of the entropically driven binding of Gal to ECroL was explained in the following way. The increase of entropy (more positive) upon Gal binding is due to release of the tightly bound water molecules 589 and 609 (Figure 30), which might still not be fully compensated by opposing factors contributing to decrease in entropy. Thus, Gln219 underwent only a small reduction in mobility in ECorL-Gal complex. Furthermore, the number of detectable water molecules in the combining site of ECorL for Gal was about the same as that in free ECorL, which suggested that there might not be an apparent increase in the ordering of water molecules in ECorL-Gal complex compared to the unliganded state. It should be pointed out that the mobility of the side chains of Asp89 and Asn133, which also were involved in direct hydrogen bonds with the ligand, hardly changed between the unliganded lectin and the complexes. In all the models, the temperature factors of the Asp89 and Asn133 side chains were, on average, 7.5 and 5 Å^2, respectively, below the overall temperature factor of the model, which probably reflected the fact that these side chains were anchored by the neighboring calcium ion, either directly (Asn133) or through a water molecule (Asp89).[145] Thus, freezing of these two side chains might not contribute significantly to the configurational entropy of binding to ECorL.

The $-\Delta H$ values of ECorL binding to disaccharides almost doubles compared to monosaccharides.[144] This increase is not apparently supported by the crystal structure (Figure 29),[143] where the reducing end pyranoside ring projects out of the pocket into the solvent. Although inspection of the structure of the ECorL complex indicates the presence of an additional possible hydrogen bond between the glucopyranoside ring and Gln-219,[146] it would appear to be insufficient to double the binding enthalpy. The crystal structure of the lactose−ECorL complex shows three localized water molecules between the glucopyranoside ring and the surface surrounding the binding cavity. The authors suggested thst a possible network of hydrogen bonds involving these water molecules between the glucopyranoside moiety of lactose and the surface of ECorL could account for the enhanced binding enthalpies. According to Elgavish and Shaanan,[145] the binding enthalpy increases with the amount of area buried upon complex formation. Apart from the increase in buried area, the rise in binding enthalpy upon moving from monosaccharide to disaccharide complexes correlates with the addition of direct hydrogen bonds between the protein and the ligand (Figure 30). Another source of increased binding enthalpy for the disaccharides may be greater van der Waals interactions with the protein.

Bradbrook et al.[147] performed molecular dynamics (MD) simulations of ECorL binding to α-Gal and LacNAc to investigate the relationship between structure and thermodynamics.

The lectin from *Erythrina indica* (EIL) exhibits somewhat different thermodynamic binding data relative to ECorL and EIL.[33] While ECorL and ECL show enhanced $-\Delta H$ values for GalNAc and MeβGalNAc relative to MeβGal, EIL demonstrates little change in $-\Delta H$ for all three saccharides. Furthermore, EIL exhibits $-\Delta H$ values for lactose and LacNAc of −10.4 and −13.7 kcal/mol, respectively, as compared to −6.3 and −10.9 kcal/mol, respectively, for ECorL, and −6.0 and −10.9 kcal/mol, respectively, for ECL. However, the affinity of lactose for all three lectins is nearly the same, as is the case with LacNAc, even though the $-\Delta H$ values of EIL for lactose and LacNAc are greater than the other two lectins. Previous equilibrium dialysis studies with EIL and lactose resulted in a $K_a = 2.2 \times 10^3$ M^{-1} at 25 °C, a Van't Hoff ΔH value of −9.4 kcal/mol, and $T\Delta S$ of −4.9 kcal/mol,[141] which are values consistent with the ITC results.

C. Winged Bean Agglutinins

The seed of the legume winged bean (*Psophocarpus tetragonolobus*) contains two different lectins, namely, WBA I and WBA II. WBA I is a homodimeric protein with an isoelectric point of 10 that recognizes D-galactopyranosides and binds most strongly to A-pentasaccharide.[148] The 29 kDa subunit contains a single binding site. WBA II is a homodimeric glycoprotein (pI 5.5) of 54 kDa which binds to the terminal fucosylated H-antigenic determinant either on human erythrocytes or in solution.[149,150]

1. WBA I

Schwarz and co-workers[151] reported an ITC binding study of WBA I with Gal, GalNAc, MeαGal, and MeβGal. The reported binding constants ranged from

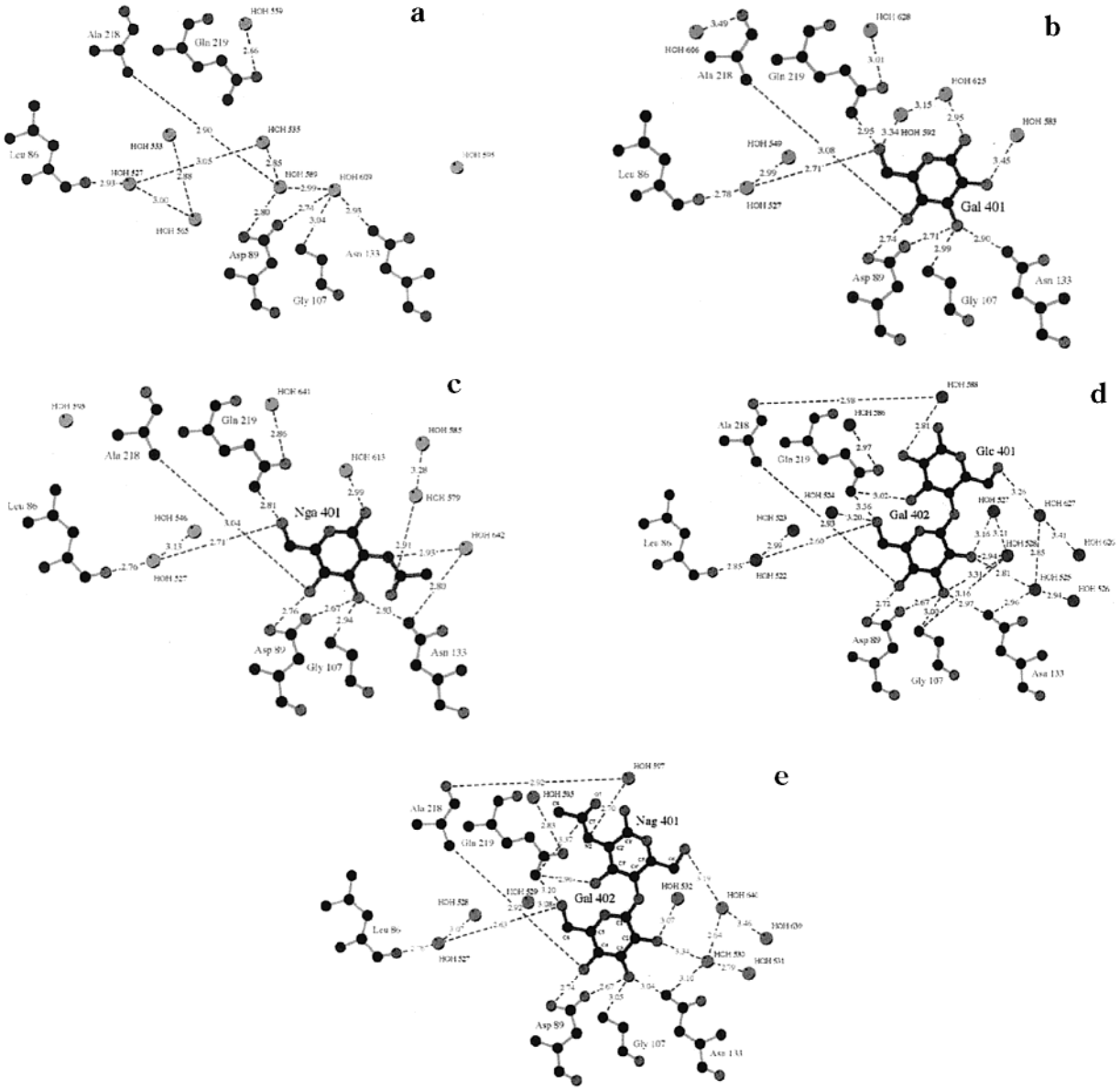


Figure 30. Schematic two-dimensional diagram of the combining sites of unligated ECorL (a) and its complexes with galactose (b) (only the β-anomer of galactose is depicted), GalNAc (c), lactose (d), and LacNAc (e). Broken lines represent hydrogen bond. HOH are water molecules.[145] (Reprinted with permission from ref 145. Copyright 1998 Academic Press.)

0.56×10^3 M^{-1} for Gal to 7.2×10^3 M^{-1} for GalNAc. The lowest binding enthalpy of −3.4 kcal/mol was obtained with MeβGal, while GalNAc produced the highest binding enthalpy of −6.7 kcal/mol. The binding reactions were essentially enthalpically driven and for the most part occurred with little change in the heat capacity.

The thermodynamics of WBA I binding to deoxy, fluorodeoxy, and methoxy derivatives of Gal were determined to assess the hydrogen bond donor−acceptor relationship between the hydroxyl groups of Gal and amino acid residues of the combining site of the lectin.[152] The binding enthalpies for various derivatives were independent of temperature and showed complementary changes with respect to binding entropies. Replacement of the hydroxyl group by fluorine or hydrogen on C3 and C4 of Gal showed that hydroxyl groups at these two positions were indispensable for binding. However, 4-methoxygalactose was found to retain its binding affinity for WBA I and jacalin, another Gal-specific lectin. The authors considered this result as an evidence of plasticity in the loci corresponding to the axially oriented C-4 hydroxyl group of Gal within the primary binding site of these two lectins.[153] The affinity for C2 derivatives of Gal decreased in the order GalNAc > 2MeOGal > 2FGal = Gal > 2HGal, which suggested that both polar and nonpolar residues surrounded the C2 locus of Gal, 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 followed the order Gal > 6FGal > D-Fuc ≫ 6MeOGal = L-Ara, indicating the presence of favorable polar interactions with a hydrogen-bond donor in the vicinity. On the basis of the results obtained with various analogues of Gal, the authors proposed a model of the combining site of WBA I complexed with Me α-Gal (Figure 31).[152]

It is known that the most lectins preferentially bind Gal moieties possessing α-linkages. As illustrated in

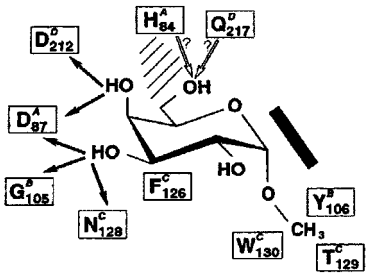


Figure 31. Schematic representation of the combining site of WBA I complexed with methyl α-D-galactopyranoside. The residues of WBA I participating in sugar binding are enclosed in rectangular boxes and are denoted by a one-letter abbreviation, subscripts refer to their positions in the sequence of the protein, while superscripts refer to the loop that it belongs to. F_{126} C is involved in stacking interactions with the hydrophobic face (*β*-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 (solid bar); van der Waals interaction (/////); hydrogen-bond-donating sugar hydroxyl group (solid arrow); hydrogen-bond-accepting sugar hydroxyl group (hollow arrow). 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.[152] (Reprinted with permission from ref 152. Copyright 1997 American Chemical Society.)

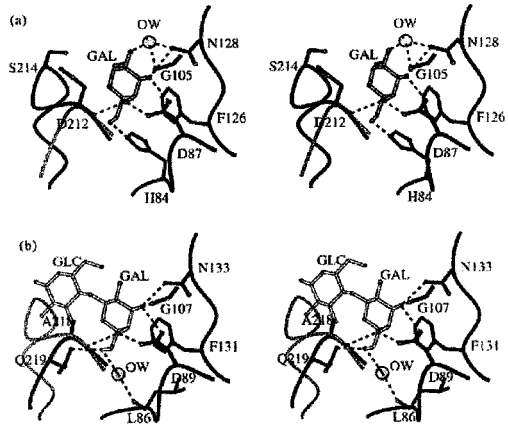


Figure 32. (a) Stereodiagram of WBAI−sugar interactions where the dotted lines represent hydrogen bonds. Loop 4 of ECorL (shown in gray) is superimposed on that of WBAI to illustrate its additional length in the latter. GAL and OW represent methyl α-D-galactose and a water molecule, respectively. (b) Stereodiagram of ECorL-lactose interactions. Loop 4 of WBAI (grey) is also shown. GAL and GLC represent the galactose and glucose residues of lactose and OW a water molecule.[154] (Reprinted with permission from ref 154. Copyright 1998 Academic Press.)

