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Thermodynamics of Multivalent Carbohydrate−Lectin Cross-Linking Interactions: Importance of Entropy in the Bind and Jump Mechanism†

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ABSTRACT: The high affinity (K_d = 0.2 nM) of the soybean agglutinin (SBA), a tetrameric GalNAc specific lectin, for a modified form of porcine submaxillary mucin, a linear glycoprotein, with a molecular mass of ~10^6 Da and ~2300 GalNAcα1-*O*-Ser/Thr residues (Tn-PSM) has been ascribed to an internal diffusion mechanism that involves binding and jumping of the lectin from GalNAc to GalNAc residue of the mucin [Dam, T. K., et al. (2007) *J. Biol. Chem. 282*, 28256−28263]. Hill plot analysis of the raw ITC data shows increasing negative cooperativity, which correlates with an increasing number of lectin−mucin cross-linking interactions and decreasing favorable binding entropies. However, the affinity of bound SBA for other Tn-PSM molecules during cross-linking is much higher than that of free SBA for GalNAcα1-*O*-Ser, a monovalent analogue. The high affinity of bound SBA for GalNAc residues on other Tn-PSM molecules appears to be due to the favorable entropy of binding associated with the internal diffusion mechanism. Furthermore, the increasing negative cooperativity of SBA binding to Tn-PSM correlates with a decreasing level of internal diffusion of the lectin on the mucin as cross-linking occurs. These findings indicate the importance of the internal diffusion mechanism in generating large, favorable entropies of binding that drive lectin−mucin cross-linking interactions. The results are important for understanding the energetics of lectin−mucin cross-linking interactions that are associated with biological signaling on the surface of cells and the role of the internal diffusion mechanism in ligand-biopolymer interactions in general.

Lectins are multivalent carbohydrate-binding proteins that are present in animals, plants, and microorganisms (*1*). The biological activities of lectins include receptor-mediated endocytosis of glycoproteins, cellular recognition and adhesion (*2*), inflammation (*3*), and cell growth and metastasis (*4*,*5*). As a consequence of their multivalent structures (*6*,*7*), lectin binding leads to cross-linking and aggregation of specific multivalent glycoprotein receptors on cells. This, in turn, results in signal transduction effects that include apoptosis of T cells (*8*,*9*), regulation of the T-cell receptor (*10*,*11*), regulation of the location and activity of the Glut-2 transporter in pancreatic β cells (*12*), and regulation cell cycling kinetics and activities of cytokine receptors (*13*). Hence, it is important to understand the mechanisms of binding and cross-linking of lectins with multivalent glycoprotein receptors to gain insight into their structure−activity properties in biological systems.

While the structures of many lectin cross-linked complexes with multivalent carbohydrates and glycoproteins have been investigated (*14*−*16*), much less is known about the mechanisms of binding of lectins to multivalent glycoproteins that lead to cross-linking of the receptors. Most glycoprotein receptors on the surface of cells possess multiple copies of a single carbohydrate epitope, which can be expressed in individual linear or branched chain structures, and/or multiple glycosylation sites in the protein (*17*). For example, CD43, a galectin-1 counter receptor on T cells (*18*), is a linear glycoprotein (mucin) that possesses approximately 80 *O*-linked chains with terminal LacNAc epitopes (*19*). The globular glycoprotein receptor CD45 RO, which is also a galectin-1 counter receptor on T cells, possesses multiple branched N-linked carbohydrates with LacNAc residues (*18*). However, the mechanisms of binding of lectins such as galectin-1 to glycoprotein receptors like CD43 and CD45 RO are not well understood.

