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Thermodynamic binding studies of bivalent oligosaccharides to galectin-1, galectin-3, and the carbohydrate recognition domain of galectin-3

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Galectins are a growing family of animal lectins with common consensus sequences that bind β-Gal and LacNAc residues. There are at present 14 members of the galectin family; however, certain galectins possess different structures as well as biological properties. Galectin-1 is a dimer of two homologous carbohydrate recognition domains (CRDs) and possesses apoptotic and proinvasive activities. Galectin-3 consists of a C-terminal CRD and an N-terminal nonlectin domain implicated in the oligomerization of the protein and is often associated with antiapoptotic activity. Because many cellular oligosaccharide receptors are multivalent, it is important to characterize the interactions of multivalent carbohydrates with galectins-1 and -3. In the present study, binding of bovine heart galectin-1 and recombinant murine galectin-3 to a series of synthetic analogs containing two LacNAc residues separated by a varying number of methylene groups, as well as biantennary analogs possessing two LacNAc residues, were examined using isothermal titration microcalorimetry (ITC) and hemagglutination inhibition measurements. The thermodynamics of binding of the multivalent carbohydrates to the C-terminal CRD domain of galectin-3 was also investigated. ITC results showed that each bivalent analog bound by both LacNAc residues to the two galectins. However, galectin-1 shows a lack of enhanced affinity for the bivalent straight chain and branched chain analogs, whereas galectin-3 shows enhanced affinity for only lacto-N-hexaose, a naturally occurring branched chain carbohydrate. The CRD domain of galectin-3 was shown to possess similar thermodynamic binding properties as the intact molecule. The results of this study have important implications for the design of carbohydrate inhibitors of the two galectins.

Key words: binding thermodynamics/galectin-1/galectin-3/multivalent carbohydrates

Introduction

Galectins are a growing class of β-galactoside-specific animal lectins (Barondes *et al*., 1994). At least 14 members of this family have been found in mammals and designated as galectin-1 through-14 (Cooper and Barondes, 1999; Hirabayashi *et al*., 1997; Visegrady *et al*., 2001; Yang *et al*., 2001). The structures of mammalian galectins can be identified as prototypes (galectins-1, -2, -5, -7, -10, and probably -11, -13, and -14) that exist as monomers or homodimers consisting of one carbohydrate recognition domain (CRD); chimera type (galectin-3) that contains a nonlectin N-terminal domain in addition to the CRD; and tandem-repeat type (galectins-4, -6, -8, and -9) composed of two different CRDs in a single polypeptide chain connected by a linker peptide (Barondes *et al*., 1994; Gabius, 1997; Kasai and Hirabayashi, 1996). The X-ray crystal structures of galectin-1 (Bourne *et al*., 1994; Liao *et al*., 1994), galectin-2 (Lobsanov *et al*., 1993), galectin-7 (Leonidas *et al*., 1998), galectin-10 (Leonidas *et al*., 1995), and the CRD domain of galectin-3 (Seetharaman *et al*., 1998) have been reported.

Galectin-1 and -3 have been among the most widely studied members of the galectins because they have been reported to have different biological activities. For example, galectin-1 induces apoptosis in a variety of cells, including immature T cells (Pace *et al*., 2000), whereas galectin-3 is associated with antiapoptotic activities (Yang *et al*., 1996). Interestingly, galectin-3 blocks the negative growth regulation of galectin-1 on neuroblastoma cells in culture (Kopitz *et al*., 2001). Hence it is important to understand the structural and functional basis for their different biological activities.

Galectin-1 has been shown by analytical sedimentation data and mass spectrometry to be a dimer in solution, and galectin-3 to be a monomer in solution (Kopitz *et al*., 2003; Morris *et al*., 2004). Galectin-1 crystallizes as a dimer in one-dimensional cross-linked complexes with a divalent complex oligosaccharide (Bourne *et al*., 1994) and forms homogeneous cross-linked complexes with asialofetuin (Gupta and Brewer, 1994). Galectin-3 precipitates as a pentamer with certain multivalent carbohydrates and glycoproteins and forms heterogeneous cross-linked complexes (Ahmad *et al*., 2004). Hence a small amount of galectin-3 pentamer is in equilibrium with the monomer in solution, and the monomer is converted to pentamer by mass-action equilibria during precipitation with multivalent oligosaccharides (Ahmad *et al*., 2004). It is also known that the N-terminal domain of galectin-3 is responsible for the oligomerization of the molecule (Birdsall *et al*., 2001; Massa *et al*., 1993; Yang *et al*., 1998).

The carbohydrate binding specificities of galectins-1 and -3 are similar in that they both bind to LacNAc and lactose

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residues (Barondes *et al.*, 1994; Gabius, 1997; Kasai and Hirabayashi, 1996). However, differences exist, including the observation that galectin-3 binds to internal and terminal LacNAc residues in polylactosamine chains, whereas galectin-1 prefers nonreducing terminal LacNAc residues (Ahmad *et al.*, 2002).