Figure 32,[154] a *β*-linkage, as in lactose and methyl *β*-Gal, leads to unacceptable steric contact with the long fourth loop of the protein, particularly Ser214 and Gly215, while an α-anomeric linkage is sterically acceptable. The crystal structure of WBA I provides a ready explanation for the increased affinities for Gal with substitutions at the C2-position such as *N*-acetylgalactosamine. Simple modeling shows that the *N*-acetyl group at the C2-position of the sugar nestles in a pocket made up of Tyr106 and Trp130. Furthermore, O7 of the acetyl group could make a hydrogen bond, with distance varying between 3.5 and 3.7 Å, with Ser214 OG belonging to the long fourth loop (Figure 32).[154]

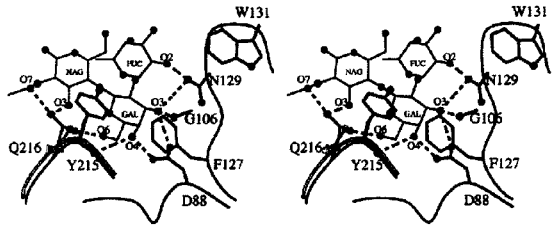


Figure 33. Stereoview of the interactions of H-type II trisaccharide in with protein atoms in WBAII. Loop D of ECorL is shown in gray.[157] (Reprinted with permission from ref 157. Copyright 2000 Academic Press.)

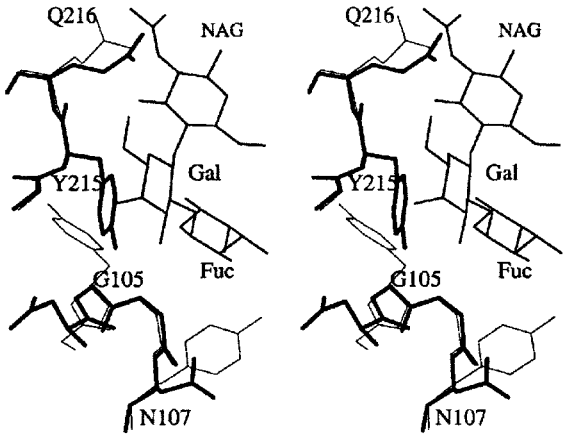


Figure 34. Stereoview of the superposition of loop B and loop D of WBAII (thick lines) and ECorL (thin lines). The modeled trisaccharide is also shown.[157] (Reprinted with permission from ref 157. Copyright 2000 Academic Press.)

2. WBA II

Binding of Fucα(1,2)Gal*β*(1,4)GlcNAc−OMe (H-type-II−OMe sugar) (Figure 35) and a series of deoxy and OMe derivatives to WBA II was examined by Srinivas et al.[155] The K_a and ΔH values of the H-type-II−OMe sugar were slightly greater than those of fucosyllactose indicating the contribution of the acetamido group in binding. The 85-fold greater affinity of the H-type-II−OMe determinant over 2-fucosyl-galactose (H-disaccharide) suggested extended site interaction with the GlcNAc residue[156] (Figure 35). The hydrogen bonds with Gln216 and the van der Waals and hydrophobic interactions with Tyr215 involving GlcNAc in the model presented by Manoj et al.[157] is in qualitative agreement with this conclusion (Figure 33). Moreover, 3b-, 4b-, 6b-, and 2c-deoxy and OMe analogues showed very reduced binding to WBA II, which suggested that the hydroxyl groups at positions 3, 4, and 6 of the galactosyl moiety and the 2-hydroxyl of the fucosyl moiety might provide the primary hydrogen-bonding interactions in the binding. The 2-deoxy fucosyl congener binds with 10-fold reduced binding affinity, while binding is altogether abolished in the corresponding 2-methoxy congener. The model (Figure 33) shows that the hydrogen bond with the fucosyl residue is lost with the 2′-deoxy analogue while a methoxy group at the same position has severe steric contacts with Trp131.[157] Differential affinities of 3a-deoxy analogue and its OMe counterpart highlight the possibility of steric hindrance by OMe group.

Binding of the fucosyl moiety, with the exception of its interaction at the 2-OH, was considered hydro-

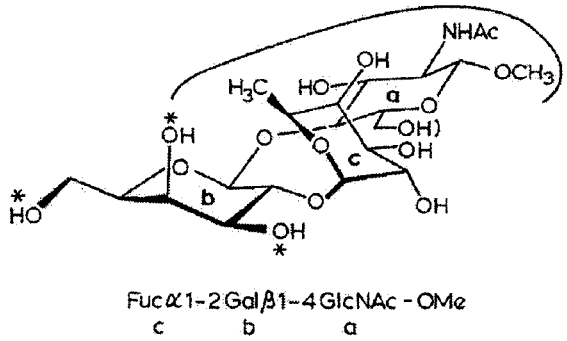


Figure 35. Structure of H-type-II−OMe sugar. The asterisks show the hydroxyl groups that may be involved in direct hydrogen bonding with WBA II, while the continuous line depicts the surface of the saccharide molecule being recognized hydrophobically.[155] (Reprinted with permission from ref 155. Copyright 1999 FEBS Society.)

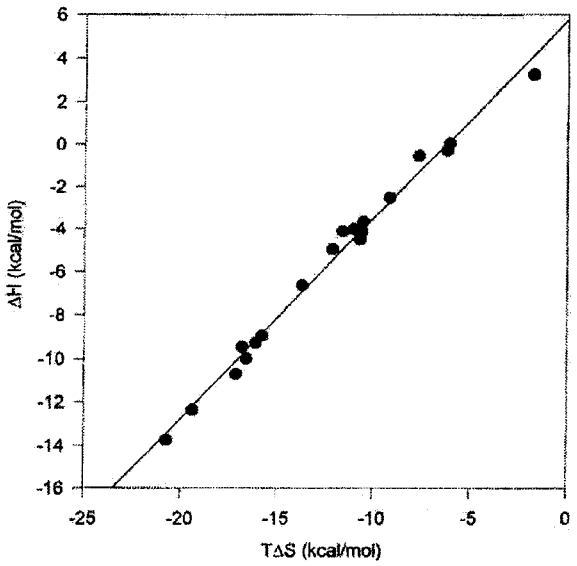


Figure 36. Enthalpy−entropy compensation plot of WBA II−sugar interactions. The slope of this plot is 0.90, and the correlation coefficient is 0.98.[155] (Reprinted with permission from ref 155. Copyright 1999 FEBS Society.)

phobic in nature.[155] The slope of the enthalpy−entropy compensation plot derived from the thermodynamic binding data of H-type-II−OMe sugar and its various analogues to WBA II was 0.9 (Figure 36). The authors concluded that the binding events were dominated by entropic terms and that the energetics of binding of WBA II to fucosylated saccharides was relatively more hydrophobically driven. The enthalpies of binding showed a distinct change with temperature. A significant change in the heat capacity (−150 to −300 cal/mol/K) upon binding of a H-type-II−OMe sugar and some of its analogues was reported.

The model of the complex of WBA II and H-type-II−OMe[157] provides a rationale for the unique binding specificity of the lectin. The prominent role of Tyr215 in generating its specificity becomes evident when the sugar binding site of WBA II is compared with those of ECorL[143,145] and WBAI.[154,158] The H-type-II−OMe oligosaccharide cannot bind to WBA I due to a steric clash of the GlcNAc residue with large loop D of the lectin. Also, the lectin does not have a pocket for the fucosyl residue. Though the sugar binding sites of WBAII and ECorL are very similar, there are important differences in side chains (Figure 34). The major difference is Tyr215 in WBA II which is an Ala in ECorL. When the H-type-II oligosaccharide is docked into the two binding sites and minimized, the sugar buries 61 Å^2 of the hydrophobic surface area of the Tyr215 in WBAII while the hydrophobic surface area of the Ala buried in ECorL is 37 Å^2. The relative affinity of 2-fucosyllactose to ECorL is four times that of Gal.[144] The corresponding number is 500 for WBA II. There is a tyrosyl residue in ECorL at the position corresponding to Gly105 in WBAII, but this aromatic ring does not interact with the sugar molecule. Thus, the presence of a tyrosyl residue at 215 and the possibility of a water bridge involving Asn107 provide a rationale for the enhancement in affinity of WBAII for the H-type-II-fucosylated oligosaccharide.[157]

D. *Ricinus communis* Agglutinin (RCA)

Ricin and *Ricinus communis* agglutinin (RCA), two Gal-specific lectins, are present in the seeds of *Ricinus communis*, the castor bean plant. Ricin, a potent inhibitor of protein synthesis in eukaryotic cells, is a 60 kDa disulfide-linked As-sB-type heterodimeric protein, the A-chain of which is an RNA *N*-glycosidase while the B-chain is a Gal-specific lectin.[159] RCA, on the other hand, is a 120 kDa tetramer consisting of two As-sB-type dimers which associate noncovalently. Both natural and recombinant A-chain of RCA inhibit protein synthesis in cell-free systems. Although the native agglutinin exhibits a strong hemagglutinating activity in comparison with ricin and also binds to other eukaryotic cells, it does not inhibit cellular protein synthesis.[160]

ITC studies by Sharma et al.[161] show that each molecule of tetrameric RCA has two equivalent and noninteracting binding sites. The ΔH values for binding of these sugars range from −5.2 kcal/mol for MumbβGal to −12 kcal/mol for ThiodiGal. The binding interactions are largely enthalpically driven. The enthalpy of binding of the various sugars does not vary significantly with temperature, indicating that the $\Delta C_p = 0$ which argues against solvent rearrangement. However, the authors commented that the insignificant change in heat capacities, together with the observation of enthalpy−entropy compensation, suggested that rearrangement of water molecules played an important role in the binding of sugars to RCA.

The binding constants for different sugars range from 2.2×10^3 M^{-1} for Gal to 4.84×10^4 M^{-1} for LacNAc. The order of binding affinity is LacNAc > lactose > Thiodigal > MumbβGal > MeβGal > MumbβGalNac > MeαGal > MumbαGal > Gal. In addition, ITC data show that the enthalpy of binding, ΔH, for lactose, LacNAc and ThiodiGal is higher than the value observed for the corresponding monosaccharide, MeβGal, by −2.7, −1.1, and −6.8 kcal/mol, respectively. This indicates that the second hexapyranoside of these saccharides binds to a site adjacent to the Gal binding site. Although ThiodiGal differs from lactose in several respects including the linkage, their overall topographies are strikingly similar. This explains the preferential binding of Thiodigal over MeβGal. The binding of LacNac is slightly entropically favored compared with that of lactose. This

suggests that the acetamido group of the reducing end sugar GlcNAc in LacNAc may be involved in nonpolar interactions in the combining site of RCA. Similarly to LacNAc, the methyl group of MeβGal is involved in a more favorable interaction than the methyl group of MeαGal in the binding pocket of RCA. MumbβGal is a better ligand than MumbαGal, which suggests that the bulky 4-methylumbelliferyl group may be accommodated in a hydrophobic pocket in the combining site of the lectin. Lack of such an interaction for this group in the α-configuration may account for the poor binding of 4-MumbαGal compared with the β-anomer.[161]

The ITC data show that each native (As-sB)$_2$-type tetrameric RCA molecule has two identical and independent sugar-binding sites. Each B-chain in a 120 kDa RCA binds to only one mono- or disaccharide. Earlier studies using batch calorimetry indicated the presence of more than one binding site on RCA for lactose with different thermal stabilities.[162] Studies on binding of simple sugars to the agglutinin using equilibrium dialysis[163] and fluorescence polarization[164] have shown that each molecule of tetrameric RCA possesses two identical and independent sugar-binding sites. In contrast, using the same techniques, Houston and Dooley[165] concluded that each B-chain of RCA has two identical and independent sugar-binding sites, i.e., there are a total of four sugar-binding sites on the tetrameric agglutinin. Lord and co-workers[166] addressed this problem using site-directed mutagenesis, which led them to conclude that there are no more than two binding sites on each molecule of the agglutinin.