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Recently, the thermodynamics of binding of the soybean agglutinin (SBA),[1] a tetrameric Gal/GalNAc specific lectin, to enzymatically and chemically modified forms of porcine submaxillary mucin (PSM), a large linear glycoprotein, were investigated using isothermal titration microcalorimetry (ITC) (*20*). SBA was shown to bind with a K_d of 0.2 nM to a form of PSM possessing ~2300 α-GalNAc residues and a molecular mass of ~10^6 Da (Tn-PSM) (Figure 1C), which is ~10^6-fold greater affinity compared to that of GalNAcα1-*O*-Ser (Table 1), a monovalent analogue (*20*). The naturally occurring mucin (Fd-PSM) (Figure 1B), which possesses longer chain carbohydrates, bound with ~100-fold lower affinity than Tn-PSM, while two shorter chain analogues of Tn-PSM (Figure 1D,E) bound with progressively lower affinities. The results listed in Table 1 from that study include the complete thermodynamics and stoichiometries of binding of SBA to the PSM analogues, which showed that the affinity of SBA increased with increasing polypeptide chain lengths of the mucins (*20*). Large favorable entropies of binding were associated with the enhanced affinities of the mucins (*20*).

Importantly, the thermodynamic data and dependence of affinity on the length of the mucin polypeptide chains suggested a dynamic binding mechanism in which lectin molecules bind and jump from carbohydrate epitope to epitope along the peptide backbone of the mucin polypeptide chain (*20*). This mechanism was previously suggested for lectins binding to multivalent carbohydrates (*21*) and to the multivalent globular glycoprotein asialofetuin (ASF) (*22*). This mechanism is also similar to the mechanism of binding of proteins to DNA (*23*).

This article presents an analysis of the thermodynamics of SBA cross-linking with the mucins in Figure 1. The results suggest that entropy effects associated with the bind and jump mechanism play a major role in driving cross-linking interactions. These findings have important implications for understanding the structure−activity properties of mucins as glycoprotein receptors, and ligand−biopolymer interactions in general.

STRUCTURES OF THE MUCINS

The structures of the PSM analogues in Figure 1 have been previously described in detail (*20*,*24*). Briefly, PSM is composed of a very large central *O*-glycosylated domain flanked by small Cys-rich globular domains at both the N- and C-termini (*25*). The *O*-glycosylated domain consists of approximately 100 81-residue tandem repeats with the sequence shown in Figure 1A (*26*). Each tandem repeat contains 31 Ser/Thr *O*-glycosylation sites, and ~75% or more of the Ser and Thr residues are glycosylated to varying extents (*24*). Hence, there are ~2300 carbohydrate chains in the *O*-glycosylated domain of PSM. The carbohydrate side chains described for PSM range from the monosaccharide GalNAcα1-*O*-Ser/Thr, the pancarcinoma carbohydrate antigen Tn, to the core 1 blood group A tetrasaccharide α-GalNAc(1−3)[α-Fuc(1−2)]-β-Gal(1−3)-α-GalNAc-*O*-Ser/Thr (*27*). Each of the monothrough tetrasaccharides is potentially glycosylated by an NeuNGl residue attached α(2−6) to the peptide-linked GalNAc, and therefore, up to eight possible oligosaccharides are found in PSM.