Because a large number of cell surface carbohydrates are branched chain structures and often multivalent in their interactions with lectins (Brewer, 1996), it is important to characterize the binding of multivalent carbohydrates with galectins-1 and -3. Hughes and co-workers (Sato and Hughes, 1992), using hapten inhibition, reported binding of bi- and trivalent oligosaccharides to galectin-3. In addition, galectin-1 and galectin-3 from human lung show differential binding toward multivalent mucin type oligosaccharides by hapten inhibition (Sparrow *et al.*, 1987). Galectin-3 has recently been shown to precipitate with certain biantennary branched chain carbohydrates (the 2,4-, 2,6-, 3,6-, and 4,6-pentasaccharides in Figure 1) (Ahmad *et al.*, 2004). To date, however, their has been little thermodynamic data reported for the binding of galectins-1 and -3 to multivalent carbohydrates. Such data are also important in terms of designing carbohydrate inhibitors, such as cluster glycosides of the two galectins.

Isothermal titration calorimetry (ITC) has increasingly been used to determine the thermodynamics of binding of carbohydrates to lectins including multivalent carbohydrates (Dam and Brewer, 2002). In the present study, ITC and hemagglutination inhibition measurements are used to investigate the binding of galectins-1 and -3 to a series of divalent LacNAc analogs possessing linear or branched chain structures (Figure 1). We also report the thermodynamics of binding of the CRD of galectin-3 to carbohydrates to assess the role of the N-terminal domain of the lectin in binding.

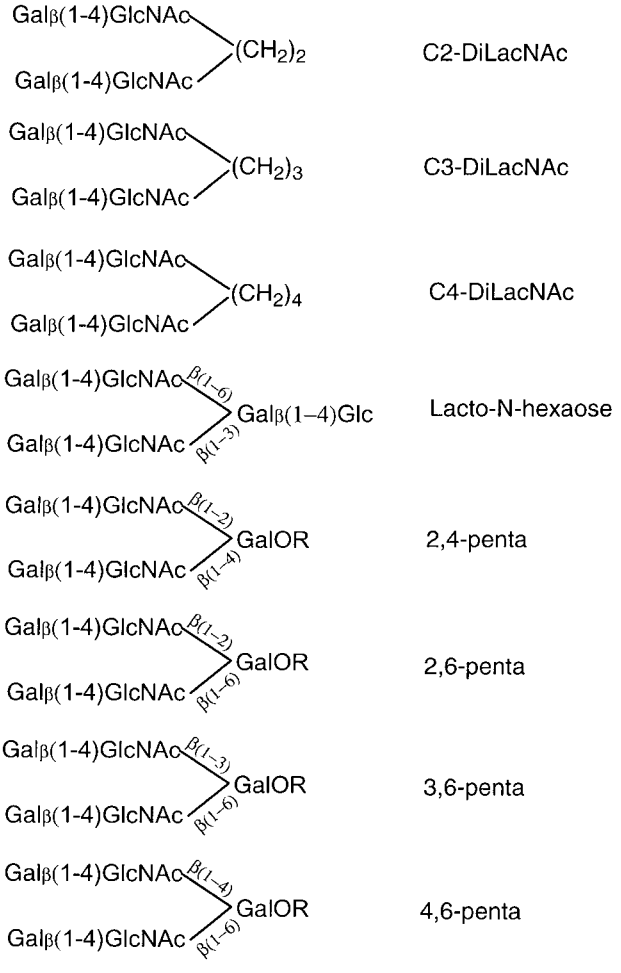

Fig. 1. Structures of the oligosaccharides. The aglycon moiety R in the 2,3-, 2,4-, 3,6-, and 2,6-pentasaccharides is $-(CH_2)_5COOCH_3$. The core galactose residue in these pentasaccharides is in the β-anomeric configuration.

Results

Hemagglutination inhibition studies

Hemagglutination inhibition data for galectins-1 and -3 in the presence of a series of structurally bivalent oligosaccharides including those in Figure 1 are shown in Table I. The inhibitory potencies of C2-, C3-, and C4-DiLacNAc for galectins-1 and -3 are similar to that of LacNAc II for both lectins. The inhibition data for the 2,4-, 2,6-, 3,6-, and 4,6-pentasaccharides in Figure 1 and lacto-N-hexaose in the presence of galectin-1 was also similar to that of LacNAc II. Small increases in the inhibition potencies for lacto-N-hexaose and some of the branched chain pentasaccharides in the presence of galectin-3 are suggested by the data in Table I. However, to accurately determine the affinities of these carbohydrates as well as their thermodynamics of binding to both galectins, ITC measurements were performed.

Thermodynamics of binding of divalent LacNAc oligosaccharides to galectin-1

ITC-derived thermodynamic binding data for galectin-1 at 287 K and 300 K are shown in Table II. The K_a values of

Table I. Relative inhibitory potencies of synthetic and a natural bivalent oligosaccharides for bovine heart galectin-1 and murine recombinant galectin-3

	Relative inhibitory potency[a]	
Saccharides	Galectin-1	Galectin-3
LacNAc II	1.0 (0.41)	1.0 (0.41)
C2 Di-LacNAc	1.4 (0.29)	1.9 (0.22)
C3 Di-LacNAc	1.4 (0.30)	1.5 (0.28)
C4 Di-LacNAc	1.2 (0.34)	1.6 (0.25)
Lacto-N-hexaose	1.5 (0.27)	2.6 (0.16)
2,4-Pentasaccharide	0.70 (0.58)	1.5 (0.28)
2,6-Pentasaccharide	0.80 (0.51)	2.5 (0.17)
3,6-Pentasaccharide	0.85 (0.48)	2.3 (0.17)
4,6-Pentasaccharide	0.70 (0.58)	3.0 (0.14)

[a]Respect to LacNAc-II; concentration (mM) of sugar required for inhibition is shown in parentheses.