E. Abrin II

Abrin II, a type II ribosome-inactivating protein (RIP) from *Abrus precatorius* seeds, consists of two subunits, the A-subunit (M_r 30 000) and the B-subunit (M_r 33 000), which are connected via a single disulfide bond. The A-subunit is an *N*-glycosidase and inactivates eukaryotic protein synthesis by cleaving the base adenine-4324 from the 28 S rRNA.[167] The B-subunit is a lectin, which binds to the cell-surface receptor that contains terminal Gal residues, thus facilitating the entry of the toxic A-subunit into the cell.[167−169] A thermodynamic binding study of abrin II showed that two lactose molecules bind to one molecule of abrin II with an association constant of 2.98×10^3 M^{-1} and a ΔH of -8 kcal/mol.[170]

F. Peanut Agglutinin

Peanut (*Arachis hypogea*) agglutinin (PNA) is a homotetrameric nonglycosylated lectin. Reddy et al.[171] reported the K_a values of PNA with lactose (1.99×10^3 M^{-1}), MeαGal (3.08×10^3 M^{-1}), MeβGal (1.87×10^3 M^{-1}), and T-antigen (Galβ(1,3) GalNAc) (23.7×10^3 M^{-1}). The X-ray crystal structures of PNA complexed with lactose and T-antigen showed identical direct contacts between the lectin and the ligands. However, in the PNA-T-antigen complex, two additional water bridges between the O atom of the acetamido group of the sugar and the lectin were found (Figure 37) which were implicated for the higher affinity of T-antigen compared to lactose.[172]

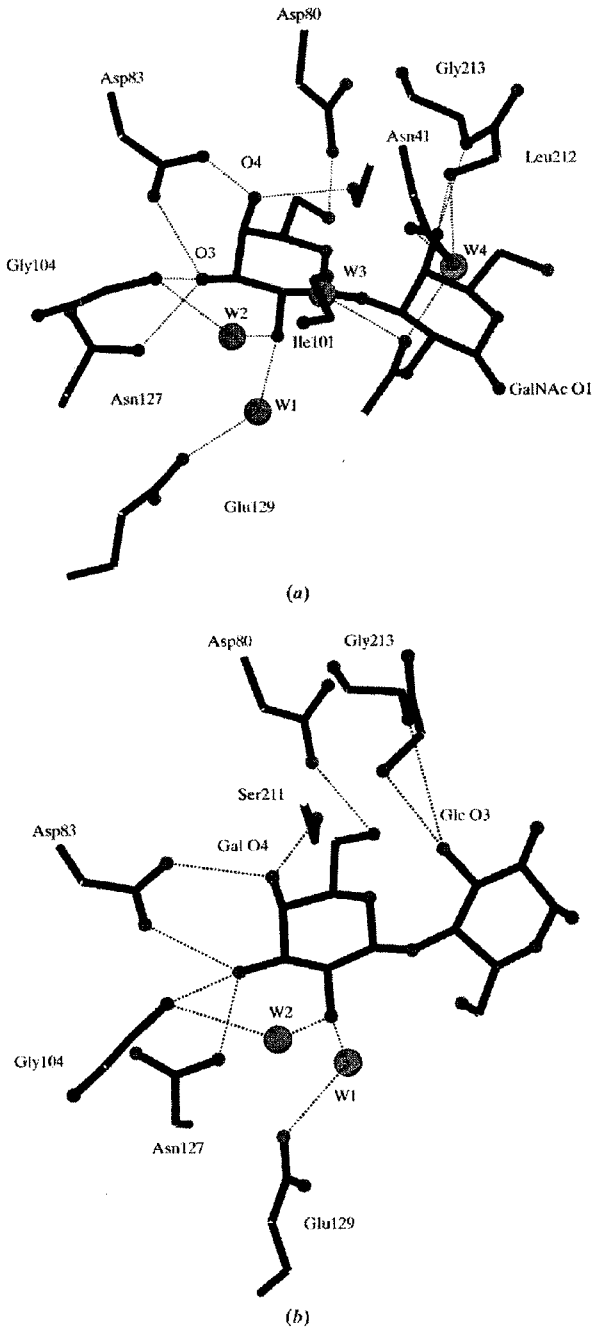


Figure 37. Interactions of peanut lectin (PNA) with (a) T-antigen and (b) lactose.[172] (Reprinted with permission from ref 172. Copyright 2001 International Union of Crystallography.)

V. Solvent Effects in the Thermodynamics of Binding

The direct involvement of solvent water is particularly important in protein−carbohydrate interactions. The importance of solvation can be demonstrated by comparing binding interactions in D_2O and H_2O or in terms of the effect of osmotic stress on the differential uptake of water molecules,[9] as presented below.

A. Studies with Deoxy and Other Sugars

The effects of solvation in carbohydrate binding have been studied with ConA and DGL.[173] Binding

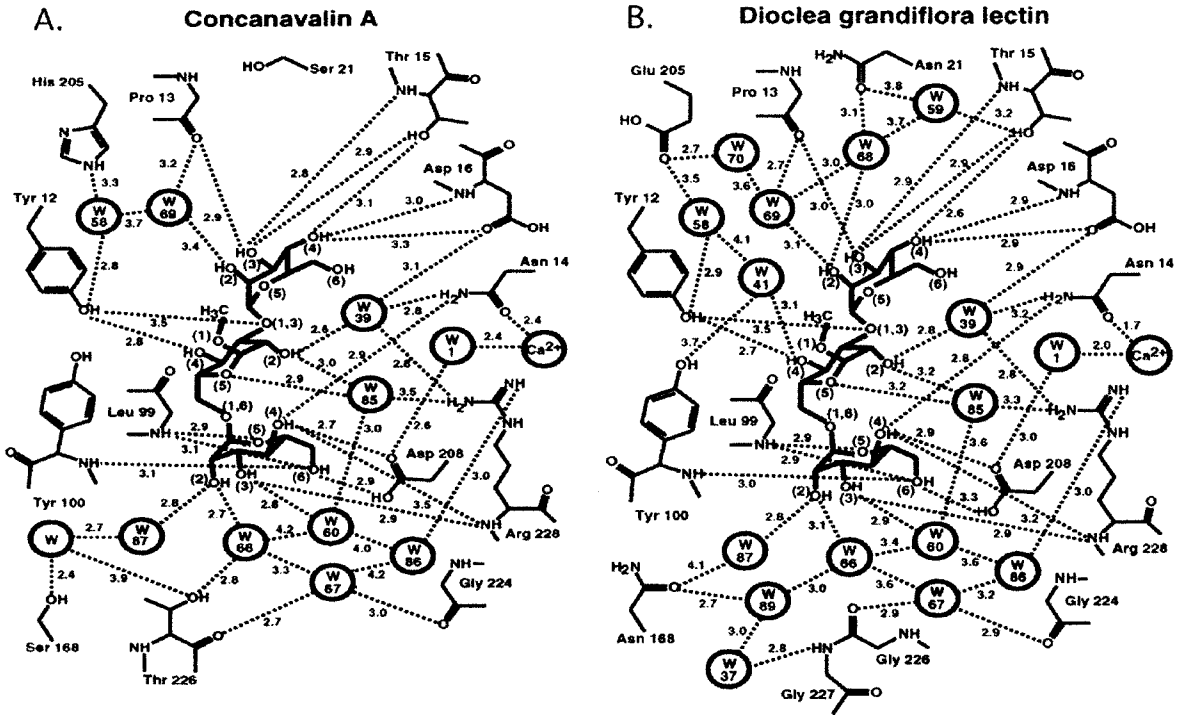


Figure 38. Schematic representation of the hydrogen-bond interactions between the trimannoside ligand and the surrounding amino acid residues and ordered water molecules in ConA and in DGL. The dashed lines represent hydrogen bonds, and the distances are labeled in Å. Water 39 has a strong electron density, is held by the side chains of Asn 14, Asp 16, and Arg 228, and makes a direct hydrogen bond with the trimannoside ligand, and its position is strictly conserved between DGL and ConA. The remaining ordered water molecules possess weaker electron density, which may indicate a decrease in binding strength between themselves and the protein.[173] (Reprinted with permission from ref 173. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

of trimannoside (**1**) and its deoxy analogues **2**−**11** (Figure 8) to ConA and DGL in hydrogen oxide (H_2O) and deuterium oxide (D_2O) showed primary solvent isotope effects in the ΔH values. The solvent isotope effect in ΔH for DGL binding to **1** is greater than that for ConA. ΔG values for the two lectins binding to **1** do not significantly change in H_2O and D_2O. The X-ray crystal structures of ConA[96] and DGL[105] complexed with the trimannoside (**1**) are similar, and the location and conformation of the bound trimannoside as well as its hydrogen-bonding interactions are nearly identical in both lectin complexes. However, differences exist in the location of two loops outside of the respective binding sites containing residues 114−125 and residues 222−227. The latter residues affect the location of a network of hydrogen-bonded water molecules which interact with the trisaccharide (Figure 38). Differences in the arrangement of ordered water molecules in the binding site of the two lectins may account for their differences in the $\Delta\Delta H$ (H_2O-D_2O) values.[173]

*1. Correlation of the $\Delta\Delta H$ (H_2O–D_2O) Data for Analogues **2**–**11** with Differences in the Location of Ordered Water in the DGL and ConA Complexes with Trimannoside **1***

The X-ray structures (Figure 38) of ConA[96] and DGL[105] with **1** reveal differences in the location of ordered water in the following three regions of the two complexes. The first region is associated with the 2-hydroxyl group on the α(1,3) Man of **1**. The $\Delta\Delta H$ (H_2O-D_2O) data for the α(1,3) deoxy analogues of **1** show that only the 2-deoxy analogue (**2**) exhibits a

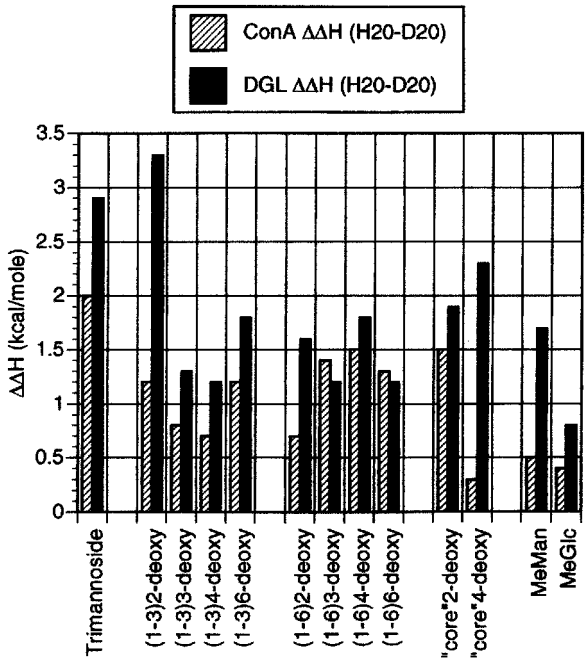


Figure 39. Plot of $\Delta\Delta H$ (H_2O-D_2O) (kcal mol^{-1}) data for DGL and ConA with trimannoside **1**, analogues **2**−**11**, MeαMan, and MeαGlc in Tables 1 and 2, respectively.[173] (Reprinted with permission from ref 173. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

large variation in the $\Delta\Delta H$ (H_2O-D_2O) for DGL and ConA (Figure 39).[173] The second region with altered order water in the two complexes is that associated with the 4-hydroxyl group of the central Man residue of **1**. The $\Delta\Delta H$ (H_2O-D_2O) data for the two deoxy

analogues of the central Man residue of **1** show that the 4-deoxy analogue (**11**) exhibits a significant difference in this parameter for DGL relative to that for ConA (Figure 39). The third area of the X-ray crystal structures of the DGL and ConA complexes which differ in ordered water structures is near the 2-hydroxyl on the α(1,6)-arm of **1**. The $\Delta\Delta H$ (H_2O-D_2O) data for DGL and ConA binding to the deoxy analogues of the α(1,6)-arm of **1** show that the 2-deoxy derivative (**6**) possesses the largest difference in their respective values (Figure 39).

*2. Correlation of ΔΔH (H_2O–D_2O) Values of Deoxy Analogues of **1** with the Number and Strength of Solvent Hydrogen Bonds to Hydroxyl Groups of Trimannoside **1** in DGL and ConA*

The magnitude of the $\Delta\Delta H$ (H_2O-D_2O) values of DGL are greater than that of ConA for deoxy analogues **2** (2-deoxy on α(1,3)), **6** (2-deoxy on α(1,6)), and **11** (core 4-deoxy) (Figure 39).[173] The number of water molecules and corresponding hydrogen bonds connected to the 2-hydroxyl group of α(1,3)Man, core 4-hydroxyl group, and 2-hydroxyl group of α(1,6)Man are more in the DGL complex than those in ConA complex (Figure 38). This shows a correlation with the numbers and strength of water molecules interacting with the corresponding hydroxyl groups of the trimannoside in the respective complexes and the magnitude of the $\Delta\Delta H$ (H_2O-D_2O) values.