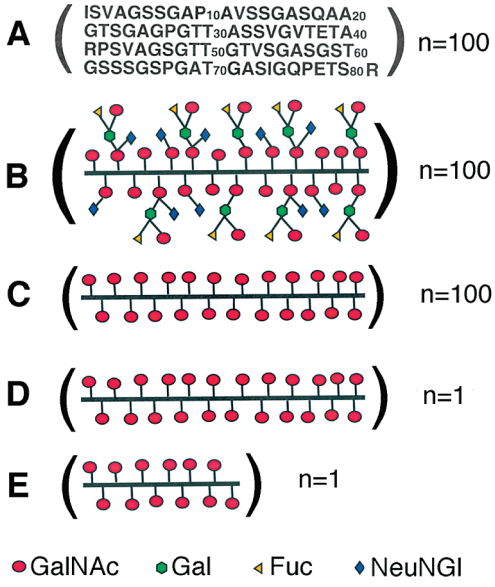

FIGURE 1: Structural representations of (A) the amino acid sequence of the 100-repeat 81-residue polypeptide *O*-glycosylation domain of intact PSM, (B) the fully carbohydrate-decorated form (described in the text) of the 100-repeat 81-residue polypeptide *O*-glycosylation domain of PSM (Fd-PSM), (C) the 100-repeat 81-residue polypeptide *O*-glycosylation domain of PSM containing only peptide-linked α-GalNAc residues (Tn-PSM), (D) the single 81-residue polypeptide *O*-glycosylation domain of PSM containing peptide-linked α-GalNAc residues (81-mer Tn-PSM), and (E) the 38- or 40-residue polypeptide (s) derived from the 81-residue polypeptide *O*-glycosylation domain of PSM containing peptide-linked α-GalNAc residues (38/40-mer Tn-PSM).

HILL PLOTS OF RAW ITC DATA

The Hill plot, in which log(concentration of free ligand) is plotted versus log(fraction of bound protein)/(fraction of free protein), has been used to investigate cooperativity in a variety of ligand−protein systems (cf. refs *28* and *29*). Such plots may typically show evidence for positive or negative cooperative in the binding of monovalent ligands to multisubunit proteins. Ligand binding without cooperativity gives rise to a linear Hill plot with a slope of 1.0. Ligand binding with positive cooperativity gives rise to Hill plots with slopes of >1.0, while ligand binding with negative cooperativity gives Hill plots with slopes of <1.0. Thus, the Hill plot has the advantage of assigning numerical values to the degree of cooperativity of the system, as compared to the Scatchard plot (*30*). The Hill plot also has the advantage of being a logarithmic representation which allows plotting of all theoretically obtainable data, unlike double-reciprocal or half-reciprocal plots that often have

[1]Abbreviations: SBA, soybean agglutinin; ConA, concanavalin A; DGL, *Dioclea grandiflora* lectin; PSM, porcine submaxillary mucin; Fd-PSM, fully carbohydrate-decorated porcine submaxillary mucin; Tn-PSM, porcine submaxillary mucin containing GalNAcα1-*O*-Ser/Thr residues; 81-mer Tn-PSM, 81-residue amino acid repeat of the domain of porcine submaxillary mucin containing GalNAcα1-*O*-Ser/Thr residues; 38/40-mer Tn-PSM, 38- or 40-residue amino acid cleavage product of 81-mer Tn-PSM containing GalNAcα1-*O*-Ser/Thr residues; Tn-antigen, GalNAcα1-*O*-Ser/Thr; ASF, asialofetuin; GalNAc, *N*-acetyl-D-galactosamine; Fuc, L-fucose; Gal, D-galactose; NeuNGl, *N*-glycoloylneuraminic acid or sialic acid; LacNAc, *N*-acetyl-D-lactosamine; ITC, isothermal titration microcalorimetry; NMR, nuclear magnetic resonance.

Table 1: Thermodynamic Binding Parameters for SBA at pH 7.2 and 27 °C (*20*)

ligand	K_d[a] (μM)	K(rel)[b]	$-\Delta G$[c] (kcal/mol)	$-\Delta H$[d] (kcal/mol)	$-T\Delta S$[e] (kcal/mol)	n[f]
GalNAcα1-*O*-Ser	170	1	5.2	7.9	2.7	1.0
38/40-mer Tn-PSM	1.4	120	8.0	32.2	24.2	0.2
81-mer Tn-PSM	0.06	2800	9.8	56.1	46.3	0.12
Tn-PSM	0.0002	850000	13.1	4310	4297	0.0018
Fd-PSM	0.024	7100	10.4	703	693	0.008

[a] Errors in K_d range from 1 to 7%. [b] Relative to GalNAcα1-*O*-Ser. [c] Errors in ΔG are less than 2%. [d] Errors in ΔH are 1−4%. [e] Eerors in $T\Delta S$ are 1−7%. [f] Errors in n are less than 4%.

open upper limits on the abscissa and ordinate (*31*). Hill plots can also reveal cooperativity associated with binding of multivalent ligands to noncooperative multisubunit proteins such as concanavalin A (ConA) and *Dioclea grandiflora* lectin (DGL) (*21*). In using Hill plot analysis of the binding of multivalent sugars, the term for the fraction of bound ligand, X_b/M_t, is corrected for the valency of the sugar to give $X_b \times$ (functional valency of sugar)$/M_t$, which is a modification of the classical Hill plot.