Table II. Thermodynamic binding parameters of bovine heart galectin-1 and murine Recombinant galectin-3 to synthetic and a natural bivalent oligosaccharide

Saccharides	K_a[a] ($M^{-1} \times 10^{-4}$)	$-\Delta H$[b] (kcal mol^{-1})	$-\Delta G$[c] (kcal mol^{-1})	$-T\Delta S$[d] (kcal mol^{-1})	n[e]
Bovine heart galectin-1 300 K					
LacNAc II[f]	1.1	9.2	5.5	3.7	1.01
C2-DiLacNAc	0.74	12.1	5.3	6.8	0.55
C3-DiLacNAc	1.5	16.0	5.7	10.3	0.60
C4-DiLacNAc	1.2	17.3	5.6	11.7	0.55
Bovine heart galectin-1 287 K					
LacNAc II[f]	2.4	8.7	5.7	3.0	0.99
Lacto-N-hexaose	1.8	13.7	5.6	8.1	0.66
2,4-Pentasaccharide	3.1	13.3	5.9	7.4	0.48
2,6-Pentasaccharide	1.2	12.4	5.3	7.1	0.45
3,6-Pentasaccharide	2.5	12.6	5.8	6.9	0.46
4,6-Pentasaccharide	5.1	13.5	6.2	7.3	0.46
Murine recombinant galectin-3 300 K					
LacNAc II[f]	1.9	7.5	5.9	1.6	1.01
C2-DiLacNAc	2.1	12.8	5.9	6.9	0.76
C3-DiLacNAc	2.3	17.2	6.0	11.2	0.53
C4-DiLacNAc	2.2	16.2	5.9	10.3	0.57
Lacto-N-hexaose	6.5	13.9	6.6	7.3	0.68
2,4-Pentasaccharide	2.6	17.8	6.0	11.8	0.51
2,6-Pentasaccharide	2.2	20.8	5.9	14.9	0.49
3,6-Pentasaccharide	2.7	20.2	6.0	14.2	0.51
4,6-Pentasaccharide	2.5	19.6	6.0	13.6	0.52
Murine recombinant galectin-3 287 K					
LacNAc II[f]	3.7	9.7	6.0	3.7	0.95
Lacto-N-hexaose	14.7	12.2	6.8	5.4	0.63
2,4-Pentasaccharide	6.1	21.4	6.3	15.1	0.46
2,6-Pentasaccharide	6.1	19.8	6.3	13.5	0.46
3,6-Pentasaccharide	5.3	19.4	6.2	13.2	0.48
4,6-Pentasaccharide	5.9	18.5	6.2	12.3	0.50

[a]Errors in K_a range from 1% to 7%.
[b]Errors in ΔH are 1% to 4%.
[c]Errors in ΔG are less than 2%.
[d]Errors in $T\Delta S$ are 1% to 7%.
[e]Errors in n are less than 4%.
[f]From Ahmad *et al.* (2002).

C2-, C3-, and C4-DiLacNAc at 300 K are 0.74×10^{-4} M^{-1}, 1.5×10^{-4} M^{-1}, and 1.2×10^{-4} M^{-1}, respectively, as compared to 1.1×10^{-4} M^{-1} for LacNAc II. The enthalpy of binding (ΔH) as well as the entropy ($T\Delta S$) increase with increased spacing of the two LacNAc moieties in C2- to C-4 DiLacNAc. ΔH increased from -12.1 kcal mol^{-1} for C2-DiLacNAc to -17.3 kcal mol^{-1} for C4-DiLacNAc, and $T\Delta S$ increased from -6.8 kcal mol^{-1} to -11.7 kcal mol^{-1}, respectively. These values can be compared to ΔH and ΔS values of -9.2 kcal mol^{-1} and -3.7 kcal mol^{-1}, respectively, for LacNAc II. The n values (stoichiometry of binding) of C2-, C3-, and C4-DiLacNAc to galectin-1 were 0.55, 0.60, and 0.55, respectively.

ITC data for the binding of galectin-1 to the pentasaccharides in Figure 1 as well as lacto-N-hexaose were performed at 287 K to enhance their affinities for the protein and maintain an ITC c value between 1 and 200 for accurate measurements. Table II shows that the K_a values for the pentasaccharides were similar to that of LacNAc II (2.4×10^{-4} M^{-1}). There were increases in the enthalpy and the entropy of binding values similar to those observed at 300 K for C2- to C4-DiLacNAc. ΔH values varied between -12.4 to -13.7 kcal mol^{-1} for the four pentasaccharides. The n values of the branched chain oligosaccharides were close to 0.5, with the exception of lacto-N-hexaose, which showed a value of 0.66.

