Glycoconjugate Journal 17, 543–551, 2000

Affinity enhancement by multivalent lectin–carbohydrate interaction

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The binding of simple carbohydrate ligands by proteins often requires affinity enhancement to attain biologically relevant strength. This is especially true for endocytotic receptors and the molecules that engage in the first-line of defense. For such purposes, nature often utilizes a mode of affinity enhancement that arises from multiple interactions between the binding proteins and the carbohydrate ligands, which we term glycoside cluster effect. In this review article we give a number of examples and describe important factors in the multi-valent interactions that govern the degree of affinity enhancement.

Keywords: **glycoside cluster effect, multi-valent interaction, C-type lectins, affinity enhancement**

Abbreviations: **CRD, carbohydrate-recognition domain; ASGP-R, asialoglycoprotein receptor; RHL, rat hepatic lectin; MBP, mannose-binding protein; SP, alveolar surfactant protein; BSA, bovine serum albumin; ah, 6-aminohexyl.**

Introduction

Since the discovery of mammalian hepatic lectin by Ashwell and Morell about three decades ago, the number and the type of animal lectins have increased dramatically. The major animal lectin types that we know at the moment include C-type lectins, galectins (S-type) and siglecs (I-type), and the sugar-binding characteristics of some members from each family are quite well documented [1]. The basic binding site architecture of both C-type lectins and siglecs is small, accommodating only a single monosaccharide unit, which is sialic acid for siglecs and either Gal- or Man-related structures for C-type lectins. The sugar structure recognized by galectins is a disaccharide, lactose or N-acetyl-lactosamine, which can be internally located in the sugar chain.

The binding affinities of these lectins for carbohydrate ligands as assessed by physical methods and by inhibition assays are quite low, K_D generally ranging between 1–0.1 mM. There are cases, however, in which the binding affinity is enhanced by the extended sugar structure interacting with other areas of protein surface, as well as by the protein–protein interaction. The binding affinity can also be dramatically increased by a concomitant clustering of lectin binding sites and carbohydrate recognition units. In this article, we will present mostly our own experimental results to discuss important factors that govern the degree of affinity enhancement in the multivalent interactions. We call this affinity enhancement due to multivalent interaction, which is substantially larger than the effect of the increased concentration, the *glycoside cluster effect* [2]. In this article the focus will be mainly on certain C-type lectins with a brief discussion on the dimeric galectins.

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C-type lectins

All C-type lectins contain a globular domain called a *carbohydrate recognition domain* (CRD) that shares certain degree of sequence identity, but more importantly these domains appear to share structural similarity. The C-type CRD requires calcium ion to maintain sugar-binding ability, whence the name C-type was derived [3]. Although characterized initially as a sugar-binding domain, these domains are now known as C-type lectin-like domains [4] and can bind other classes of ligand, which may or may not require calcium. The proteins that contain a C-type lectin-like domain can be subdivided into several groups based on the other domains to which it is connected, as shown in Figure 1 [5]. Four of these subgroups have at least one member in which the biological function is well characterized as sugar-dependent. These are: Group II, type II transmembrane proteins in which the extracellular domain is composed of a C-terminal C-type CRD and a heptad stalk; Group III (collectins), soluble proteins having a C-terminal C-type CRD connected to a collagenous stalk; Group IV (selectins), type I transmembrane proteins in which the extracellular domain is composed of an N-terminal C-type CRD, an EGF domain and