When the raw ITC binding data in Table 1 (*20*) are subjected to Hill plot analysis, for example, SBA binding to 38/40-mer Tn-PSM, the results are shown in Figure 2a, which shows increasing curvature with an increased level of binding. For increased clarity, the data are shown as progressive three-point tangent slopes in Figure 2b, and a bar graph in Figure 2c. The data in Figure 2a−c show increasing negative cooperativity with an increased level of binding of SBA to 38/40-mer Tn-PSM. Similar results are observed for the binding of SBA to Tn-PSM and 81-mer Tn-PSM. The ITC-derived Hill plot for the binding of SBA to GalNAcα1-*O*-Ser in Figure 2d shows a straight line with a slope of 0.93, indicating essentially no binding cooperativity. Thus, the increasing negative cooperativity observed for the binding of SBA to the mucins is due to the ligands and not the lectin. Similar Hill plots have been observed for the binding of ConA and DGL to synthetic bi-, tri-, and tetraantennary N-linked carbohydrates (*21*), and the binding of galectin-1, -2, -3, -4, -5, and -7 to ASF (*22*).

STOICHIOMETRY OF BINDING OF SBA TO MUCINS

The ITC data in Table 1 provide the n value, the number of binding sites per SBA subunit, for each complex. We have previously shown that the reciprocal of n ($1/n$) represents the functional valence of the carbohydrate or glycoprotein binding to the lectin in the case where the number of binding sites per subunit of the lectin is known (*32*). In the case of SBA, the number of binding sites for GalNAc residues per subunit is one as determined by ITC (*20*) and X-ray crystallography (*33*). Using the $1/n$ value for Tn-PSM in Table 1 indicates that ~540 GalNAc residues of a total of ~2300 GalNAc residues of the mucin bind to SBA under saturation conditions. SBA is a tetramer with four binding sites per molecule (*33*). Stoichiometric analysis indicates that all four sites of SBA are occupied at the end of the ITC experiment and, therefore, that SBA is completely cross-linked with Tn-PSM in solution. This is also true for the ITC experiments for SBA binding to the shorter mucin fragments.

INCREASING NEGATIVE COOPERATIVITY CORRELATES WITH A DECREASING FAVORABLE ENTROPY OF BINDING

The ΔH values for SBA binding to Tn-PSM and its fragments have been shown to be proportional to the number of GalNAc residues in the mucins that bind to the lectin (*20*). For example, ΔH is −4310 kcal/mol for SBA binding to Tn-PSM as compared to −7.9 kcal/mol for GalNAcα1-*O*-Ser (Table 1). If ΔH for Tn-PSM is divided by the $1/n$ value of 540 α-GalNAc residues in the mucin that bind to the lectin, the ΔH per α-GalNAc residue is −8.0 kcal/mol, which is similar to that of GalNAcα1-*O*-Ser. This indicates that each α-GalNAc residue of Tn-PSM that binds to SBA possesses essentially the same ΔH as GalNAcα1-*O*-Ser. The observed ΔH for SBA binding to Tn-PSM is thus the sum of the individual ΔH values of the α-GalNAc binding residues of the mucin.