*3. Correlation of the ΔΔH (H_2O–D_2O) Data for MeαMan and MeαGlc with Differences in the Location of Ordered Water in the DGL and ConA Complexes with Trimannoside **1***

MeαMan and MeαGlc (Figure 39) also show a correlation with the altered ordered water structures observed in the binding site regions of the DGL and ConA complexed with the trimannoside (Figure 38).[173] Since MeαMan occupies the same site as the α(1,6) Man residue of **1** and makes similar contacts with ConA (2), it is reasonable to assume that the altered ordered water near the 2-hydroxyl of the α-(1,6) Man residue of **1** in the DGL and ConA complexes is present in their respective complexes with the monosaccharide. The $\Delta\Delta H$ (H_2O-D_2O) value for DGL binding to MeαMan is higher than that for ConA, which is consistent with altered solvation of these two lectin complexes. Furthermore, the $\Delta\Delta H$ (H_2O-D_2O) values for DGL binding to MeαMan and MeαGlc are substantially different, while the corresponding values for ConA binding to the two monosaccharides are almost similar (Figure 39). Since the two sugars differ in the orientation of their 2-hydroxyl groups (axial and equatorial, respectively), these results are consistent with altered solvation of the two monosaccharide complexes in both lectins, specifically at the 2-axial hydroxyl group of Man in both lectins.

*4. Lack of Correlation of Altered Water Structures in the DGL and ConA Complexes with the Core Trimannoside and ΔΔH Values in H_2O for Binding of Both Lectins to the Deoxy Analogues of Trimannoside **1***

The ITC solvent isotope data with analogues **2**, **6,** and **11**[173] confirm that differences in the ordered water structures observed in the X-ray crystal complexes of ConA and DGL with the trimannoside exist in their corresponding solution complexes. However, a lack of correlation was found between the $\Delta\Delta H$ values of the deoxy analogues of **1** with both lectins and the altered water structure of the two complexes with **1**.

The $\Delta\Delta H$ for the loss of 2-, 3-, 4-, or 6-hydroxyl groups at α(1,6) Man is ∼3 kcal/mol in H_2O and ∼2.0 kcal/mol in D_2O greater in DGL than ConA.[173] However, as shown in Figure 38, no significant difference in the water structure in the region of 3-, 4-, and 6-hydroxyl groups of α(1,6) Man was found in either complexes.[105] The 3-hydroxyl of the α(1,6) Man of **1** is in contact with W60 in both complexes, while the 4- and 6-hydroxyl groups are not directly bonded to water molecules. Thus, the altered water structure near the α(1,6) Man of **1** does not appear to account for the higher $\Delta\Delta H$ (relative to **1**) values of deoxy analogues **7**, **8**, and **9** (both in H_2O and D_2O) for DGL compared to ConA. On the other hand, significant differences in ordered water exist between DGL and ConA at the 2-hydroxyl group of α(1,3)Man and the 4-hydroxyl group of core Man, yet the $\Delta\Delta H$ values of α(1,3)2-deoxy (**2**) and "core" 4-deoxy (**11**) (relative to **1**) in H_2O are almost similar for both the lectins. Thus, the results indicate that altered structural water in these two regions of DGL and ConA complexes with **1** do not correlate with the $\Delta\Delta H$ values in H_2O of both lectins for **2** and **11**.

B. Relative Contribution of Solvent to the Enthalpy of Binding of Saccharides to ConA

The thermodynamics of binding of carbohydrates to ConA and DGL were also investigated in H_2O and D_2O by Chervenak and Toone.[174] The enthalpy of binding in D_2O was 400−1800 cal/mol less negative than the enthalpy in H_2O. Binding free energy remained unchanged due to the offsetting change in the entropy. A strong correlation between the differential enthalpy of binding and ΔC_p for binding was observed, with a slope of 5K. The authors concluded that solvent reorganization provided 25−100% of the observed enthalpy of binding of carbohydrates to the lectins.

C. ITC Measurements of Carbohydrate Binding to ConA Under Osmotic Stress

The number of water molecules involved in the binding of ConA to trimannoside (**1**), Manα(1,3)Man, Manα(1,6) Man, and D-mannopyranoside was determined by ITC under osmotic stress using glycerol and ethylene glycol.[95] The osmotic release of water molecules from the binding sites caused a concomitant decrease in the binding free energy (Figure 41). This observation, as the authors suggested, indicated the importance of water mediation in sugar binding by the lectin. The number of solute-excluding water molecules coupled to the binding of sugars to ConA as a function of osmotic stress was found to be 5, 3, 3, and 1 for Man, Manα(1,3) Man, Manα(1,6) Man, and trimannoside, respectively. The slope of the enthalpy−entropy compensation plot was greater than unity, both in the presence and absence of osmolyte. Enthalpy−entropy compensation was also reported in other solvent systems[173,174] (Figure 40).

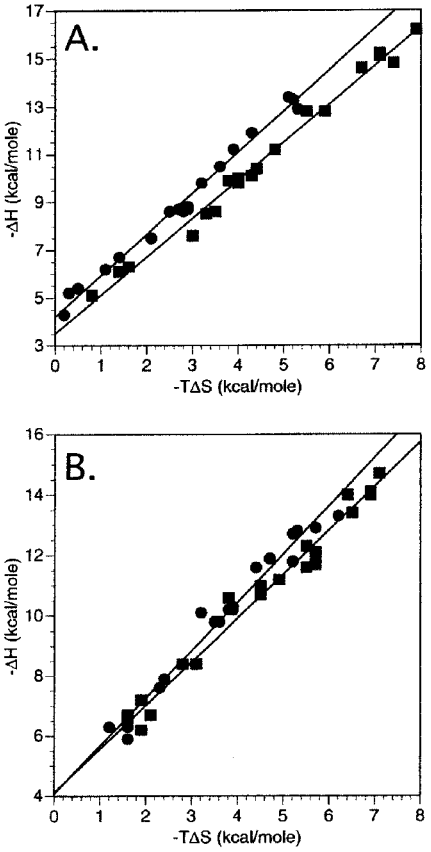


Figure 40. Plots of $-\Delta H$ versus $-T\Delta S$ for the binding of (A) DGL to the carbohydrates in Table 1 in H_2O (■) and D_2O (●) and (B) ConA (■) to the carbohydrates in Table 2 in H_2O (■) and D_2O (●). The solid lines are fits of the data.[173] (Reprinted with permission from ref 173. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

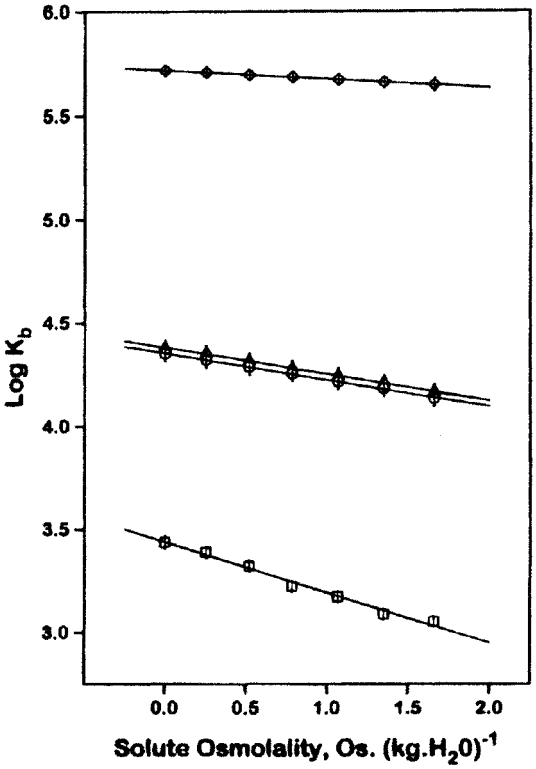


Figure 41. Osmotic sensitivity of the logarithm of binding constant as a function of neutral solute osmolality for the binding of mannose (lower data set), Manα(1,3)Man, Man(α1,6)Man (middle data set), and trimannoside (upper data set) to ConA under ethylene glycol stress at 283.2 K. The straight lines were obtained by linear regression analysis of the data using Origin and have slopes of −0.245 with a correlation coefficient to −0.993 for mannose, −0.127 with a correlation coefficient to −0.999 for Man(α1,3)man, −0.127 with a correlation coefficient to −0.999 for Man(α1,6)Man, −0.043 with a correlation coefficient to −0.999 for trimannoside binding to ConA. The data points represent average values of four independent measurements. The standard deviations were well within the size of the data points.[95] (Reprinted with permission from ref 95. Copyright 1998 American Chemical Society.)

VI. Multivalent Carbohydrate–Lectin Interactions

Many carbohydrate-mediated biological processes require the formation of specific, high-affinity complexes.[175−177] Most lectin−carbohydrate interactions, however, show weak affinities for monovalent sugars and broad specificities for simple oligosaccharides. Since lectins are oligomeric proteins and carbohydrate ligands of cell-surface glycolipids and glycoproteins are multivalent, branched chain molecules,[178] they are engaged in high-affinity multivalent interactions[179,180] that are associated with biological signaling mechanisms.[1,176,181−187] A number of studies have demonstrated that multivalent carbohydrate ligands and synthetic "cluster" analogues possess higher affinities for specific lectins.[185,188−194] However, only during the past few years have thermodynamic studies of the enhanced affinities of multivalent carbohydrate−lectin interactions been investigated. These ITC studies have provided important new insights as reviewed below.

Table 2. Thermodynamic Binding Parameters[a] for ConA with Multivalent Sugars at 27 °C[197]

	K_a ($M^{-1} \times 10^{-4}$)	$-\Delta G$ (kcal/mol)	$-\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	n (no. sites/monomer)
MeαMan	1.2	5.6	8.4	2.8	1.0
21	2.2	6.0	12.7	6.7	0.59
22	2.5	6.0	11.4	5.4	0.67
23	5.3	6.5	15.2	8.7	0.54
p-APMan	1.3	5.6	7.8	2.2	1.0
24	4.7	6.4	17.0	10.6	0.52
25	5.4	6.5	16.6	10.1	0.52
26	6.8	6.6	14.3	7.7	0.60
TriMan	39	7.6	14.7	7.1	1.0
27	286	8.8	23.1	14.3	0.53
28	250	8.7	26.2	17.5	0.53
29	420	9.0	29.0	20.0	0.51
30	1350	9.7	53.0	43.3	0.26

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A. Studies with ConA and DGL

Multivalent neoglycoconjugates ranging from clusters and oligomer to macromolecular polydispersed systems such as glycopolymers and glycodendrimers have been designed and synthesized to overcome the intrinsic low affinity of carbohydrate ligands (cf. ref 195). Man-containing ligands are involved in a number of important biological events; therefore, multivalent neoglycoconjugates with terminal Man residues are attractive candidates for antiadhesiontherapy and other biologically important purposes. Since many of the natural Man receptors, including the Man binding lectin ConA and DGL, possess greater affinity for the trimannoside [3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside] compared to monosaccharide Man, incorporation of trimannoside into multivalent ligands is expected to further enhance the binding affinity. Multivalent ligands bearing terminal Man or trimannoside residues showed increased affinities for ConA and DGL as assessed by enzyme-linked lectin assay[195,196] and hemagglutination inhibition.[201] For the ligands used in this model, hemagglutination inhibition results were found to be consistent with ITC-derived K_a values. To gain insight into the thermodynamic basis of affinity enhancement, binding of synthetic dimeric analogues of α-D-mannopyranoside (Figure 42 a and b) and di-, tri-, and tetrameric analogues of 3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (Figure 42 c) to ConA and DGL was studied by ITC.[197] The results show that ITC can be used to determine the functional valence of multivalent carbohydrates for ConA and DGL and the thermodynamic basis for the enhanced affinities of the multivalent sugars, as discussed below.