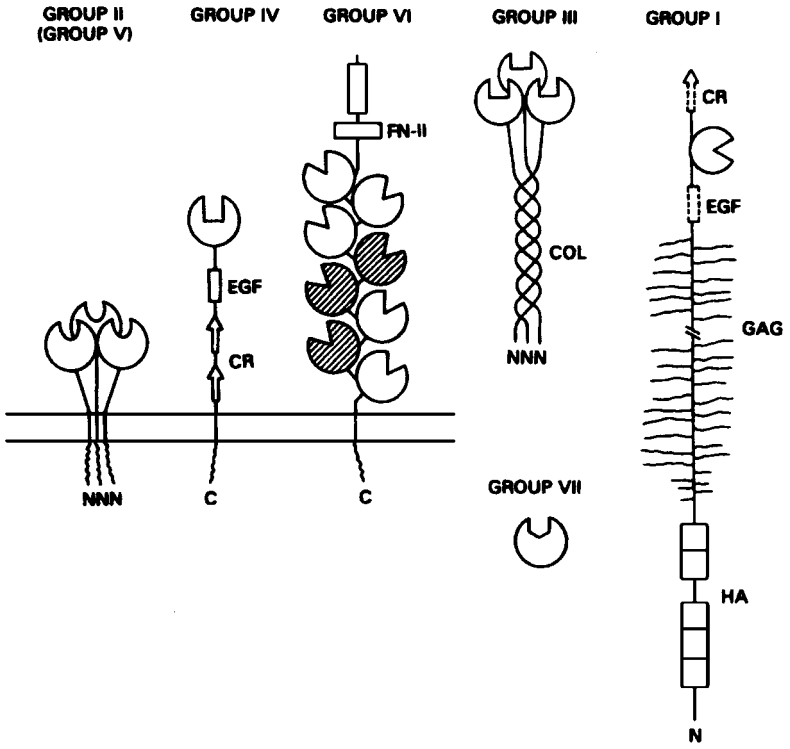


Figure 1. Subgroups of the C-type lectin family. Courtesy of K. Drickamer, *Curr. Opin. Struct. Biol.* (1993) **3**: 393, by permission of Current Biology, LTD.

multiple complement repeat units; and Group VI, type I transmembrane proteins having multiple C-type CRDs.

Group II C-type lectins

Both the Gal/GalNAc-binding mammalian hepatic lectin, also known as asialoglycoprotein receptor (ASGP-R), and the GlcNAc-binding avian hepatic lectin belong to this family, as well as more recently discovered natural killer cell C-type lectin-like proteins. Both hepatic lectins are endocytotic receptors with a binding affinity for multivalent ligands that is so high ($K_D \leq$ nM) that the ligand–receptor complex can remain associated during the endocytosis *via* coated pits and subsequent travel to the endosomes [6]. The heptad stalk of hepatic lecins promotes trimerization, and the trimers further dimerize to form stable hexamers. On the hepatocyte cell surface, hexamers are further packed densely to form a large array of binding sites, which is the foundation of immensely enhanced affinity.

The importance of multivalent interaction in generating high affinity was first demonstrated by Ashwell and coworkers, who used a number of desialylated serum glycoproteins having various numbers of exposed Gal residues, to show that a 4-fold increase in the number of terminal Gal residues on the protein can elicit more than 10 000-fold higher affinity toward rabbit hepatic lectin on the plasma membrane [7]. We used a variety of bovine serum albumin (BSA)-based neoglycoproteins [8] that carry various numbers of a single sugar species, usually a monosaccharide or a disaccharide, to unequivocally demonstrate that the hepatic lectin recognizes terminal Gal and GalNAc residues and the presence of the penultimate sugar is not important. A linear increase in the density of the terminal Gal/GalNAc residues produces an exponential increase in affinity (Figure 2) [9].

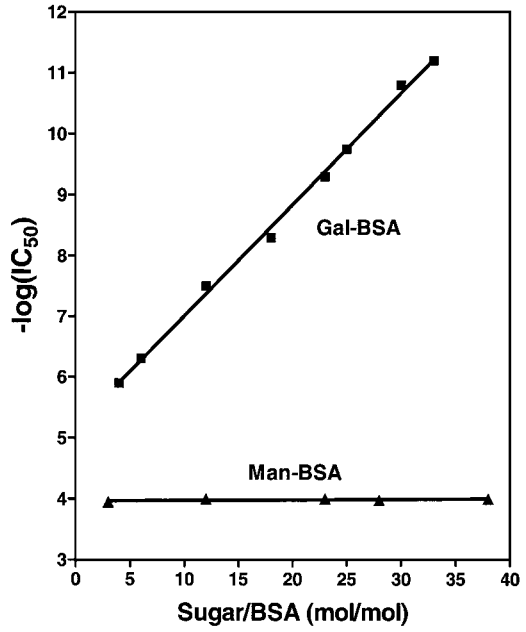


Figure 2. Inhibition of ^{125}I-ASOR binding to the rabbit liver plasma membrane by Gal-BSA and Man-BSA at various sugar coupling levels.

Surprisingly a very large affinity enhancement can also be achieved by much smaller molecules having just two or three terminal Gal residues [10]. Table 1 column 2 shows IC_{50} values of Gal-terminated, complex-type glycopeptides and synthetic oligosaccharides that represent terminal structures of the complex-type N-glycans (See Figure 3 for structures). The inhibition assays were carried out using concentrations of both the lectin and the reference ligand [usually ^{125}I-asialoorosomucoid (ASOR)] well below the dissociation constant. Under such conditions, IC_{50} approaches K_D [11]. In addition, we calculated K_D by a curve-fitting program [12] for a number of inhibitors. For this purpose, about 10 inhibition data points (within the range of 10% to 90% inhibition) were fitted with the predetermined K_D of the reference ligand (obtained by the curve-fitting of its binding isotherm). The K_D values thus obtained are generally only slightly lower than the corresponding IC_{50} values, the largest difference being $\sim$10 fold. The following conclusions can be made from the hepatocyte inhibition data presented in Table 1.