The $T\Delta S$ values for SBA binding to Tn-PSM and its fragments, on the other hand, have been shown to be nonproportional to the number of GalNAc residues in the mucins that bind to the lectin (*20*). For example, $T\Delta S$ is −4297 kcal/mol for SBA binding to Tn-PSM as compared to −2.7 kcal/mol for GalNAcα1-*O*-Ser (Table 1). If $T\Delta S$ for Tn-PSM is divided by the $1/n$ value of 540, the resulting $T\Delta S$ per α-GalNAc residue is −7.96 kcal/mol, which is more unfavorable than −2.7 kcal/mol for GalNAcα1-*O*-Ser (Table 1). If $T\Delta S$ were proportional to the number of binding epitopes in Tn-PSM, the observed $T\Delta S$ would be −1458 kcal/mol, and ΔG would be −2852 kcal/mol, an impossibly large value. The observation that $T\Delta S$, unlike ΔH, does not scale in proportion to the number of binding epitopes has been previously realized in the binding of ConA and DGL to bi-, tri-, and tetraantennary carbohydrates (*21*) and galectin-1, -2, -3, -4, -5, and -7 to ASF, a multivalent glycoprotein (*22*). The nonproportional behavior of $T\Delta S$ is characteristic of multiple lectin molecules binding to different epitopes of a multivalent carbohydrate, as opposed to a single lectin molecule binding to multiple epitopes of a single multivalent carbohydrate (*34*). In the latter case, both ΔH and $T\Delta S$ increase in proportion to the number of binding epitopes in the multivalent ligand, with concomitantly larger increases in affinity (*34*).

Importantly, the increasing negative cooperativity observed for the binding of SBA to the mucins in the Hill plots must be due to the nonproportional $T\Delta S$ for these interactions and not the proportional ΔH contributions, which remains a fixed value per GalNAc residue with an increasing level of SBA binding.

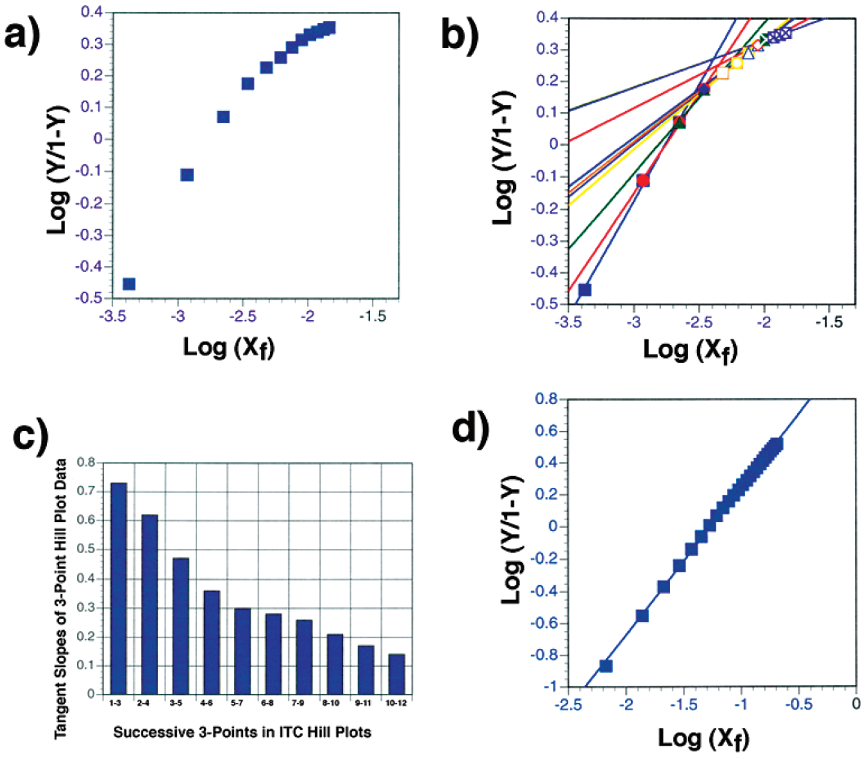

FIGURE 2: (a) Hill plot of ITC data for SBA binding to 38/40-mer Tn-PSM. (b) Tangent slopes of progressive three-point intervals of the Hill plot for SBA binding to 38/40-mer Tn-PSM. (c) Bar graphs of the three-point tangent slopes of the ITC data for Hill plots of SBA binding to 38/40-mer Tn-PSM. (d) Hill plot of the ITC data of SBA binding to GalNAcα1-*O*-Ser (slope of 0.93).