Thermodynamics of binding of divalent LacNAc oligosaccharides to galectin-3

Table II shows ITC data for the binding of galectin-3 to the oligosaccharides in Figure 1. There were little differences in the K_a values at 300 K of C2- to C4-DiLacNAc, and all were similar to that of LacNAc II. ΔH showed an increase from -7.5 kcal mol^{-1} for LacNAc II to -12.8 for C2-DiLacNAc, -17.2 kcal mol^{-1} for C3-DiLacNAc, and -16.2 kcal mol^{-1} for C4-DiLacNAc. The *n* values for the three analogs were close to 0.5.

K_a values at 300 K for the pentasaccharides in Figure 1 were similar to that of LacNAc II. The pentasaccharides possessed larger $-\Delta H$ and $-T\Delta S$ values relative to LacNAc II. The highest affinity branched chain carbohydrate was lacto-N-hexaose, which showed a threefold enhancement relative to LacNAc II. ITC data was also obtained at 287 K for lacto-N-hexaose and the pentasaccharides to compare data with that for galectin-1. The highest affinity ligand was lacto-N-hexaose (14.7×10^4 M^{-1}), which was nearly fourfold greater than LacNAc II. The K_a values of galectins-1 and -3 for the 4,6-pentasaccharide at 287 K were similar, relative to LacNAc II, but the remaining three pentasaccharides showed slightly enhanced affinity at 287 K for galectin-3 relative to LacNAc II.

Thermodynamics of binding of divalent oligosaccharides to galectin-3 CRD

ITC thermodynamic binding data at 287 K for the binding of a variety of carbohydrates to the galectin-3 CRD are shown in Table III. K_a, ΔH, and TΔS values for the binding of LacNAc I and II and dithiogalactoside were similar to the intact galectin-3 molecule (Ahmad *et al.*, 2002). The thermodynamic binding parameters of 2,6-sialyl diLacNAc were similar to that of LacNAc II, indicating that galectin-3 CRD can recognize internal binding epitopes as well as the nonreducing terminal ones. The binding data for lacto-N-tetraose and Di-LacNAc did not show much change compared to that of LacNAc II.

The binding of galectin-3 CRD to the structurally divalent carbohydrates in Figure 1 was also compared to that of intact galectin-3. The binding data for these ligands show a large increase in the enthalpy as with the intact molecule (Table II). The association constants were also similar to that of intact molecule. Lacto-N-hexaose was observed to possess the highest K_a value, which was three times greater than LacNAc II. The ΔH versus TΔS plot (Figure 2C) for the CRD of galectin-3 binding to the carbohydrates in Table III was similar in slope and correlation coefficient to similar plots for galectin-1 (Figure 2A) and galectin-3 (Figure 2B) binding to the carbohydrates in Table II at 287 K.

Discussion

This study investigates the effects of varying the distance separating two nonreducing LacNAc residues in a molecule and the distance separating two LacNAc residues in biantennary branched chain carbohydrates on their binding to galectins-1 and -3. This is important in terms of understanding the interactions of the two galectins with multivalent carbohydrate receptors, as well as the design of carbohydrate inhibitors of the proteins.

Table III. Thermodynamic binding parameters of the CRD of murine recombinant galectin-3 with saccharides at 287 K

Sugars	K_a[a] ($M^{-1} \times 10^{-4}$)	$-\Delta H$[b] (kcal mol^{-1})	$-\Delta G$[c] (kcal mol^{-1})	$-T\Delta S$[d] (kcal mol^{-1})	*n*[e]
LacNAc I	1.9	7.6	5.6	2.0	0.96
LacNAc II	4.1	9.7	6.0	3.6	1.01
Methyl β-LacNAc II	2.3	11.5	5.7	5.8	0.71
Dithiogalactoside	2.4	8.6	5.7	2.9	0.96
Lacto-neo-tetraose	4.8	9.0	6.1	2.9	0.98
Di-LacNAc	7.5	8.8	6.4	2.4	1.03
2,6-sialyl diLacNAc	6.2	10.0	6.3	3.7	0.97
Lacto-N-hexaose	12.1	13.7	6.7	7.1	0.70
C2-DiLacNAc	5.1	21.7	6.2	15.5	0.53
C4-DiLacNAc	5.9	21.0	6.2	14.7	0.51
2,4-Pentasaccharide	7.2	19.6	6.4	13.2	0.50
2,6-Pentasaccharide	4.9	18.5	6.1	12.4	0.49
3,6-Pentasaccharide	7.7	21.2	6.4	14.8	0.53
4,6-Pentasaccharide	5.6	15.5	6.2	9.2	0.47

[a]Errors in K_a range from 1% to 7%.
[b]Errors in ΔH are 1% to 4%.
[c]Errors in ΔG are less than 2%.
[d]Errors in TΔS are 1% to 7%.
[e]Errors in *n* are less than 4%.