1) The highest affinity increments generated by the best divalent and trivalent structures are close to the maximally attainable enhancement, if one assumes that each Gal-binding site generates an affinity corresponding to $K_D \simeq 0.6$ mM.
2) The addition of the fourth Gal residue, as in the tetraantennary structure, does not generate appreciable additional enhancement.
3) Within the same valency group, K_D can vary by more than 100-fold (e.g., compare NONA I vs NONA II).

These and other observations on the structure–affinity relationship led us to hypothesize that the basic binding unit

Table 1. The IC_{50} values of oligosaccharides and glycopeptides.

	IC_{50}, μM	
	Rabbit hepatocyte	*Rabbit/Rat soluble lectin*
monovalent		
galactosides[1]	400–700	400–800
divalent		
Penta-2,4[2]	0.3	30
Penta-2,6	4.5	70
BI-GP	2.4	ND[3]
trivalent		
NONA-I	0.007	0.9
NONA-II	0.15	2.2
TRI-GP	0.006	ND
tetravalent		
UNDECA	0.0034	3.0

[1]Several galactosides used as inhibitors include methyl α/β-D-galactopyranoside and Galβ(1,4)GlcNAcβ(1,2)Man.
[2] See Figure 3 for structures.
[3]ND: not determined.

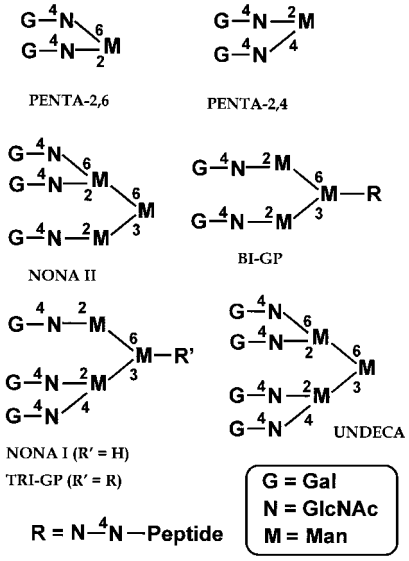


Figure 3. Structures of Gal-terminated oligosaccharides and glycopeptides.

of rat hepatic lectin on the hepatocyte surface is composed of three binding sites, and they are held in a configuration complimentary to the terminal Gal residues of NONA I (which represents the non-reducing terminal structure of the most common complex-type triantennary glycan) in its preferred conformation. This conformation places the three terminal Gal residues separated spatially by 15, 20, and 25 Å [13]. The fact that Penta-2,4 and NONA I produced affinity enhancements that are maximally (or close to maximally) attainable means that the binding event elicits little penalty in the entropic energy, suggesting that hepatic lectin subunits are held rigidly by a force that maintains this complementary configuration.

There are other experimental data to suggest that there is rigid organization of hepatic lectin subunits on rat hepatocyte surface. Three isomeric photoaffinity-labeling reagents, based on the TRI-GP structure, labeled the major (RHL-l) and the minor (RHL-2/3) subunits of hepatic lectin on the rat hepatocyte surface in a very specific manner; i.e., only one specific arm of the TRI-GP structure engages RHL-2/3, while the other two arms engage only RHL-1 [14]. Although the RHL-1 subunit is more abundant than RHL-2/3 by 2 to 4 fold, it was labeled on the hepatocyte surface or plasma membrane much less intensely by reagents involving large molecule, such as radioiodination catalyzed by lactoperoxidase and photoaffinity labeling by certain affinity labeling reagents [15,16]. Such results suggest that the hepatic lectin subunits are held tightly together so as to make the RHL-1 subunit somewhat cryptic. Moreover, we demonstrated that a single molecule of ASOR ($\sim$40 kDa) can eclipse a number of potential TRI-GP binding sites, which suggests that many RHL hexamers are tightly packed on the hepatocyte cell surface [17].