DECREASING FAVORABLE ENTROPY OF BINDING AND THE BIND AND JUMP MECHANISM

The initial binding of SBA to the mucins must involve very favorable $T\Delta S$ contributions since the observed K_d values are averages of very high affinities and then lower affinities with increasing negative cooperativities. For example, if the first SBA molecule that binds to Tn-PSM (Figure 3A) possesses a K_d of 0.2 nM (or likely higher) (Table 1), ΔH for this interaction would be −8.0 kcal/mol (per GalNAc residue) and $T\Delta S$ would be approximately 5.2 kcal/mol, as compared to −2.7 kcal/mol for SBA binding to GalNAcα1-*O*-Ser (Table 1). (The difference between $T\Delta S$ values reflects the difference in $-\Delta G$ values for SBA binding to Tn-PSM and GalNAcα1-*O*-Ser.) The highly favorable initial $T\Delta S$ for SBA binding to Tn-PSM can be understood in terms of the dynamic bind and jump mechanism for SBA binding to the mucin (Figure 3A), as previously suggested (*20*). In essence, SBA binds and jumps from GalNac residue to GalNAc residue along the polypeptide chain of the mucin, resulting in an increased residence time of the lectin on the mucin and a longer macroscopic off rate. Since the dissociation constant, K_d, is the ratio of the reverse rate constant (off rate) to the forward rate constant, the macroscopic K_d becomes much lower (greater affinity). This mechanism has been suggested to explain the higher affinity of SBA for Tn-PSM as compared to the shorter mucin fragments in Table 1 (*20*). This mechanism is also well-known for the binding of protein ligands to DNA, which has been called the bind and slide or bind and hop mechanism (*23*).

The increasing negative cooperativity of SBA binding to the mucins with an increasing concentration in the initial binding interactions can be explained on the basis of increasing the density of lectin molecules on the mucins, which would shorten their one-dimensional diffusion paths, as previously suggested (*20*), and hence lower their favorable entropies of binding (Figure 3B).

SBA−MUCIN CROSS-LINKING INTERACTIONS CORRELATE WITH AN INCREASING UNFAVORABLE ENTROPY OF BINDING

Stoichiometric analysis indicates that an increased level of binding of SBA to the mucins beyond the first subunit of the tetrameric lectin involves cross-linking interactions (Figure 3C), as discussed above. The Hill plots show increasing negative cooperativity under conditions of SBA−mucin cross-linking, and therefore decreasing favorable $T\Delta S$ contributions. Hence, SBA−mucin cross-linking interactions are entropically less favorable as compared to the initial binding of the lectin to the mucin in panels A and B of Figure 3.

However, the affinity of bound SBA for GalNAc residues on other mucin molecules during cross-linking is very high compared to that of free SBA for GalNAcα1-*O*-Ser. For example, the observed K_d of SBA for Tn-PSM is 0.2 nM (Table 1), at which 50% of the lectin is bound to the mucin. Under these conditions, at least two of the four subunits of SBA are cross-linked with Tn-PSM, and therefore, the affinity of the lectin is nearly 10^6-fold greater than that of free SBA for GalNAcα1-*O*-Ser (K_d = 0.17 mM) (Table 1). The high affinity of SBA in these cross-linked complexes is not due to the ΔH of binding, which is fixed at −8.0 kcal/mol per binding GalNAc residue in Tn-PSM, but instead must be due to favorable $T\Delta S$ contributions per GalNAc residue. Since favorable entropies of binding of SBA to Tn-PSM are ascribed to the bind and jump mechanism, it follows that SBA is dynamically bound to