1. ITC Measurements of K_a and n Values of Multivalent Carbohydrates with ConA and DGL

ConA and DGL are dimers at pH 5.0 and at low salt concentrations (less than 0.15 M NaCl). Precipitation was either arrested or slowed when titration was performed under these conditions with low concentration of lectins and multivalent ligands. Analogues **21**−**23** have 2−4-fold higher K_a values for ConA relative to MeαMan (Table 2) and 4- to 20-fold higher affinities for DGL (Table 3).[197] Importantly, the *n* values for **21**−**23** binding to ConA are considerably lower than 1.0, with values of 0.59, 0.67, and 0.54, respectively. The *n* values for **21**−**23** binding to DGL are 0.61, 0.70, and 0.56, respectively. The lowest *n* values are for **23** which possesses the highest K_a values of the three analogues for each lectin. Since the theoretical value of *n* for divalent binding of a carbohydrate to ConA is $n = 1.0/2 = 0.5$, the *n* values for **23** of 0.54 and 0.56 for ConA and DGL indicate that it predominately exists in divalent cross-linked complexes with each lectin. The somewhat higher values of *n* for **21** and **22** indicate a lower percentage of cross-linked molecules in solution relative to that for **23**. Analogues **24**−**26** show 4−5-fold higher K_a values for ConA (Table 2) relative to MeαMan and 2−5-fold higher K_a values for DGL (Table 3) relative to MeαMan. The *n* values for **24**−**26** binding to ConA are also lower than 1.0, with values of 0.52, 0.52, and 0.60, respectively. The *n* values for **24**−**26** binding to DGL are 0.60, 0.57, and 0.70, respectively. Analogues **27** and **28** show 7- and 6-fold higher K_a values for ConA, respectively, relative to monovalent TriMan (Table 2). The same analogues show 5-fold greater K_a values for DGL (Table 3) relative to TriMan. The *n* values for **27** and **28** binding to ConA are 0.53 for both analogues and for binding to DGL are 0.50 and 0.51, respectively. Analogues **29** and **30** show 11- and 35-fold higher K_a values for ConA, respectively, and 8- and 53-fold higher K_a values for DGL relative to the trimannoside. Importantly, *n* values for **30** binding to ConA and DGL are 0.26 and 0.25, respectively. These values are consistent with the theoretical value of a tetravalent carbohydrate binding to either lectin which is $n = 1.0/4 = 0.25$. The functional valency of a ligand can be determined from the ITC-derived *n* value.[197]

*2. Functional Valency of **29** Differs from Its Structural Valency for ConA and DGL*

The value of *n* for the binding of **29** to ConA is 0.51 instead of the predicted value of 0.33 based on the structural valency of the triantennary analogue which possesses three trimannoside residues.[197] Thus, **29** is functionally bivalent in binding to ConA as indicated by its ITC-derived value of *n*. On the other hand, the *n* value for **29** binding to DGL is 0.40, which is less than 0.50 for divalent binding but higher than 0.33 for trivalent binding. This difference in functional valency of **29** for ConA and DGL is interesting in light of the similarity in overall structures of the two lectins.[105] There has been a report of differences in the binding of divalent *C*-glycosides to ConA and DGL.[184] Determination of *n* by ITC reveals that the functional valency of a multivalent ligand may or may not be similar to its structural valency.

3. ΔH Increases in Direct Proportion to the Valency of Multivalent Carbohydrate Analogues Binding to ConA and DGL

The results by Dam et al.[197] demonstrate that for higher affinity multivalent analogues, the observed value of Δ*H per mole* of the analogue is approximately the sum of the Δ*H* values of the individual epitopes. Similar observations, as discussed below, have been made for the binding of a trivalent system of receptor and ligand derived from vancomycin and D-Ala-D-Ala[198,199] (Tables 7 and 8) and for the interaction of divalent *C*-glycosides to ConA[184] (Table 4).

4. TΔS Does Not Directly Increase in Proportion to the Valency of High-Affinity Carbohydrates Binding to ConA and DGL

Studies have shown that the enhancements in affinity of multivalent ligands are much greater when the receptor possesses multiple binding sites. An example is the binding of a triantennary complex carbohydrate to the hepatic asialoglycoprotein receptor which has a $\sim 10^{-9}$ M inhibition constant relative to the $\sim 10^{-3}$ M inhibition constant of the corresponding monovalent oligosaccharide.[200] Even more dramatic is the increase in affinity to $\sim 10^{17}$ M^{-1} of a trivalent derivative of vancomycin binding to a trivalent derivative of D-Ala-D-Ala in which the affinity

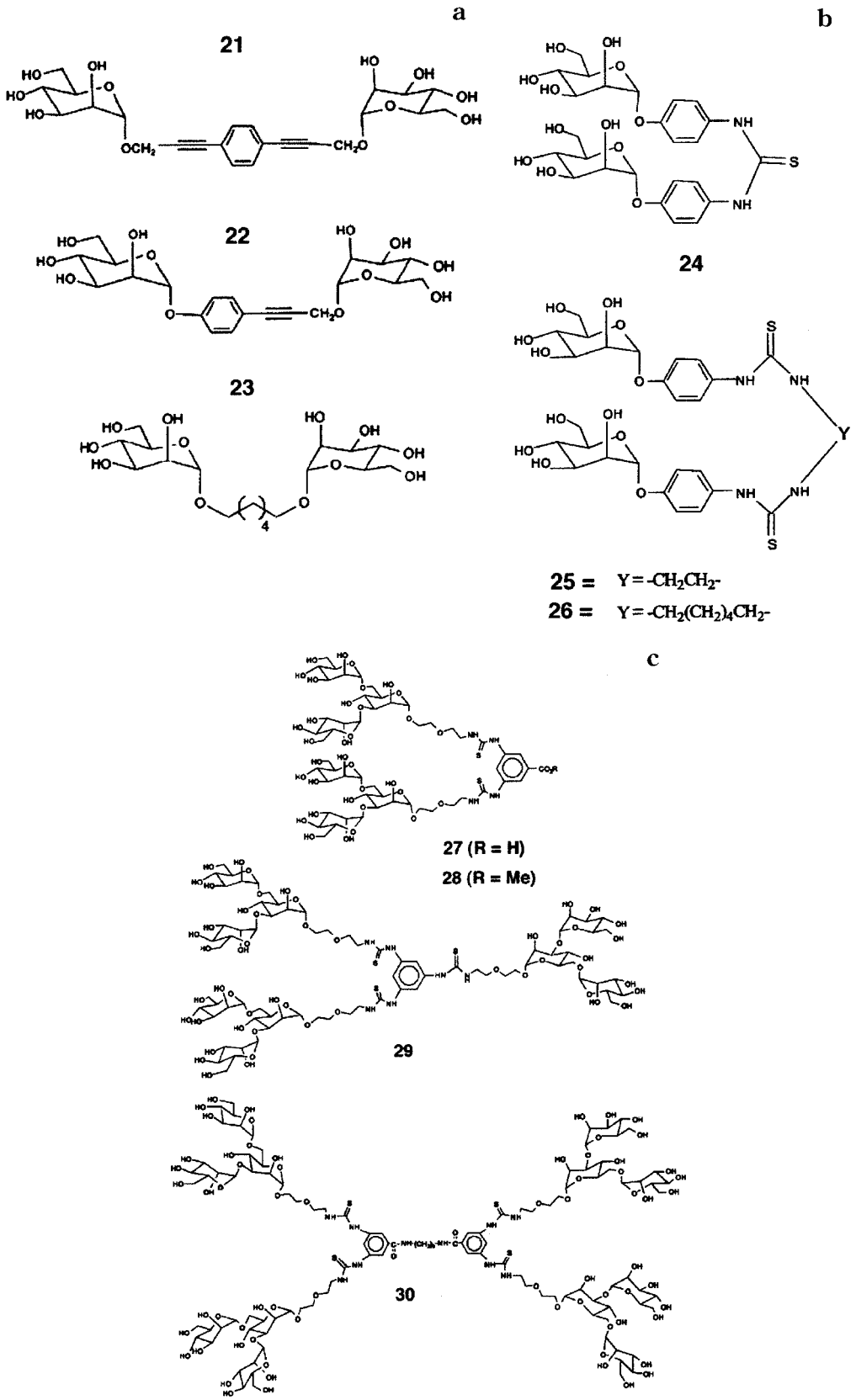

Figure 42. (a−c) Ligands used by Dam et al.[197] (Reprinted with permission from ref 197. Copyright 2000 American Society for Biochemistry and Molecular Biology.)

of the corresponding monovalent analogues is ~10^6 M^{-1}.[198] In the latter study, thermodynamic measurements showed that both ΔH and $T\Delta S$ scaled proportionally to the number of binding epitopes in the molecules (discussed below). These thermodynamic findings are characteristics of the binding of a multivalent ligand to a single multivalent receptor molecule.

However, the results of Dam et al.[197] show that when separate molecules of ConA or DGL bind to

Table 3. Thermodynamic Binding Parameters[a] for *Dioclea grandiflora* Lectin with Multivalent Sugars at 27 °C[197]

	K_a ($M^{-1} \times 10^{-4}$)	$-\Delta G$ (kcal/mol)	$-\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	n (no. sites/monomer)
MeαMan	0.46	4.9	8.2	3.3	1.0
21	2.0	5.9	11.2	5.3	0.61
22	1.6	5.7	11.0	5.3	0.70
23	10.6	6.8	14.8	8.0	0.56
p-APMan	0.7	5.2	7.3	2.1	1.0
24	1.6	5.7	14.3	8.6	0.60
25	2.5	6.0	14.8	8.8	0.57
26	3.7	6.2	12.0	5.8	0.70
TriMan	122	8.3	16.2	7.9	1.0
27	600	9.3	24.7	15.4	0.50
28	590	9.2	27.5	18.3	0.51
29	1000	9.6	32.2	22.6	0.40
30	6500	10.6	58.7	48.1	0.25

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Table 4. Thermodynamic Parameters[a] for ConA-C-Glycoside Complexation[184]

carbohydrate	protein	K_{eq} ($M^{-1} \times 10^{-3}$)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	n
MeαGlc	tetramer	2.4	−4.6	−5.3	−0.7	1.00
MeαMan	tetramer	7.6	−5.3	−6.8	−1.5	1.02
15	tetramer	4.5	−5.0	−5.9	−0.9	1.08
16	tetramer	5.2	−5.1	−5.9	−0.8	1.00
17/18	tetramer			no binding detected		
19	tetramer	4.7	−5.0	−8.5	−3.5	0.50
20	tetramer	29.0	−6.1	−11.1	−5.0	0.51
19	dimer	3.6	−4.8	−14.5	−9.7	0.49
20	dimer	5.0	−5.1	−13.8	−8.7	0.52

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Table 5. Binding[a] of Dendritic Ligands to ConA[205]

ligand	K_{eq} (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	n
monovalent (**31**)	11 820	−5.5	−6.4	−0.9	1
divalent (**32**)	18 782	−5.8	−8.9	−3.1	1
trivalent (**33**)	3 734	−4.9	−8.6	−3.7	0.8
tetravalent (**34**)	9 640	−5.4	−4.9	+0.5	1
hexavalent (**35**)	7 504	−5.3	−3.8	+1.5	1

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Table 6. ITC Results[a] for Peptide- and TEG-Linked Glycodendrimers[206]

ligand	K_a (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
		peptide-linked		
monovalent (**36**)	9 200	−5.4	−7.4	−2.0
bivalent (**37**)	8 000	−5.3	−7.5	−2.2
trivalent (**38**)	7 900	−5.3	−7.8	−2.5
tetravalent (**39**)	7 500	−5.3	−4.2	+1.1
hexavalent (**40**)	ND			
		TEG-linked		
monovalent (**41**)	8 000	−5.3	−6.4	−1.1
bivalent (**42**)	8 600	−5.3	−7.7	−2.3
trivalent (**43**)	4 700	−5.0	−7.1	−2.1
tetravalent (**44**)	620 00	−6.6	−2.3	+4.3
hexavalent (**45**)	1 500 000	−8.5	−1.3	+7.2

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different epitopes of single multivalent carbohydrates, ΔH scales proportionally but $T\Delta S$ does not scale proportionally to the number of carbohydrate epitopes. Data for ΔH and $T\Delta S$ for ConA and DGL, respectively, binding to multivalent analogues **27**, **28,** and **30** are shown in Tables 2 and 3. While ΔH scales proportionally, $T\Delta S$ is much more negative than if it proportionally scaled to the number epitopes in the carbohydrates. For example, the observed $T\Delta S$ value for tetravalent **30** is −43.3 kcal/mol, not −28.4 kcal/mol, if it scaled with the $T\Delta S$ value of −7.1 kcal/mol for TriMan (Table 2). The resulting ΔG value of **30** would also be much greater if $T\Delta S$ scaled with valency since the difference between ΔH and $T\Delta S$ would be greater. These results demonstrate the differences in the thermodynamics of binding of a multivalent ligand binding to a receptor with multiple binding sites (e.g., hepatic asialoglycoprotein receptor) and to separate receptors not possessing clustered binding sites (ConA and DGL). In the latter

Table 7. Thermodynamic Parameters[a] of Binding of Vancomycin Derivatives to Depsipeptide/Peptide Ligands at 298 K[198]

receptor	ligand	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
$R_t\mathbf{V}_3$	$R'_t\mathbf{L}'_3$	4×10^{-11}	−22.4	−39.9	−17.4
$R_t\mathbf{V}_3$	$R'_t\mathbf{L}'$	1.1	−8.1	−12.4	−4.3
$R_t\mathbf{V}_3$	L	2.7	−7.6	−12.0	−4.4
vancomycin	$R'_t\mathbf{L}'_3$	0.34	−8.8	−17.5	−8.7
vancomycin	L	1.6	−7.88	−11.98	−4.1

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Table 8. Thermodynamic Parameters[a] of Binding of Vancomycin Derivatives to Depsipeptide/Peptide Ligands at 298 K[199]

receptor	ligand	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
V^4	Ac_2KDADLac	1700	−3.8	−3.7	+0.1
V−R_d-V	Lac−R'_d−Lac	42.6	−6.0	−6.5	−0.5

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case(s), the distances between binding sites on ConA and DGL are too great to be spanned by a single multivalent carbohydrate.