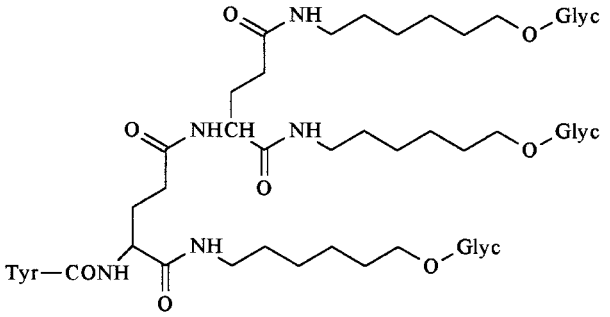
How much affinity enhancement will there be, if the lectin subunits are not so rigidly and complementarily organized? The detergent-solubilized and affinity-purified lectins from

both rat/rabbit liver and chicken liver appear to have the same basic subunit organization as on the hepatocyte surface, i.e., they exist as hexamers in the detergent micelle. It appears, however, the force which was responsible in keeping subunits in tight and rigid organization is no longer fully operative, since all subunits are now equally accessible to 'large' and 'small' reagents alike [16]. As shown in the last column of Table 1, the same set of oligosaccharides still produced respectable affinity enhancement, but the magnitude is greatly diminished. Note also that the IC_{50} values among divalent and trivalent structures are much closer to each other, i.e., Penta-2,4 and NONA I structures no longer possess the special advantage over other oligosaccharide structures. It appears that, in contrast to the hepatocyte system where the binding of Penta-2,4 and NONA I elicits little entropic penalty, the binding of the same two ligands by the solubilized RHL, in which the subunits are more flexibly organized, would impose a configurational restriction on subunit organization. This would result in negative entropic contribution to the binding energy, and hence a much lower degree of affinity enhancement.

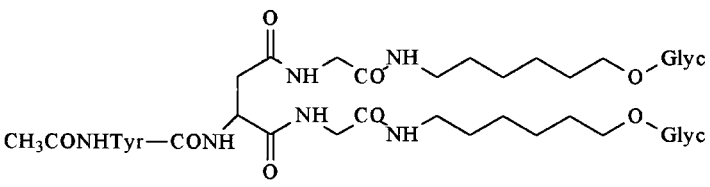
We also have examples of rigidly organized hepatic lectin on hepatocyte surface binding to flexible multivalent ligands. We synthesized a series of divalent and trivalent glycosides that contain only one specific sugar, Gal (Lac) or GalNAc for mammalian hepatic lectins and GlcNAc for chicken hepatic lectin [18,19]. As shown in Figure 4, the divalent structures are based on aspartic acid and the trivalent structures on γ-glutamylglutamic acid to provide branching points. For the divalent structures, the arm connecting the sugar residue to the carboxylic acid of Asp was varied by addition of glycyl residues as well as by changing the aglycon.

Results of inhibition assays using these synthetic structures with the hepatocyte system are shown in Table 2, columm 2 [18,19]. The affinity enhancement observed with these compounds are quite reasonable, although lower than that observed for Penta-2,4 and NONA I. These data and more extensive variation of arm with divalent GlcNAc analogs using the chicken hepatocyte system [18] indicated that the optimal arm length is G-ah (glycyl aminohexyl) for a monosaccharide and ah (aminohexyl) for a disaccharide. The derivatives of aspartate with these arm–sugar combinations give maximal terminal sugar-to-sugar spatial separation of ~ 30 Å, which should allow simultaneous occupation of binding sites separated by 15, 20 and 25 Å. Structures with much shorter or much longer arms both had decreased affinity enhancement. The table also shows that GalNAc-based multivalent structures give much higher degree of affinity enhancement than the corresponding Gal-based structures. This is expected, since GalNAc itself binds 50–80 fold tighter to the rat hepatic lectin binding site than Gal or Lac.

Columm 3 of Table 2 shows that the degree of affinity enhancement decreases further, if both lectin and ligands are flexible. Since ΔH of the interaction is not expected to change



YEE(ah-Glyc)3 (***Trivalent***)



NAcYD(Gah-Glyc)2 (***Divalent***)

Figure 4. Structural designs of synthetic divalent and trivalent neoglycopeptides.

Table 2. The IC_{50} values of synthetic di- and tri-valent inhibitors[1].

	IC_{50}, μM	
Inhibitor	*Rat hepatocyte*	*Rat soluble lectin*
Gal-terminated		
monovalent		
Lac	300	600
Lac-ah	40	190
divalent		
$D(ah\text{-}Lac)_2$	0.6	7
$YD(ah\text{-}Lac)_2$	4	8
$D(Gah\text{-}Lac)_2$	7	30
trivalent		
$YEE(ah\text{-}Lac)_3$	0.05	3
GalNAc-terminated		
monovalent		
GalNAc	4	14
GalNAc-ah	4	5
divalent		
$D(Gah\text{-}GalNAc)_2$	0.004	0.08
trivalent		
$YEE(ah\text{-}GalNAc)_3$	0.0002	0.004

[1]Data from ref. [19]. For divalent and trivalent structures, see Figure 4.

significantly with subunit organization, the decreased affinity as compared to the rat hepatocyte experiment (colunm 2) is likely to have arisen almost entirely from the negative effect of ΔS.