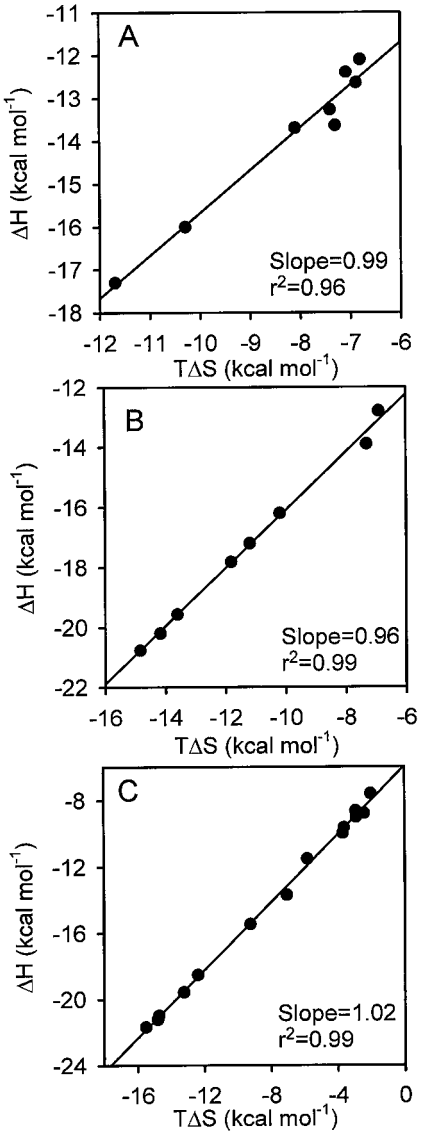

Fig. 2. Entropy enthalpy compensation plots for galectin-1 (**A**), galectin-3 (**B**), and galectin-3 CRD (**C**). Slope and correlation coefficient (r^2) have been shown in each plot.

Binding of linear and branched chain diLacNAc analogs to galectins-1 and -3

To determine the effects of varying the distance between two LacNAc residues in a molecule on their binding interactions with galectins-1 and -3, C2-, C3- and C4-DiLacNAc were synthesized that possess 2-, 3-, and 4-methylene groups, respectively, between two nonreducing LacNAc residues. Likewise, to determine the effects of varying the structures of biantennary LacNAc analogs on their binding interactions with the two galectins, the 2,3-, 2,4-, 3,6-, and 4,6-pentasaccharides in Figure 1 were tested. Hemagglutination inhibition data in Table I, however, show little difference in the inhibition potencies of the linear chain analogs for the two galectins relative to LacNAc II. Hemagglutination inhibition data for the biantennary analogs also show no enhanced inhibition potencies for galectin-1 but suggest some smaller inhibition enhancements for certain biantennary analogs for galectin-3, as was noted for wedge-like glycodendrimers (André *et al.*, 2001). To determine if both types of analogs bind as bivalent carbohydrates as well as their thermodynamics of binding to the two galectins, ITC experiments were performed.

It has been previously shown that the ITC-derived *n* value, the number of binding sites per monomer of protein, is inversely proportional to the functional valency of carbohydrates (Dam *et al.*, 2000). For example, bivalent carbohydrates binding to the lectins Concanavalin A and *Dioclea grandiflora* show *n* values close to 0.5. In these cases, the X-ray crystal structures of the lectins show a single binding site per monomer in both cases (Naismith and Field, 1996; Rozwarski *et al.*, 1998), and therefore the reduced *n* value reflects doubling of the number of binding epitopes per molecule of the ligands. The X-ray crystal structures of galectin-1 (Liao *et al.*, 1994) and the CRD domain of galectin-3 (Seetharaman *et al.*, 1998) show that only one molecule of LacNAc II is associated with each monomer of the two proteins. Table II shows that the *n* values obtained by ITC for LacNAc II is close to 1.0 for galectins-1 and -3, as previously reported (Ahmad *et al.*, 2002), in agreement with the X-ray crystal data. Table II also shows that the *n* values for the linear and branched chain diLacNAc analogs are close to 0.5 for both galectins. Thus the analogs are functionally bivalent, which agrees with their structural valency, and therefore both LacNAc residues in each analog are involved in binding to the two galectins. Importantly, there are cases in which the functional valency of multivalent carbohydrates are less than their structural valency (Dam *et al.*, 2000).

The ITC-derived K_a values at 287 K and 300 K in Table II for C2-, C3-, and C4-DiLacNAc were similar to that of LacNAc II for both galectins, respectively. These results agree with the hemagglutination inhibition data in Table I. The binding enthalpies, $-\Delta H$, of the two galectins to the linear spacer analogs increased by nearly a factor of two relative to LacNAc II. These increases, however, were offset by increases in the entropy of binding, $T\Delta S$, such that no increases occurred in their K_a values. These results contrast with the two- to fourfold and 4–20-fold increases in affinities of the plant lectins Concanavalin A and *D. grandiflora* lectin, respectively, for a series of linear spacer analogs containing two nonreducing mannopyranoside residues, relative to methyl α-D-mannopyranoside (Dam *et al.*, 2000).

The K_a values at 287 K in Table II for lacto-N-hexaose and the 2,4-, 2,6-, 3,6-, and 4,6-pentasaccharides in Figure 1 for galectin-1 are very similar to that of LacNAc II, which agrees with the hemagglutination inhibition data in Table I. The binding enthalpies, $-\Delta H$, of the branched chain analogs were close to those of the linear spacer analogs. The *n* values for these analogs were close to 0.5 indicating their functional bivalency.