Subsequent Hill and Scatchard plot analysis of the ITC raw data of ConA and DGL binding to the above multivalent sugars revealed negative cooperativity in their binding.[201] The slope of the Hill plots becomes increasingly negative as the binding progressed, consistent with decreasing affinity of the individual sugar epitopes with increased lectin binding. This was experimentally demonstrated by measuring the microscopic binding thermodynamics of individual epitopes of multivalent sugars through reverse ITC in which ConA was titrated into the sample cell containing the multivalent sugar.[202] It was shown that the value of K_a of the first epitope of a bivalent sugar was over a magnitude greater than the second epitope. $T\Delta S$ of the second epitope was more unfavorable than that of the first one, whereas the ΔH values of the individual epitopes were the same.

5. ITC Studies of High Man Oligosaccharides Binding to ConA

ITC studies of high-Man oligosaccharides and glycopeptides binding to ConA provided insights into multivalent carobohydrate−lectin interactions.[203] The Man8 and Man9 glycopeptides showed higher affinities for tetrameric ConA as compared to dimeric ConA, indicating the important role of the valency of the lectin in affinity enhancements of multivalent carbohydrates.[203] Fractional n values of 0.64 and 0.59 were also obtained for Man5 and Man9 oligosaccharides, respectively, binding to dimeric acetyl-ConA.[92]

6. ITC Studies of Peptide Mimetics Binding to ConA

ConA has also been used to study the multivalency of sugar mimicking peptides. In a recent study, ITC demonstrated that the K_a of a ConA binding peptide, which is shown to bind to the carbohydrate binding site of the lectin through some common amino acid residues, increased severalfold when the peptide was presented in a multivalent format.[204]

7. Additional ITC Studies of ConA

ConA has been the subject of additional ITC studies with other synthetic ligands. Weatherman et al.[184] investigated the binding of *C*-glycosides (Figure 43) to dimeric and tetrameric ConA by ITC. Bivalent Glc and Man derivatives **19** and **20** exhibited different binding properties (Table 4) compared to the corresponding monovalent **15** and **16** (monovalent **17** and **18** were reported not to interact with the lectin). Affinities of **19** and **20** for dimeric and tetrameric ConA were comparable to that of monovalent **15** and **16**. Only **20** showed a modest enhancement of affinity for the tetrameric ConA. The n values of the bivalent ligands were 0.49 and 0.52, and the ΔH values were 2-fold higher than the corresponding monovalent ligands accompanied by large entropic losses (Table 4). These observations are similar to those of Dam et al.[197] To explain the low binding stoichiometry, the authors[184] argued against cross-linking of the lectins by bivalent ligands (intermolecular binding) and suggested that ConA contained a second class of binding sites not found on the lectin from *Dioclea grandiflora* (DGL). However, structural[96,105] and thermodynamic studies[106] indicate that both ConA and DGL possess one carbohydrate binding site per monomer.

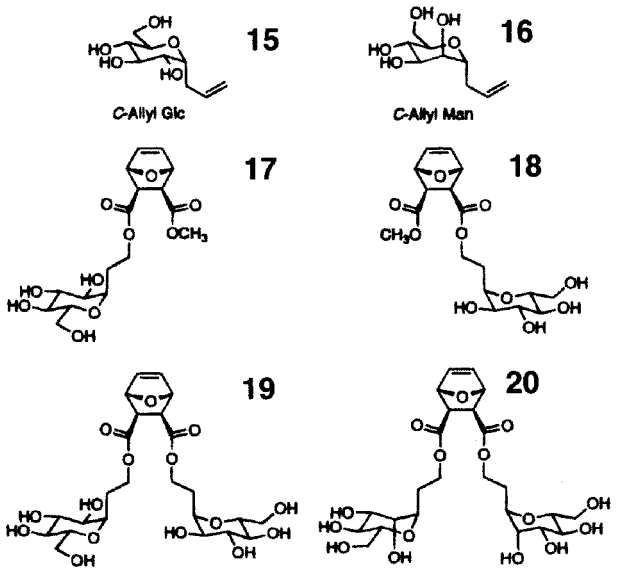

Figure 43. Ligands used by Weatherman et al.[184] (Reprinted with permission from ref 184. Copyright 1996 American Chemical Society.)

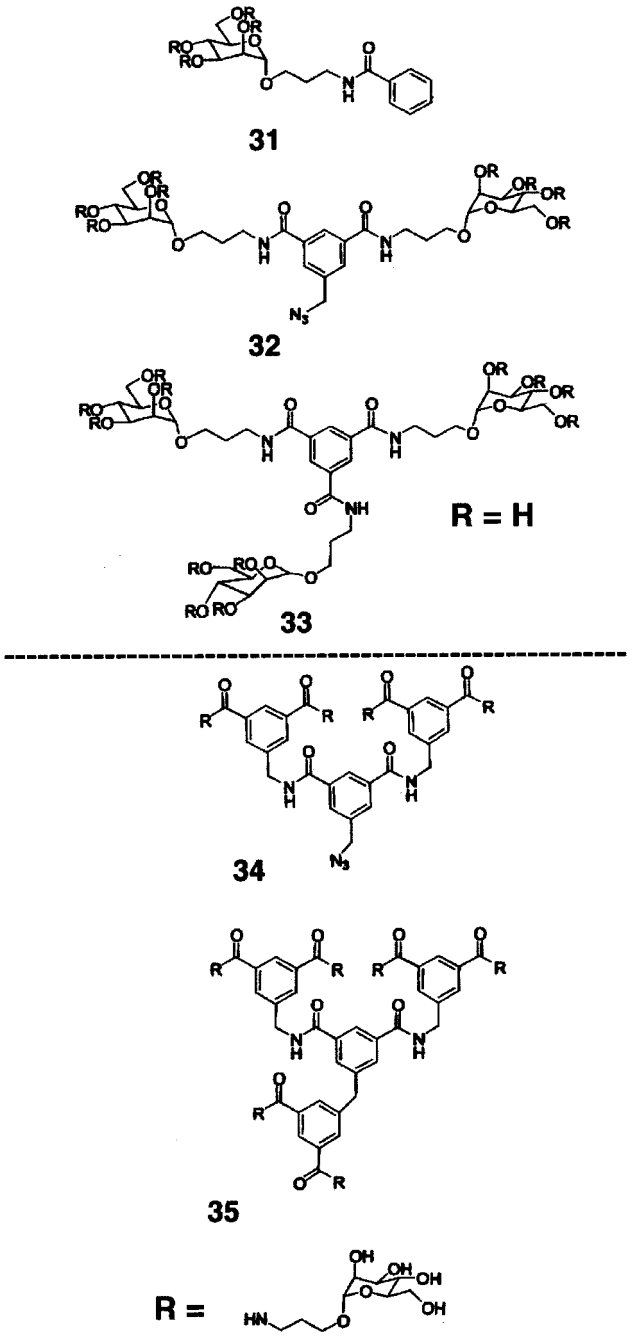

Figure 44. Ligands used by Dimick et al.[205] (Reprinted with permission from ref 205. Copyright 1999 American Chemical Society.)

A different picture of the thermodynamics of the binding of multivalent carbohydrate analogues to ConA was reported by Dimick et al.,[205] in which a series of multivalent dendritic saccharides have been used (Figure 44). Although a 30- and 13-fold enhancement in affinities was observed with **34** and **35**, respectively, by agglutination assay, ITC with a range of multivalent ligands revealed no enhancement in binding free energies. Mono- (**31**), bi- (**32**), tri- (**33**), tetra- (**34**), and hexavalent (**35**) ligands (Figure 44) as well as MeαMan showed almost the same ΔG values, whereas the binding enthalpies became more negative from mono- to bi- and trivalent ligands, but tetra- and hexavalent lgands showed comparatively positive enthalpies (Table 5). The authors concluded that by ITC there was no true affinity enhancements due to multivalency and that the enhancements with **34** and **35** observed by agglutination were not real affinities but artifacts of this type of assay.

In a subsequent study, different sets of synthetic multivalent carbohydrates were used by the same group[206] to study the thermodynamics of multivalent binding to ConA. Structurally, these ligands are closely related to those used by Dimick et al.[205] except for the spacer regions (Figure 45). The first series of ligands contained a semirigid glycylglycine spacer (**36**−**40**), and the second series (**41**−**45**) contained a flexible tetraethylene glycol spacer. Ligands **36**−**40** did not show any significant enhancement in any binding study. Ligands **41**−**45**, especially **44** and **45**, showed marked enhancement in both agglutination and ITC studies, which is interesting in light of the same group's previous result[205] as both agglutination and ITC now showed consistent enhancement. The three sets of ligands (**31**−**35**, **36**−**40,** and **41**−**45**) are structurally similar differing only in the spacers. The most obvious reason for the divergent results obtained with these three different sets seems to be the varied structures of the spacers. As in their previous study,[205] the ITC-derived ΔH values (Table 6) showed increasingly positive values with increased valency, irrespective of affinity enhancement.[206] The $T\Delta S$ values were much more positive for the tetra- and hexavalent ligands. According to the authors' explanation, the reported ΔH represents the enthalpy of protein−carbohydrate interaction and the enthalpy of nonspecific aggregation. Similarly, the reported $T\Delta S$ represents the entropic contributions of these two processes. It thus becomes difficult to discern the individual contribution of these two simultaneous events to the overall ΔH and $T\Delta S$ values. The authors suggested that the steadily diminishing enthalpy of binding was the thermodynamic signature of an endothermic nonspecific aggregation process. In specific carbohydrate−lectin interactions, the origin of such nonspecific aggregation is not well understood. In their first paper on this topic,[205] affinity enhancements of their multivalent carbohydrates were observed by an agglutination assay but not by ITC. Hence, the authors found ITC reliable and questioned the ability of the agglutination assay to measure the relative affinities of carbohydrates for lectins. However, the same ligands with different spacer groups showed measurable affinity enhancement by ITC but not in an enzyme-linked lectin assay (ELLA). Interestingly, the authors suggested that ELLA, not ITC, might more faithfully report the actual protein−carbohydrate affinities than other assays. There are several reports in the literature, one of them from this group,[207] where ELLA faithfully reported the enhancement in affinity in multivalent binding.[195,196,208]

B. Studies with Vancomycin

Vancomycin (V) is a glycopeptide antibiotic that is active against Gram-positive bacteria. It binds to the carboxy-terminal D-Ala-D-Ala (DADA) of the bacterial cell wall mucopeptide precursors and disrupts the

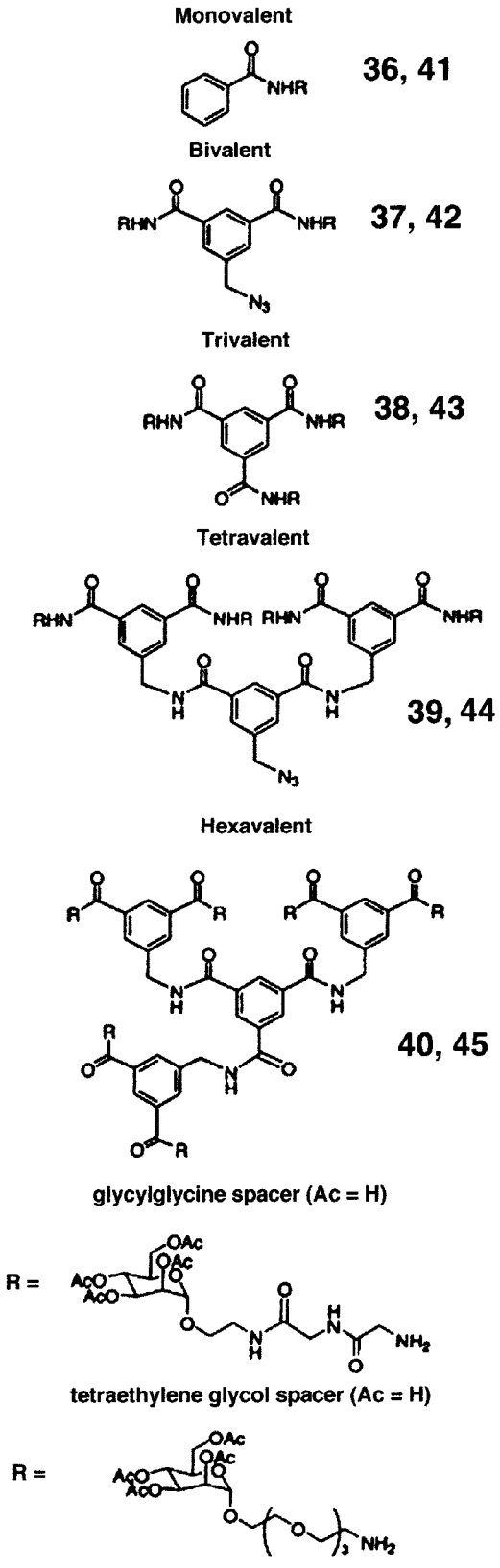

Figure 45. Ligands used by Corbell et al.[206] **36**−**40** are with a glycylglycine spacer, where as **41**−**45** are with a TEG spacer. (Reprinted with permission from ref 206. Copyright 2000 Elsevier Science.)