Group III C-type lectins

Lectins of this group are also known as collectins because they contain a prominent collagen stalk in addition to the lectin domain. Members of this family so far discovered all appear to function as innate defense molecules [19], and the binding of the ligand is either purely through carbohydrate, as in the case of mannose-binding proteins (MBPs), or partially via sugars as in the case of surfactant protein A (SP-A) and SP-D. The basic polypeptide construct of the subunits of this group is, from the N-terminal end, a short cysteine-rich domain followed by collagen domain, a short heptad and the C-terminal C-type CRD. Due to the presence of collagenous domain and heptad, the basic organizational unit is a trimer, as shown in Figure 1. The trimers are further bundled together at the N-terminal end to form aggregates. In the case of MBP and SP-A, the aggregates take the form of 'bouquet of flower' of up to six trimeric units, whereas in bovine conglutinin and SP-D, four trimers aggregate to form 'cruci-form structure (Figure 5). The flower-bouquet structure of MBP strikingly resembles that of Clq, the first component of the complement cascade, and in fact, MBP can initiate the complement cascade and serve as an opsonin just as Clq does. However, the event that triggers these biological responses is the direct binding of MBP to the target organism via sugar recognition, and does not require intermediary binding to IgG.

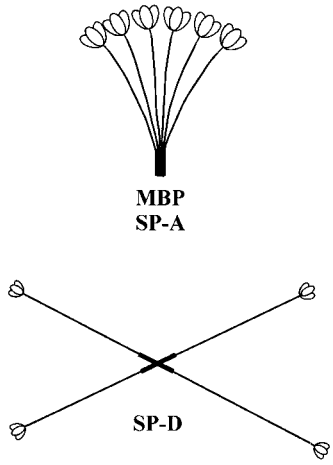


Figure 5. Two types of quaternary assembly of collectin subunits.

We carried out a number of studies on the binding specificity, as well as the glycoside cluster effect, using a fragment of MBP that was composed of entire CRD and the heptad neck region [20]. This molecularly-engineered fragment also formed a stable trimer. Although known as mannose-binding protein, MBP is capable of binding also GlcNAc, L-Fuc and Glc with nearly equal affinity [21]. This broad sugar-binding capacity, which is a great asset for a defense molecule, is accomplished by direct coordination of a calcium ion with two vicinal, equatorial hydroxyl groups of sugars [22]. Together with hydrogen bonding to the same two OH groups, the bulk of the binding energy can be generated by just these two OH groups.

Although the binding energy generated at each site is rather low ($IC_{50} \sim 1$ mM), up to 18 binding sites on the native MBP should generate very high affinity when bound to the surface of invading organisms. Interestingly, the X-ray structure of the trimer shows the three CRDs to be spread out in such a way that the binding sites are far apart (~ 53 Å for rat MBP trimer), and all sites are pointing upward away from the stalk (Figure 6) [23]. Such a configuration of binding sites would be very suited for latching on to the target sugars which may be well-separated on organisms. BSA neoglycoproteins with increasing numbers of Man showed exponential enhancement on affinity (Figure 7), as was observed for hepatic lectins [21]. However, the slope value for MBP was smaller than that for the mammalian hepatic lectin, and the maximum affinity leveled off around 30 nM, when more than 24 Man residues were present per molecule of BSA. Since the per-site dissociation constant for Man is $\sim$1 mM, the maximally enhanced affinity (K_D) by the occupation of two sites would be ~ 1 μM, and that generated by the occupation of three sites would be ~ 1 nM. The observed maximal IC_{50} of 30 nM by the highly mannosylated BSA derivatives lies between 1 μM and 1 nM. This suggests that all three binding sites of the MBP trimer are probably occupied in this binding event, but the affinity enhancement is less than optimal, perhaps due to the binding-site configuration of the MBP trimer being somewhat

Figure 6. A model of MBP-trimer interacting with oligo-Man glycopeptides, made by superposition of monomer-oligosaccharide complex structure onto each monomer unit of the trimer structure. Note the location and the orientation of the sugar-binding sites. Courtesy of W.I. Weis and K. Drickamer, *Structure* (1994) **2**: 1227, by permission of Current Biology, LTD.