The K_a values of galectin-3 at 300 K in Table II for the four pentasaccharides were similar to that of LacNAc II; however, the K_a value of lacto-N-hexaose was nearly threefold greater than LacNAc II. The K_a values at 287 K for the pentasaccharides were slightly greater than that of LacNAc II; however, the value for lacto-N-hexaose was nearly

fourfold greater than that of LacNAc II. The $-\Delta H$ values for the pentasaccharides at 287 K (the ITC *c* value is more optimal than at 300 K) shows a near doubling of that for LacNAc II, as expected for divalent oligosaccharides binding to a lectin (Dam *et al*., 2000). The observation that galectin-3 precipitates as a pentamer to a greater extent with the 3,6-pentasaccharide, followed by the 4,6-, 2,6-, and 2,4-pentasaccharides (Ahmad *et al*., 2004), is thus not due to differences in their K_a values but rather to the stability and solubility of their respective cross-linked complexes. It is also interesting to note that although the K_a values for the four pentasaccharides are similar for both galectin-1 and -3, the four pentasaccharides show differential inhibition activities toward five Gal-specific plant lectins (Gupta *et al*., 1993). Thus the architectures of the binding sites for galectins-1 and -3 appear to be more open toward binding these biantennary branch chain oligosaccharides as compared with certain Gal-specific plant lectins. However, the sixfold enhanced affinity of the plant lectin *Erythrina cristagalli* for the four pentasaccharides relative to LacNAc II (Gupta *et al*., 1993) is not present in the binding of galectins-1 and -3 to the four pentasaccharides. These results are important in terms of designing carbohydrate inhibitors to the two galectins.

The data in Table II show that lacto-N-hexaose binds with three- to fourfold higher affinity to galectin-3 relative to LacNAc II. No similar increase in affinity occurs with galectin-1. Sato and Hughes (1992) reported that binding of lacto-N-hexaose to hamster kidney galectin-3 is weaker than that of straight chain lacto-N-tetraose having the same binding epitopes. However, the data in Table II show that lacto-N-hexaose ($K_a = 6.5 \times 10^{-4}\ M^{-1}$) possesses 2.5-fold higher affinity than lacto-N-tetraose ($K_a = 2.6 \times 10^{-4}\ M^{-1}$) for galectin-3 (Ahmad *et al*., 2002), which confirms ITC studies with human galectin-3 (Bachhawat-Sikder *et al*., 2001). The latter group, however, reported an *n* value for lacto-N-hexaose of one as compared to *n* values between 0.6–0.7 in the present study with galectins-1 and -3, consistent with the structural bivalency of the oligosaccharide.

The sugar binding sites of galectin-1 and galectin-3 show some differences that may help explain the higher affinity of lacto-N-hexaose for the latter. Galectin-3 has been shown to bind to internal LacNAc residues in polylactosamine oligomers, whereas galectin-1 is constrained primarily to nonreducing terminal LacNAc residues (Ahmad *et al*., 2002). The fact that lacto-N-hexaose possesses three potential binding epitopes for galectin-3, one on each branch chain arm, and the core lactose epitope may explain some of the enhanced affinity of galectin-3 for the lacto-N-hexaose relative to galectin-1.

Thermodynamics of binding of oligosaccharides to the CRD of galectin-3

Galectin-3 is the only chimera type galectin and possesses a nonlectin N-terminal region linked to a C-terminal CRD (Barondes *et al*., 1994; Gabius, 1997).The C-terminal CRD domain of galectin-3 is homologous to that of galectin-1 and other members of the galectin family (Cooper, 2002; Jia and Wang, 1988). Indeed, the CRD alone of galectin-3 binds lactose but lacks the hemagglutination activity and cooperative binding associated with intact galectin-3 (Hsu *et al*., 1992). This suggests that the N-terminal domain is important for aggregation of the protein. Indeed purified N-terminal fragments have been found to self-associate (Mehul *et al*., 1994).

Recently, the quaternary structure of recombinant murine galectin-3 in solution was determined by sedimentation velocity and equilibrium measurements, and the results show that the lectin is predominantly a monomer (Morris *et al*., 2004). However, recent studies also show that recombinant murine galectin-3 precipitates as a pentamer with the 2,4-, 2,6-, 3,6-, and 4,6-pentasaccharides in Figure 1 (Ahmad *et al*., 2004). This indicates that the galectin-3 monomer in solution is in equilibrium with a small percent of pentamer that undergoes cross-linking and precipitation with certain multivalent carbohydrates. Hence it is important to determine the effects of the N-terminal region on the C-terminal CRD domain of galectin-3 because large changes occur in the quaternary structure of galectin-3 on binding and precipitating with multivalent carbohydrates.

Table III shows ITC data at 287 K for the binding of the CRD domain of recombinant murine galectin-3 to the listed carbohydrates. The thermodynamic data is not significantly different from that of the intact molecule (Table II) and recently reported ITC data for the intact molecule (Ahmad *et al*., 2002). Little difference in K_a values is observed for the linear spacer DiLacNAc analogs as well as the 2,4-, 2,6-, 3,6-, and 4,6-pentasaccharides relative to LacNAc II. The other thermodynamic binding parameters are also similar to those of the intact molecule. A slight enhancement in affinity is observed for DiLacNAc, the linear dimeric analog of LacNAc II, relative to LacNAc II, which was observed for the intact galectin-3 molecule (Ahmad *et al*., 2002). There is also a nearly threefold enhanced affinity toward lacto-N-hexaose, which is present for the intact molecule.