structure of the cell wall resulting in lysis. Whitesides and co-workers used V and DADA as models to study multivalent binding interactions.[198,199] Although this system does not represent typical carbohydrate−protein interactions, it provides important insights

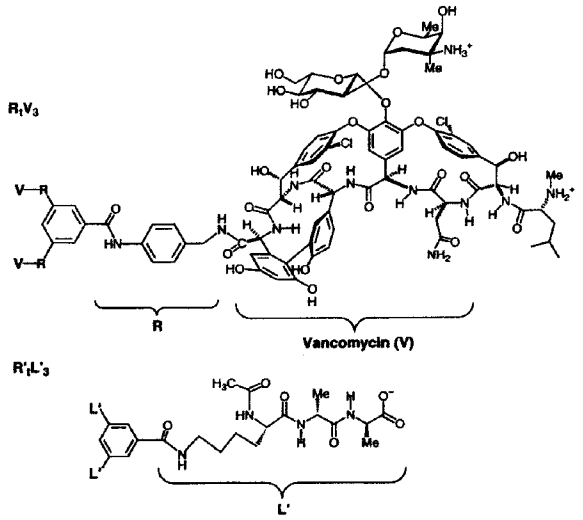

Figure 46. Structures of the trivalent derivatives of vancomycin, R_tV_3, and DADA, $R'_tL'_3$.[198] (Reprinted with permission from ref 198. Copyright 1998 American Association for the Advancement of Science.)

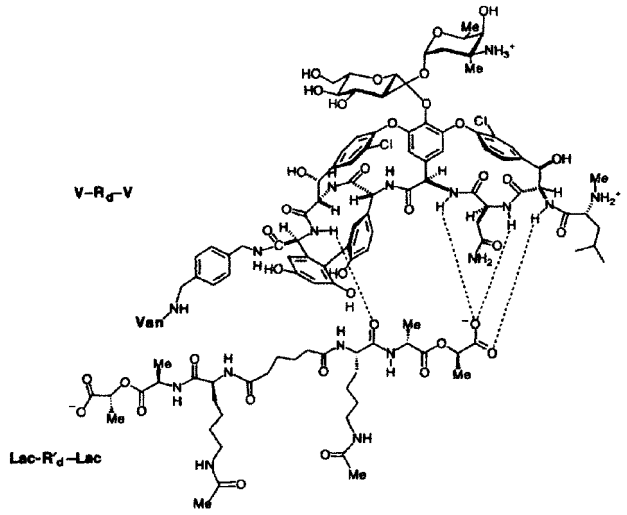

Figure 47. Structures of the dimeric vancomycin (V−R_d−V) and dimeric lactate ligand (Lac−R'_d−Lac).[199] (Reprinted with permission from ref 199. Copyright 1999 Elsevier Science.)

into the thermodynamics of multivalent ligand−receptor interactions that have been compared with recent studies involving multivalent carbohydrates and lectins. The authors described the synthesis of a trivalent system of receptor and ligand derived from vancomycin (R_tV_3) and DADA (R'_t L'_3), respectively (Figure 46), that shows exceptionally high affinity compared to monovalent binding.[198] Although the enhanced affinity was too high to determine by ITC, they reported an ITC-derived ΔH for the interaction of R_tV_3 and R'_t L'_3 (Table 7). ΔH and $T\Delta S$ for the trivalent binding were approximately three times greater than that of the monovalent ligand. In a separate study,[199] the same group reported ITC studies of the binding of monomeric V and a dimeric analogue (V−R_d−V) to lactate ligands (Figure 47). K_d and ΔH for the binding of Ac_2KDADLac to V were 1745 μM and −3.7 kcal/mol, respectively, whereas, for the binding of dimeric V−R_d−V to divalent Lac−R'_d−Lac, K_d and ΔH were found to be 43 μM and −6.5 kcal/mol, respectively (Table 8). There was no detect-

Table 9. ITC Data[a] for MBP-C[69]

ligand	log K_a	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (kcal/mol)	n
Me-O-Man	3	−3.8	−5.1	−4.7	1
Me O-GlcNAc	3	−3.8	−4.7	−3.2	1
NacYD(G-ah-Man)$_2$ (**46**)	4.6	−5.9	−7.4	−5.4	1

[a] Reprinted with permission from ref 69. Copyright 1997 American Chemical Society.

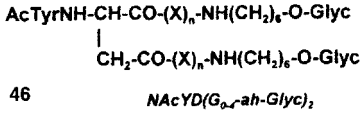

Figure 48. Bivalent ligand used in ref 69. Glyc = mannose. (Reprinted with permission from ref 69. Copyright 1997 American Chemical Society.)

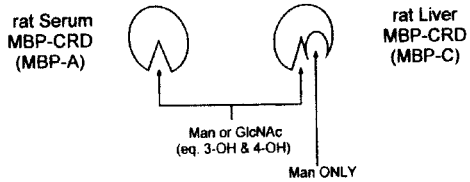

Figure 49. Schematic presentation of MBP CRD.[69] (Reprinted with permission from ref 69. Copyright 1997 American Chemical Society.)

able binding of V−R_d−V to Ac_2KDADLac, and the affinity of Lac−R'_d−Lac for V was comparable to that of Ac_2KDADLac for V. Comparing the binding data of mono- and divalent binding, the authors observed that the increased enthalpy without a large compensatory loss in entropy was the origin of enhanced affinity. Indeed, the increase in entropy was essentially directly proportional to the number of epitopes. The absence of a large loss in entropy was also observed in the trivalent ($R_t\mathbf{V}_3$ and R'_t $\mathbf{L}'_3$) system, and the phenomenon was attributed to intramolecular binding.

C. Mannose Binding Proteins

Lee and co-workers[69] studied the binding of two Man binding proteins, namely, MBP-A and MBP-C, with several monovalent and multivalent carbohydrates by different assay techniques. Thermodynamic parameters (Table 9) obtained from ITC experiments showed that the bivalent ligand **46** (Figure 48) bound to MBP-A with an affinity similar to that of monovalent ligands. The affinity of **46** for MBP-C and its $-\Delta H$ value were greater than all other tested sugars. The affinities obtained from ITC agreed reasonably well with the I_{50} values of the inhibition assay. The authors attributed the enhanced affinity of **46** to the presence of two sugar binding sites on MBP-C monomer (Figure 49). They concluded that simultaneous occupation of the two sites by Man containing flexible bivalent ligands enhanced the binding affinity by 10-fold or more.

D. Hevein

Many plants defend against pathogenic attack using proteins that are able to bind to chitin, a β(1−4)-linked GlcNAc polysaccharide, which is an important structural component of the cell wall of fungi and the exoskeleton of invertebrates. These defense proteins are generally known as the hevein domain or chitin binding motif (CBD). Most of these proteins contain a common structural motif of 30−43 residues that are rich in glycines and cysteines in highly conserved positions which are organized around a four disulfide core.[209] Apart from the rubber tree, *Hevea brasiliensis*, the hevein domain is also found in other lectins such as pseudohevein, *Urtica dioica* (UDA), wheat germ agglutinin (WGA), and Ac-AMP antimicrobial peptides. It is also present in enzymes with antifungal activity, such as class I chitinases.

Table 10. Thermodynamic Parameters[a] for Binding of $(GlcNAc)_{2-5}$ to Hevein[209]

ligand	K_a (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol K^{-1})
$(GlcNAc)_2$	616	−3.8	−6.3	−8.4
$(GlcNAc)_3$	8525	−5.4	−8.3	−9.9
$(GlcNAc)_4$	10,850	−5.5	−9.5	−13.4
$(GlcNAc)_5$	474,000	−7.8	−9.6	−6.3

[a] Reprinted with permission from ref 209. Copyright 2000 Elsevier Science.

Table 11. Thermodynamic Parameters[a] for Binding of $(GlcNAc)_{1-5}$ to Wheat Germ Agglutinin (WGA) and *Urtica diocia* Agglutinin (UDA)[210,214]

ligand	K_a ($M^{-1} \times 10^{-3}$)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
		WGA		
GlcNAc	0.41	−3.7	−6.1	−2.4
$(GlcNAc)_2$	5.3	−5.1	−15.6	−10.5
$(GlcNAc)_3$	11.1	−5.5	−19.4	−13.9
$(GlcNAc)_4$	12.3	−5.6	−19.2	−13.6
$(GlcNAc)_5$	19.1	−5.8	−18.2	−12.4
		UDA		
$(GlcNAc)_2$	0.8	−3.9	−4.7	−0.8
$(GlcNAc)_3$	6.2	−5.1	−6.3	−1.2
$(GlcNAc)_4$	14.4	−5.6	−5.1	+0.5
$(GlcNAc)_5$	26.5	−5.9	−5.1	+0.8

[a] Reprinted with permission from ref 210 and 214. Copyright 1992 American Chemical Society and 1998 Kluwer Academic Publishers.

The thermodynamics of association of WGA with GlcNAc and its β(1,4) oligomers have been investigated by Bains and co-workers[210] using ITC. Association constants of 0.4. 5.3, 11.1, 12.3, and 19.1 mM^{-1} and ΔH values of −6.1, −15.6, −19.4, −19.3, and −18.2 kcal/mol were obtained for the titration of WGA with GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$, respectively. The $T\Delta S$ values were all negative. The magnitude of the enthalpy change and the free energy change increased as the number of GlcNAc residues increased (Table 11) in the oligosaccharide up to three residues. There was little further increase in these thermodynamic parameters with the tetrasaccharide. These results supported the "three subsite" structural binding model proposed by Allen et al.[211]

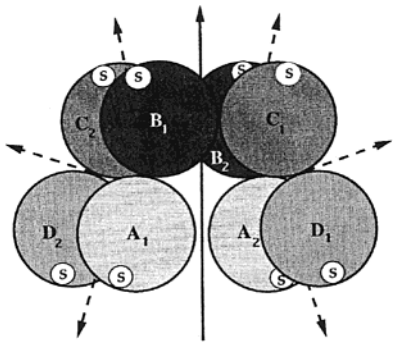

Figure 50. Schematic representation of the WGA dimer. Domains are shown as large shaded circles and labeled A1, B1, C1, D1, etc. The position of the molecular 2-fold axis is indicated by an arrow. Dotted arrows represent the two types of pseudo-2-fold axes generated in the dimer interface between domains of different dimers. "S" refers to the aromatic sugar binding pocket.[212] (Reprinted with permission from ref 212. Copyright 1996 Cold Spring Harbor Laboratory Press.)

The WGA monomer consists of four similar disulfide-rich 43-amino acid residue domains (A, B, C, D) arranged in tandem and dimerizes in a "head to tail" fashion, forming an extensive monomer/monomer interface. This interface accommodates eight independent saccharide binding sites (four unique sites) between contacting domains (Figure 50) characterized by a cluster of 2−3 aromatic amino acids.[212] Each unique site is referred to by the domain that contributes the aromatic pocket. Because 2-fold-related sites are equivalent, there are two of each type of site present in the dimer: A1 or A2, $B1_{C2}$ or $B2_{C1}$, $C1_{B2}$ or $C2_{B1}$, $D1_{A2}$ or $D2_{A1}$. The subscripts designate the domain that contributes the polar region on the opposing monomer. Because the D-domain has no polar region, the binding site of domain A1 or A2 consists of an aromatic pocket alone. X-ray crystallography revealed that all four unique sites are functional. Further analysis of the WGA-$(GlcNAc)_2$ complex by hydropathic interactions (HINT) modeling program showed that the sites are nonidentical in terms of affinity. Presumably, two of these have affinities too weak to be detectable in solution. The magnitudes of the HINT scores confirm that the *N*-acetyl group is responsible for most of the binding through two strong hydrogen bonds (Figure 51). In all four binding configurations, hydrogen-bonding and polar van der Waals interactions constitute the largest contribution to overall binding consistent with the negative values for the enthalpy and entropy changes as obtained with thermodynamic studies.[210]

Using ITC, Rice[213] measured the binding constants of $(GlcNAc)_3$ and $(GlcNAc)_4$ to a single domain of WGA, produced by recombinant techniques. The values obtained were almost 10 times lower than those of published binding constants. This suggests that high-affinity binding requires additional contacts across the dimer interface.