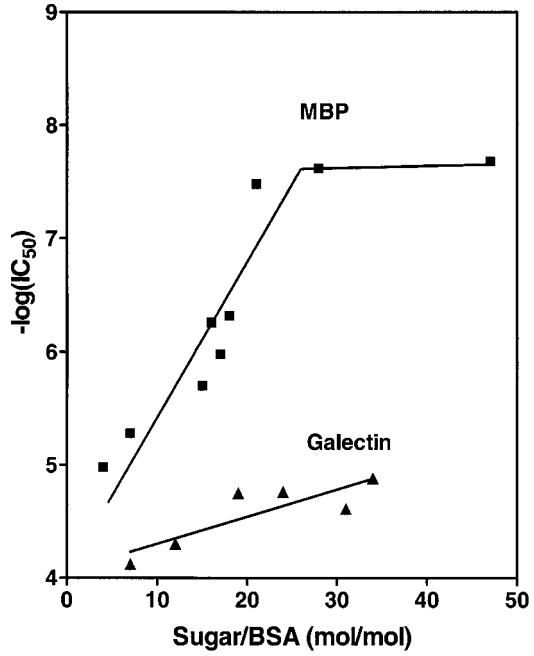


Figure 7. Inhibition data of lectin binding by neoglycoproteins; $-\text{Log}(IC_{50})$ (*y*-axis) is plotted against the inhibitor sugar density (*x*-axis). ■, MBP-trimer binding to ^{125}-I-Man_{28}-BSA. Inhibitors are Man-BSAs carrying various numbers of Man residues. ▲, binding of human splenic galectin to lactose-modified Sepharose beads. Inhibitors are Lac-BSAs carrying various numbers of lactose residues. For detail, see Lee *et al.* (1991) and Lee *et al.* (1990), respectively.

flexible. A steeper increase in the affinity of RHL on the hepatic plasma membrane and the observation that the curve never leveled off (Figure 2) agree well with our hypothesis of a tight organization of binding sites and clustering of many trimeric or hexameric units of RHL on the cell membrane.

Since the three binding-sites of MBP trimer are more widely separated than those of hepatic lectins, none of the naturally derived oligo-mannose type glycans, where the outermost sugar spans are probably either comparable to or less than those of NONA I, showed any glycoside cluster effect [24]. In fact, none of the divalent and trivalent synthetic structures of the type shown in Figure 4 is physically capable of binding simultaneously to the two or three sites of MBP. Furthermore, when we synthesized and tested a divalent mannoside of much wider span, $[\text{Man-O-}(CH_2)_6\text{NH-CO}(CH_2)_5\text{NH-CO}(CH_2)_2\text{-CONH}(CH_2)_2\text{O}(CH_2)_2]_2\text{O}$, with an expected Man-to-Man distance of more than 60 Å, its IC_{50} was only 2-fold lower than simple mannosides, indicating that no affinity-enhancement (R.T. Lee and Y.C. Lee, unpublished result). Perhaps a very high entropic energy expenditure associated with tying up two ends of a long and flexible molecule negates any gain in the binding free-energy, especially since the binding energy generated at each site is quite small.

In another example, Glick *et al.* [25] reported that divalent glycosides of sialic acid of various designs and spans all failed to produce any affinity-enhancement when tested on a soluble trimeric ectodomain of influenza virus hemagglutinin, although the X-ray structure of the trimer [26] suggested that some of their glycosides should have been able to occupy two sites of the hemagglutinin simultaneously.

Group IV and Group VI lectins

Just a few comments will be offered on the possible role of the cluster effect for these lectins.

Three selectins belong to Group IV C-type lectin family. Selectins appear not to self-associate in any significant degree and appear to utilize mechanisms other than glycoside cluster effect, such as extended binding area and possible protein–protein interactions, to increase the monomeric binding affinity to the level of K_D of μM or lower [27]. Since the ligand binding by this group of immunologically important molecules requires relatively fast on-off rate, the individual binding event probably does not require glycoside cluster effect. A sort of clustering effect is probably operating on the cell surface, not for the purpose of increasing binding affinity, but for the purpose of producing the lymphocyte rolling phenomenon [28].

Group VI, trans-membrane lectin family is an interesting group of cell surface receptors. A prominent feature of the protein backbone construct of this group is the presence of tandemly-linked multiple (8 or more) C-type CRDs [29]. At the moment only one member of this group, macrophage mannose-binding receptor, is a truly carbohydrate-binding protein. A receptor for secretory phospholipase A_2 is an important non-lectin member of this group [30]. Macrophage mannose receptor, like MBP, has broad sugar-binding specificity, and is a defense molecule capable of ingesting microorganisms, such as yeast. For the tight binding of mannan and yeast, several of the 8 CRDs are required [31].