Interestingly, Figure 2 shows that the ΔH versus $T\Delta S$ plot for the CRD of galectin-3 binding to the carbohydrates in Table III is similar to similar plots for galectin-1 and galectin-3 binding to the carbohydrates in Table II. This also suggest little change in the carbohydrate binding activity of the CRD domain of galectin-3 relative to intact galectin-3 as well as galectin-1.

The present results indicate that the N-terminal domain has little effect on the carbohydrate binding properties of the CRD domain of intact, monomeric galectin-3 in solution.

Summary

The observations that galectins-1 and -3 do not possess substantial enhanced affinities for straight chain and branch chain bivalent LacNAc analogs has important implications for the design of carbohydrate inhibitors of these proteins. So-called cluster glycosides have been synthesized and shown to possess enhanced affinities for certain lectins. For example, the mannose-specific *D. grandiflora* shows 20-fold enhanced affinity for a synthetic divalent mannose carbohydrate and nearly 60-fold enhanced affinity for a tetravalent trimannoside analog (Dam *et al*., 2000). The absence of substantial enhanced affinities of galectins-1 and -3 for the divalent LacNAc analogs suggest

that carbohydrate analogs possessing higher valencies will not exhibit much greater affinities than LacNAc. Multivalent analogs of lacto-N-hexaose may also not possess much greater affinities than the parent molecule. Higher-affinity interactions of these two galectins and possible other members of the galectin family may depend on cross-linking interactions with separate carbohydrate epitopes on the surface of a matrix (extracellular matrix) or cell surface. Synthetic analogs that incorporate these design principles may give rise to higher affinities for galectins, including those in the present study. Alternatively, sugar analogs, which possess synthetic side chains, have been recently shown to possess enhanced affinity of the parent sugars for specific galectins, as in the case of galectin-3 (Sorme *et al.*, 2003).

Materials and methods

Materials

Bovine heart was purchased from Pel-Freez Biologicals (Rogers, AR). Fetuin, LacNAc II (Galβ1,4GlcNAc), dithiogalactoside, and lacto-N-tetraose were obtained from Sigma Chemical (St. Louis, MO). LacNAc I (Galβ1,3GlcNAc) and methyl β-LacNAc I were obtained from Toronto Research Chemicals (Toronto, Canada). Lacto-N-hexaose was purchased from V-labs (Covington, LA). Synthesis of C2-DiLacNAc, C3-DiLacNAc, and C4-DiLacNAc have been reported (Halasayam *et al.*, 2003), as have been the 2,4-, 2,6-, 3,6-, and 4,6-pentasaccharides (Sabesan *et al.*, 1992). DiLacNAc, 2,6-sialyl diLacNAc, and methyl β-LacNAc II were chemically and/or enzymatically synthesized and will be reported elsewhere. The structures and purity of carbohydrates were established by ^{1}H nuclear magnetic resonance spectroscopy at 500 MHz. All other reagents were of analytical grade.

Purification and expression of the proteins

Fetuin was desialylated and purified as described earlier (Gupta and Brewer, 1994). Asialofetuin-Sepharose was prepared by a published procedure (Parikh *et al.*, 1974). Galectin-1 from bovine heart was purified using the procedure for the isolation of calf spleen galectin-1 in our laboratory (Gupta and Brewer, 1994). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the affinity-purified lectin was found to contain one additional band. Affinity-purified lectin was fractionated into two clear peaks, I and II, by gel chromatography using Sephadex G-50 column in the presence of 0.05 mM lactose. The second peak corresponded to galectin-1 and was found to be pure by SDS–PAGE. Fractions containing the second peak were pooled and dialyzed extensively against phosphate buffered saline containing 10 mM β-mercaptoethanol, and stored at 4°C.

Recombinant murine galectin-3 was purified after recombinant expression, using the plasmid prCBP35 kindly provided by J. L. Wang (East Lansing, MI) and *Escherichia coli* JA221 cells (Agrwal *et al.*, 1993). Galectin-3 thus produced was found to be pure by SDS–PAGE. Galectin-3 batches were found to have different *n* values by ITC using LacNAc II as a ligand. Therefore each batch of galectin-3 was subjected to several affinity chromatography cycles on ASF-Sepharose until an *n* value of 1 was achieved by ITC. Galectin-3 samples were tested with LacNAc II before and after an ITC experiment with a new saccharide. Galectin-3's CRD was produced by collagenase treatment of the intact molecule and purified on a asialofetuin-Sepharose column. The purified galectin-3 CRD moved as a single band on SDS–PAGE.

Monomeric lectin concentrations were determined spectrophotometrically at 280 nm using the specific extinction coefficient of the three proteins ($E^{1\%}_{1\ cm}$). A value of 5.4 ($E^{1\%}_{1\ cm}$) was used for galectin-1, 6.1 ($E^{1\%}_{1\ cm}$) for galectin-3, and 5.5 ($E^{1\%}_{1\ cm}$) for galectin-3 CRD as determined from ITC experiments. Molecular masses of the galectins were determined by matrix-assisted laser desorption ionization and electrospray ionization mass spectrometry and were found to be 14,500 Da for galectin-1, 27,500 Da for galectin-3, and 14,500 Da for galectin-3 CRD. The carbohydrate concentration was measured by the phenol–sulfuric acid method (Dubois *et al.*, 1956; Saha and Brewer, 1994).