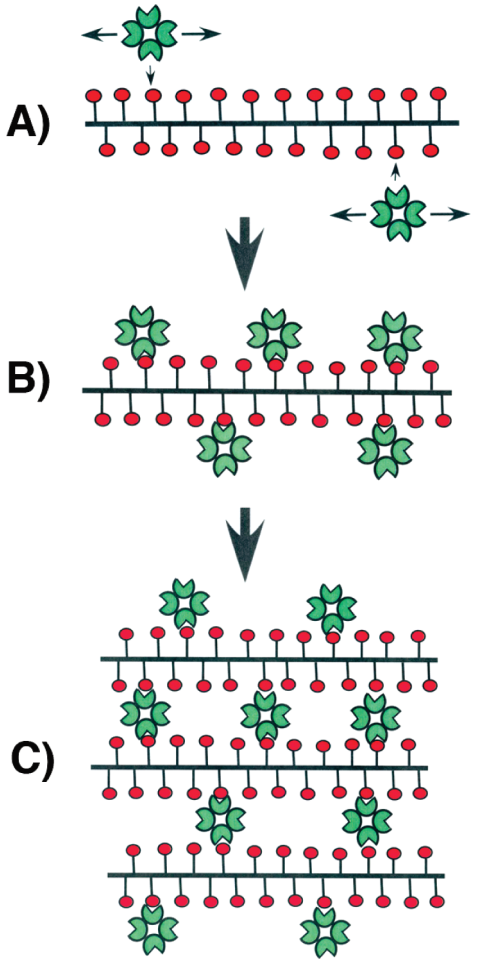

FIGURE 3: Schematic representations of (A) SBA binding at low fractional occupany to Tn-PSM, (B) SBA binding at higher fractional occupancy to Tn-PSM, and (C) SBA binding at high fractional occupancy with cross-linking of Tn-PSM.

Tn-PSM in the process of cross-linking. As SBA undergoes further cross-linking, increasing negative cooperativity suggests a reduction in the level of internal diffusion of SBA on Tn-PSM as cross-linking restricts the movement of lectin molecules. Hence, high-affinity cross-linking of Tn-PSM by SBA is driven by the highly favorable entropy of binding of the lectin to the mucin that is associated with the bind and jump mechanism.

Similar increasing negative cooperativity effects have been observed in the binding of ConA and DGL to bi-, tri-, and tetraantennary carbohydrates (*21*) and galectin-1, -2, -3, -4, -5, and -7 to ASF (*22*), and a great deal of these effects are likely to be due to cross-linking. Indeed, recent experiments with so-called monovalent galectins such as truncated galectin-3 and -5 show evidence of cross-linking activities.

ESTIMATED RANGE OF MICROSCOPIC K_D VALUES FOR SBA BINDING TO TN-PSM

Figure 4 shows a hypothetical plot of the microscopic K_d values versus the fractional occupancy of SBA binding to Tn-PSM. The horizontal dashed line is the observed K_d value of 0.2 nM obtained from the ITC experiments (Table 1). However, due to increasing negative cooperativity, the microscopic K_d values for SBA binding to Tn-PSM increase (decreasing affinity)

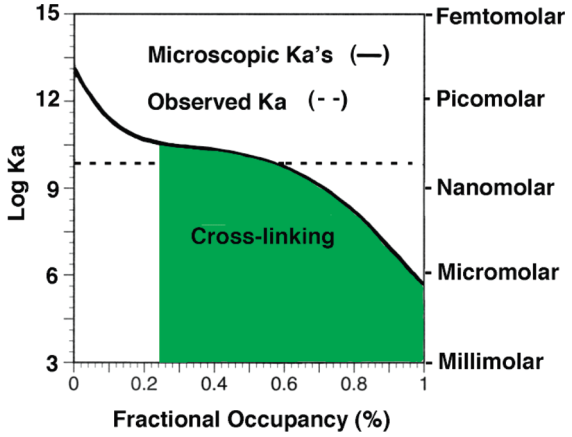

FIGURE 4: Hypothetical plot (—) of the microscopic K_d values of SBA binding to Tn-PSM from 0 to 100% occupancy (0−540 GalNAc residues). The dashed line represents the observed K_d value of 0.2 nM for SBA binding to Tn-PSM in Table 1.