Of the two designs of clustering sugar-binding sites adopted by defense molecules, i.e., the trimeric CRDs further bundled together *via* a long and somewhat flexible stalk as found in MBP and the sequential arrangement of multiple CRDs as found on the macrophage receptor, the former appears to be more easily adaptable to various sugarbinding orientations. Exactly what advantage, if any, the multiple CRD design has is an interesting question.

Galectins

Galectins constitute yet another group of lectins on which CRDs share different sequence homology from that of the C-type lectins [32]. The tertiary structure of galectin CRD assumes a β-sheet sandwich that resembles remarkably that of legume lectins, although there is no significant amino acid sequence similarity [33]. All galectins bind β-galactosides, the preferred disaccharide sequence being lactose and N-acetyl lactosamine. Galectins can be subdivided into three groups on the basis of the polypeptide construct: 1) galectins consisting entirely of CRD which forms stable dimers, 2) galectin in which the CRD is attached at its N-terminal end to a highly sequence-repetitive domain, 3) galectins that contain two tandemly connected CRDs. Here we limit our discussion to the non-covalent dimeric type.

The tertiary and quaternary structures of galectin dimers resemble that of the canonical dimer of the legume lectins in which two monomers associate non-covalently at one end of the molecule that are on the opposite side from the location of the carbohydrate-binding site [33] (Figure 8). For this reason, the two sugar-binding sites are quite far apart (~ 50 Å) located at the opposite ends of an oblong dimer, and more importantly these sites are facing in opposite directions. Such a binding site arrangement is dramatically different from that of MBP and many membrane-associated proteins, e.g., hepatic lectins, where the binding sites are generally pointing toward the same direction. The opposite orientation of the two sites makes it virtually impossible for a synthetic divalent structure of the designs described earlier in this review, to bind to the two sites simultaneously, even if the linking arm is sufficiently long. As to neoglycoproteins, e.g., highly lactose-substituted BSA derivative, although it is larger than the dimeric galectin, the globular nature of both the ligand and lectin makes it sterically improbable to have two lactose residues occupying the two sites simultaneously. As expected, experimentally the binding of Lac-BSAs by a dimeric galectin did not exhibit density-dependent affinity enhancement seen in the MBP and hepatic lectin experiments (Figure 7) [34].

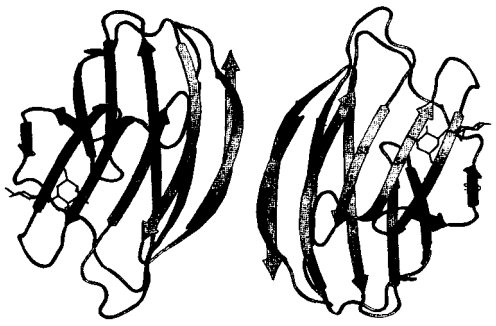


Figure 8. Ribbon diagram of human L-14-II-lactose complex. Courtesy of Y.D. Lobsanov, M.A. Gitt, H. Leffler, S.H. Barondes, and J.M. Rini, *J. Biol. Chem.* (1993) **268**: 27034, by permission of American Society for Biochemistry and Molecular Biology, Inc.

However, the bipolar binding site arrangement of dimeric galectins and legume lectins is most suited for the cross-linking type of interaction. In fact both legume lectins and dimeric galectins are known for their ability to form extensively cross-linked network resulting in crystalline precipitin. However, this type of cross-linked network is fundamentally different from multivalent interaction of MBP and hepatic lectins. A molecule or a binding unit of MBP and hepatic lectins interacts with a single multivalent ligand molecule at multiple sites, whereas each molecule of galectin or legume lectin interacts with a multivalent ligand only at one site. To illustrate this point, the network of galectin-asialofetuin interaction proposed by Brewer and coworkers [35] is shown in Figure 9. Each galectin dimer interacts with two separate asialofetuin molecules, and if limited to a single dimer of galectin the interaction does not result in affinity enhancement. However, an expansive cross-linking of galectin with a large number of multivalent ligand molecules would be expected to result in affinity enhancement because of the concerted binding event. Once the network is formed, the overall dissociation rate would be expected to be very much slower than the individual dissociation rates. A recent microcalorimetric analysis of ConA binding of multivalent ligands showed that there is no increase in ΔG per unit sugar for the binding to multivalent ligands, even though the IC_{50} values showed an apparent increase in inhibitory potency. However, the IC_{50} values do correlate with the ΔS of binding [36].