Binding of UDA[214] with the same set of oligosaccharides revealed that the binding enthalpy increased from $(GlcNAc)_2$ to higher oligomer but the values were considerably smaller than that of WGA (Table 11). Relatively high ΔC_p values of the UDA−carbohydrate interactions and relatively favorable

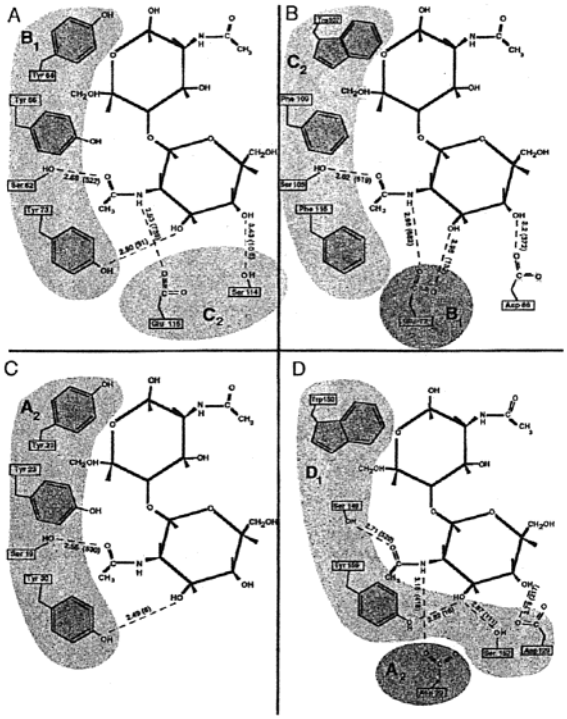

Figure 51. H-Bond network for bound $(GlcNAc)_2$ at the four unique WGA binding sites. $(GlcNAc)_2$ bound at sites (A) $B1_{C2}$, (B) $C2_{B1}$, (C) A2, and (D) $D1_{A2}$, respectively. Shaded areas depict the binding regions on different monomers. Hydrogen bonds are shown as dashed lines. Their bond lengths are given in Å, and their HINT values are shown in parentheses.[212] (Reprinted with permission from ref 212. Copyright 1996 Cold Spring Harbor Laboratory Press.)

entropy term for $(GlcNAc)_4$ and $(GlcNAc)_5$ compared to WGA suggested that binding of the higher oligosaccharides by UDA had a higher hydrophobic contribution than that of WGA. A favorable entropic term for $(GlcNAc)_4$ and $(GlcNAc)_5$ binding was also reported by Katiyar et al.[215]

UDA consists of two hevein-like domains with the same spacing of cysteine residues and several other conserved residues. The sequences of the two domains of isolectin VI (UDA-VI) show a 42% similarity.[216] The crystal structure of the isolectin VI complexed with $(GlcNAc)_3$ provides further insights into the carbohydrate binding. The arrangement of the two domains makes the shape of the molecule like a dumbbell, and the sugar binding sites are located at both ends of this molecule. The binding site in domain-1 (N-terminal domain) consists of three subsites complementary to $(GlcNAc)_3$ structure. The sugar residues B and C are bound on the indole moieties of Trp23 and Trp21 with face to face contact. Residue A binds to the third subsite of domain-1 through hydrogen bonding. On the other hand, two GlcNAc residues bind to domain-2 (C-terminal domain). In both the domains, three aromatic amino acid residues and one serine residue participate in the $(GlcNAc)_3$ binding. The higher affinity of $(GlcNAc)_3$ and $(GlcNAc)_4$ over $(GlcNAc)_2$ is attributed to the increased possibilities of better contacts between GlcNAc residues and the two binding sites of independent UDA molecules.[216] Indeed, ITC-derived K_a and ΔH values of $(GlcNAc)_3$ show a significant

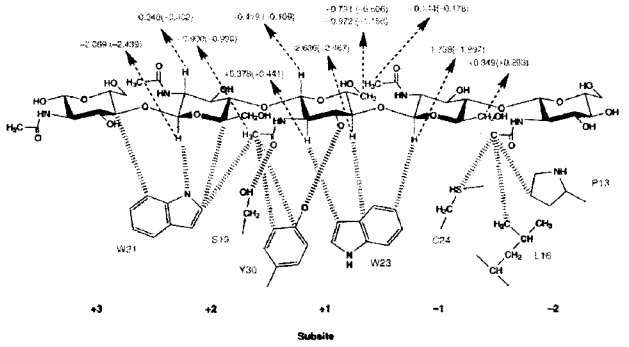

Figure 52. NMR-derived model of hevein−chitin complex.[209] (Reprinted with permission from ref 209. Copyright 2000 Elsevier Science.)

increase over those of $(GlcNAc)_2$. ΔH values of $(GlcNAc)_4$ and $(GlcNAc)_5$ do not increase further, but moderate entropy-derived enhancements in K_a were recorded[214] which suggested hydrophobic interactions with the fourth and fifth GlcNAc residues. The relative affinities of the two sugar binding sites of UDA were concluded to be different involving different amino acid residues.

Asensio et al.[209] reported ITC-derived binding parameters of hevein (from *Hevea brasiliensis*) with $(GlcNAc)_{2-5}$ oligomer. Although their enthalpy values were slightly larger than those reported in a previous report,[217] both ITC and NMR studies showed a similar trend of interaction. The affinity of the protein for the ligand increased by 1 order of magnitude per GlcNAc residue between 1 and 3. For tetrasaccharide $(GlcNAc)_4$ binding, a further increase in ΔH of about 1 kcal/mol was observed in comparison to $(GlcNAc)_3$ but the increase in K_a was negligible (Table 10). A sharp increase in the association constant was recorded with $(GlcNAc)_5$, and the ITC curve did not fit to a 1:1 stoichiometry. Ultracentrifugation experiments showed the existence of 1:1 complex (intramolecular binding) in solution up to $(GlcNAc)_3$. In contrast, $(GlcNAc)_5$ appeared to induce significantly larger complexes (intermolecular binding). The authors suggested that for oligosaccharides up to $(GlcNAc)_4$, the carbohydrate length was too short to allow the binding of more than one hevein molecules but $(GlcNAc)_5$ could probably bind two hevein molecules, thus producing a mixture of several 1:1 and 2:1 protein−carbohydrate complexes. The authors envisaged two different origins to account for the observed increase in macroscopic affinity of the protein for $(GlcNAc)_5$ compared with $(GlcNAc)_4$. First, the pentasaccharide provides a larger number of contacts to the protein (Figure 52), and therefore, an increase in affinity would be expected. Second, to some extent, two protein molecules could be bound to $(GlcNAc)_5$. According to the data presented in Table 10, entropy became increasingly unfavorable up to $(GlcNAc)_4$. For $(GlcNAc)_5$ the entropic term was found to be relatively favorable compared to $(GlcNAc)_4$. For UDA, both $(GlcNAc)_4$ and $(GlcNAc)_5$ binding was accompanied with favorable entropy. The binding of GlcNAc oligosaccharides to WGA and UDA represents another type of multivalent interaction where the multiple sugar epitopes interact with three subsites of the same carbohydrate binding site. For hevein, $(GlcNAc)_5$ have been shown to be engaged in intermolecular binding, where it is capable of connecting two different hevein molecules.

VII. Summary

This review demonstrates that ITC measurements have become an important and widely used method for investigating the thermodynamics of carbohydrate−lectin interactions. Indeed, no other method affords the ease of directly determining thermodynamic binding parameters without modifying the carbohydrates or proteins of interest. Recent improvements in commercial instruments made it possible to perform ITC experiments in the micromolar range of protein concentrations, depending on the affinity of the ligand and the magnitude of the heat taken up or released upon binding.

This review has summarized a wide range of lectin binding studies investigated by ITC during the past decade. Below, we highlight certain applications that are of general importance and which will likely find increasing usage in the future.

First and foremost is the ability to directly and accurately determine K_a values for the interactions of carbohydrates with lectins. The ability to determine K_a values for a series of unmodified carbohydrate ligands to a given lectin is important in developing structure−activity data. However, caution is required with the low affinities of many carbohydrate−lectin interactions. The unitless constant $c = K_a M_t$, where M_t is the initial protein concentration and K_a is the association constant in M^{-1}, is important in this regard. The value of c determines the shape of the binding isotherm,[16] and all experiments should be performed with c values of $1 < c < 200$. Values of c less than 1 lead to erroneous data analysis, and therefore, there is a limit to weak affinity interactions that can be investigated by ITC (typically, $K_a \sim 10^3\ M^{-1}$). Several studies mentioned in this review involve low-affinity binding data, especially with monosaccharides. ITC experiments with low-affinity ligands require high concentrations of the lectins to obtain valid c values.

The ability to determine ΔH values directly and $T\Delta S$ values from ΔH and ΔG values is also valuable in analyzing structure−activity data for carbohydrate−lectin interactions. Changes in K_a values for a series of structurally related ligands to a protein are often interpreted in terms of the size of the binding site(s) of the protein. Relative enhanced affinities of certain ligands are often taken as evidence for extended site binding interactions. However, increases in K_a or ΔG may be due to more favorable ΔH contributions or more favorable $T\Delta S$ contributions. A good example is a study of the binding of a series of oligosaccharides to ConA using ITC.[92] Most of the carbohydrates fall on a linear ΔH versus $T\Delta S$ plot except for certain $\alpha(1-2)$ Man and $\beta(1-2)$ GlcNAc oligosaccharides which show enhanced $T\Delta S$ contributions relative to the other carbohydrates. The authors suggested a sliding mechanism between adjacent Man residues of these oligosaccharides and the monosaccharide binding site

of the lectin as responsible for their enhanced affinities instead of a complementary extended binding site on ConA. Subsequent X-ray crystallographic results with ConA are consistent with this conclusion.[218]

Substantial enhancements in the K_a as well as ΔH values of an oligosaccharide binding to a lectin, relative to a monosaccharide, can be evidence for an extended binding site. An example is binding of the core trimannoside of N-linked carbohydrates to ConA.[90,92] Extended site interaction was subsequently confirmed by X-ray crystallographic studies.[96] Hence, knowledge of the K_a, ΔH, and $T\Delta S$ values for a series of structurally related carbohydrates can provide insight into the physical nature of their binding interactions with a lectin and, in certain cases, the size of the combining site of the protein.

Epitope mapping by ITC is also a powerful method of determining the binding interactions between a carbohydrate and lectin. For example, the use of deoxy analogues of a carbohydrate has long been a means of determining the potential hydrogen-bonding interactions of specific hydroxyl groups with the protein. ITC provides precise measurements of the K_a and ΔH values of the derivatives, which are required to determine the thermodynamic of binding of the analogues. Examples of ITC epitope mapping are deoxy analogues of the core trimannoside (**1**) binding to ConA,[94] DGL,[106] Diocleinae lectins,[114] Artocarpin,[132] and winged bean lectins.[152,155] Most of the data obtained with deoxy sugars have been confirmed by X-ray crystallography. ITC can also be used to elucidate the role of solvent in carbohydrate binding.[95,173,174]

Perhaps the most exciting new developments in the use of ITC are to explore the thermodynamics of binding of multivalent carbohydrates to lectins. For example, ITC measurements can determine the functional valency of a multivalent carbohydrate for a given lectin,[197] a result difficult to achieve by other methods. The same study also showed that the thermodynamic basis for the enhanced affinities of ConA and DGL for di-, tri-, and tetraantennary carbohydrate analogues, with respect to the monovalent sugar, were their more favorable entropies of binding. Inverse ITC experiments have provided direct determinations of the microscopic thermodynamic parameters of individual epitopes of multivalent carbohydrates binding to ConA.[202] These findings indicate that multivalent analogues of carbohydrates can be designed to optimize their thermodynamic binding parameters for maximum enhanced affinities and hence specificities. Future applications of ITC include determining the thermodynamics of binding of lectins to intact natural glycoconjugates.

VIII. Acknowledgment

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IX. References

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