with increasing fractional occupancy. The solid line is an estimate of the range of microscopic K_d values of SBA with increasing fractional occupany of Tn-PSM from 0 to 100%, which corresponds to 0−540 GalNAc residues. The microscopic K_d values for the binding of ConA and DGL to a tetraantennary carbohydrate were estimated to cover an ~2500-fold range from the first unbound carbohydrate epitope to the fourth epitope (*21*). Microscopic K_d values for the binding of galectin-1, -2, -3, -4, -5, and -7 to ASF were estimated to cover an ~3000−6000-fold range from the first unbound epitope to the ninth unbound epitope (*22*). The range of microscopic K_d values for SBA binding to 540 GalNAc residues of Tn-PSM is estimated to be greater than the examples mentioned above and as high as $\sim 10^5$ to $\sim 10^6$ fold. This suggests that the first few molecules of SBA binding to Tn-PSM possess microscopic K_d values on the magnitude of picomolar or less, as shown in Figure 4. These SBA molecules have very favorable entropies of binding because of their ability to bind and jump along the GalNAc residues of the Tn-PSM polypeptide chain. The $T\Delta S$ value for these molecules would be $\sim +10$ kcal/mol, as compared to -2.7 kcal/mol for the binding of free SBA to GalNAcα1-*O*-Ser. Thus, the large increase in the microscopic K_d of SBA for Tn-PSM under these conditions is due to highly favorable entropy of binding effects that drive high-affinity cross-linking interactions with an increasing concentration of the lectin.

The diminished affinity of SBA for Tn-PSM over the full range of fractional occupancy in Figure 4 is thus due to decreasing favorable $T\Delta S$ effects as the lectin undergoes an increasing amount of cross-linking with the mucin.

IMPLICATIONS

The high-affinity binding and cross-linking interactions of SBA with the mucins in this study involve increasing negative cooperativity, which extends the binding isotherms over a wide range of lectin concentrations. This suggests that lectin-induced cross-linking of mucin receptors on the surface of cells, which is associated with many biological signal transduction processes such as galectin-1-induced apoptosis of T-cells (*18*), requires a relatively large change in the concentration of lectin to

initiate signaling. This provides a level of control for this important biological signaling mechanism. In addition, the concentration required for lectin-induced cross-linking of mucin receptors would be a function of the mucin structure, including the number and composition of carbohydrate binding epitopes, the number of tandem repeat polypeptide domains, the amino acid composition of the tandem repeat domains, and the density of the carbohydrate epitopes. These structural parameters would determine the microscopic affinity and hence entropy of binding of lectins to mucins that drive cross-linking interactions via the bind and jump mechanism. All of these variations are present in the family of animal mucins, of which there are at least 17 mucin gene products (*35*).

It has recently been suggested that essentially all ligands may bind to biopolymers by the bind and jump mechanism (*36*). The results presented here provide a general model for the enhanced favorable entropies of binding of ligands to biopolymers and the effects on subsequent complex formation and activity of bound ligands.

Finally, a central question in carbohydrate biochemistry of why lectins have relatively low affinities for monovalent carbohydrate epitopes appears to be answered in part by this study. Such low-affinity interactions allow the lectin to gain large favorable entropies through multiple dissociation and rebinding to arrays of low-affinity carbohydrate epitopes. This, in turn, permits high-affinity dynamic binding, and high affinity of the bound protein for other molecules. It will be of interest to determine the effects of modulating the affinity and off rates of proteins for individual epitopes in biopolymers on this highly favorable entropy of binding mechanism.

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