Hemagglutination inhibition assays

Hemagglutination assays were performed by serial dilution technique (Osawa and Matsumoto, 1972). Rabbit erythrocytes 3% (v/v) were prepared in 20 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.2, whereas the sugar and protein solutions were prepared in 20 mM sodium phosphate buffer containing 0.15 M NaCl and 10 mM β-mercaptoethanol, pH 7.2. The final concentration of β-mercaptoethanol in the assay was 5 mM.

Isothermal titration microcalorimetry

ITC experiments were performed using an MCS Microcalorimeter from Microcal (Northampton, MA). A typical titration profile is shown in Figure 3. Protein and carbohydrate samples were prepared in 20 mM sodium phosphate buffer containing 0.15 M NaCl and 10 mM β-mercaptoethanol, pH 7.2. Protein solution (2.5 ml) was taken in a syringe and loaded into the ITC sample cell (cell volume = 1.34 ml). Reducing agents in the buffer were found to increase time for the baseline stabilization which varied from 3 to 6 h. The effect of dithiothreitol on baseline stabilization was greater than β-mercaptoethanol. Degassing of protein solutions and using fresh buffers (maximum 12 h storage time) was found to decrease the time for baseline stabilization from 4 fto 1 h. After the baseline had stabilized, 4-μl injections of the carbohydrate solution were added from the computer-controlled 100-μl rotating syringe (400 rpm) into the protein solution, and exothermic heat changes accompanying the additions were recorded. The time period between the two consecutive injections was fixed at 4 min to allow the exothermic peak to return to the baseline. Control experiments were carried out by making identical injections into the cell containing buffer with no protein.

The experimental data were fitted using software ORIGIN version 2.9 supplied by Microcal, with ΔH (enthalpy change in kcal mol^{-1}), K_a (association constant in M^{-1}), and *n* (number of binding sites/monomer) as adjustable parameters. The unitless constant $c = K_a M_t$, where M_t is the initial protein concentration, and K_a is the association

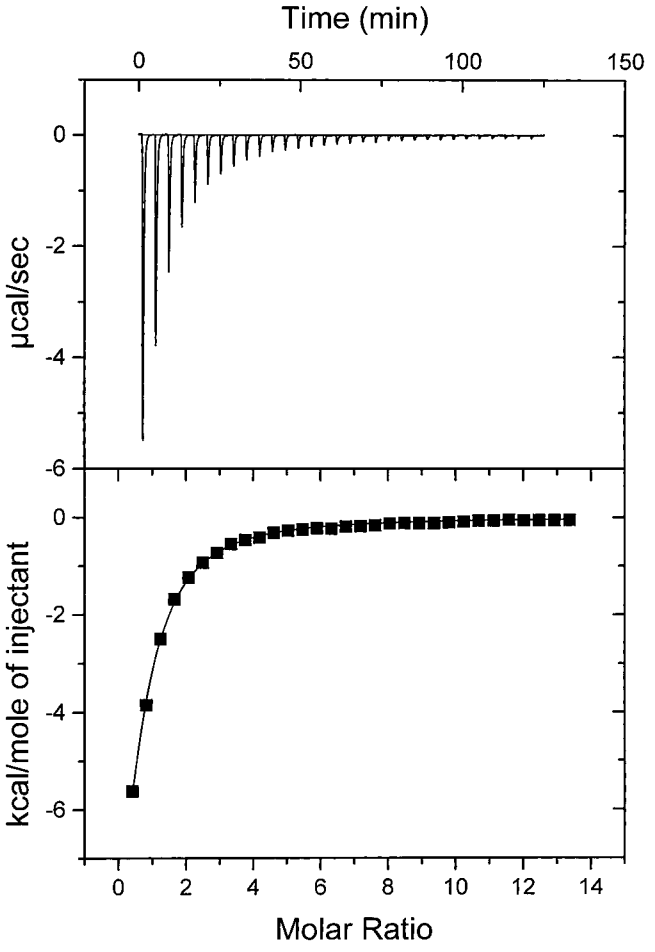

Fig. 3. ITC profile of galectin-3 (53 μM) with C2-DiLacNAc (7.4 μM) at 300 K in 20 mM sodium phosphate buffer, pH 7.20, containing 0.15 M sodium chloride and 10 mM β-mercaptoethanol.

constant in M^{-1}. The value of c is of great importance necause it determines the shape of the binding isotherm (Wiseman *et al.*, 1989). All experiments were performed with c values of $1 < c < 200$. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2′-monophosphate supplied by the manufacturer. Thermodynamic parameters were calculated by using the standard equation, $\Delta G = \Delta H - T\Delta S = -RT \ln K_a$, where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the absolute temperature in kelvin, and $R = 1.98$ cal mol^{-1} K^{-1}.

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Abbreviations

CRD, carbohydrate recognition domain; ITC, isothermal titration calorimetry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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