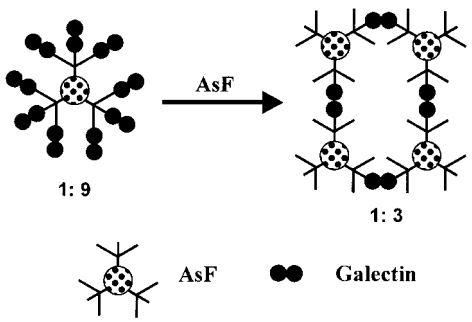


Figure 9. A model of cross-linking interaction of dimeric galectin with asialofetuin (AsF). The initial precipitin form containing AsF and galectin in 1:9 ratio was converted to a new precipitin form upon addition of more AsF which contains AsF and galectin in 1:3 ratio. Adapted from D.K. Mandal and C.F. Brewer, *Biochemistry* (1992) **31**: 8465.

Conclusions

The glycoside cluster-effect is an important mechanism for affinity-enhancement in many carbohydrate–lectin interactions. The fact that many endocytotic receptors and defense molecules employ glycoside cluster-effects attests to the effectiveness of this mode of affinity-enhancement.

To produce a strong affinity-enhancement, the configurational complementarity between the lectin binding sites and target sugar residues is of utmost importance. Mammalian hepatic lectin on the hepatocyte surface binding to certain Gal-terminated natural oligosaccharide structures represents the supreme example of high complementarity. In this case, the overall binding energy is close to the sum of the binding energy at individual sites, suggesting that this binding event pays little entropic penalty. A more recent example is a pentavalent or decavalent ligands designed and synthesized for Shiga-like toxin [37].

In contrast, it is often difficult to obtain a meaningful affinity-enhancement using flexible divalent ligands, especially if the distance between the two binding sites is long (> 45 Å). Presumably, any gain in the binding energy obtained by simultaneous occupation of two binding sites is almost totally offset by the entropical expenditure. This is particularly true for lectins, since the binding energy generated at a typical binding site is rather small.

The orientation and direction of binding sites are other important factors. When the binding sites are pointing in opposite directions, such as in the case of galectins and legume lectins, it is improbable for a molecule of lectin to bind a single multivalent ligand at multiple sites. Sterically more suitable interaction is cross-linking, and one would expect extensive cross-linking will produce affinity enhancement, probably mostly due to a decreased dissociation rate.

The recognition and binding of carbohydrate is important in many biomedical topics, e.g., the initial recognition and the binding of invading microorganisms and their toxins. Inhibitors for the binding step may be potentially important therapeutic agents. If the clustering of target sugar residues is suspected to be a significant factor in the binding event, the use of serum albumin-based neoglycoproteins as inhibitors may be a good starting option. The advantages of neoglycoproteins are: 1) The size is large enough to allow binding to lectin binding-sites, which might be more than 50 Å apart; 2) The incorporation of a large number of sugars into the albumin molecule increases the chance of sugar residues occupying two or more binding sites simultaneously; 3) The globular protein backbone gives a certain amount of rigidity to the spacial arrangement of target sugars, which will reduce the entropic energy loss.

If the presence of an unnatural backbone is not unacceptable and heterogeneity in the molecular size distribution can be tolerated, the synthetic polymers of the linear type, such as polyacrylamide, and of dendrimer type, such as commercially available Starburst, can be effective scaffolding for multivalent neoglycoconjugates. In fact, we have synthesized neoglycoconjugates of the linear polymeric type based on poly-L/D-lysine as well as polyacrylamide backbones and found them to be potent inhibitors in the hepatic lectin [38], alveolar macrophage mannose-receptor [39], and MBP [40] systems. The effectiveness of small flexible di- or trivalent molecules as inhibitors is probably limited to a few restricted cases where lectin binding sites are fairly close to each other and relatively rigidly organized, as in the case of hepatic lectins. A parasite, *Entamoeba histolytica*, expresses a cell-surface Gal/Ga1NAc-binding protein, which represents the adherence as well as the virulence factor. Like in the case of ASGP-R on the rat hepatocyte, GalNAc-containing BSA derivatives are potent inhibitors for this lectin [41]. However, in contrast to RHL none of the divalent GalNAc derivatives (up to 60 Å maximum span) showed any significant affinity-enhancement [41] (R.T. Lee, Y.C. Lee, R.L. Schnaar, and W.A. Petri, Jr., unpublished results), suggesting that the organization of the GalNAc-binding protein on the *E. histolytica* cell surface may be very fluid.

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