Entropically Assisted Carbohydrate Recognition by a Natural Killer Cell-Surface Receptor

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The nontrivial nature of carbohydrate recognition, be it in apolar or aqueous media, confronts the creation of truly effective saccharide receptors.[1] Understanding how biological systems continue to resolve this persistent problem and sculpt exquisite macromolecular assemblies that mediate events such as cell adhesion and signaling,[2] offers hope for unveiling hidden principles for carbohydrate receptor design. Presented herein is the direct thermodynamic basis of sialooligosaccharide specificity of a cell-surface protein, Siglec[3a,b] (sialic acid-binding immunoglobulin-like lectin); this reveals a hitherto under-appreciated[4] entropically assisted aspect of carbohydrate–protein recognition. The best-characterized lectins display unfavorable entropy changes upon saccharide binding.[4]

During innate immune responses to viral infection or tumorigenic processes, cells with aberrantly glycosylated surface proteins are destroyed by human natural killer (NK) cells.[5a,b] This occurs, in part, due to an attenuation of the inhibitory signals delivered by cell-surface type I transmembrane glycoprotein receptors, such as Siglec-7.[6a–h] Sialooligosaccharide binding by Siglec-7, the dominant lectin on the surface of NK cells, modulates its target-cell killing activity.[6h] Despite advances on synthetic sialooligosaccharide derivatives,[7a,b] understanding the quantitative energetics of sialooligosaccharide recognition have, heretofore, proven daunting. Even after extensive enzymatic and/or chemical treatments, removal of bound[8a,b] sialoglycoconjugates from eukaryotically expressed proteins remains sufficiently incomplete as to shield the combining site and obscure target detection. Our *E. coli*-based production of homogeneously refolded Siglec-7,[9a,b] brings the prospect of true evaluation of ligand-binding characteristics as it does not suffer from such "masking effects". As interactions essential for defining the structure of β-sandwich nucleate its folding,[10] it is necessary to determine the refolded Siglec-7 structure.[9b]

A solution far-ultra violet circular dichroism spectrum (Figure 1 A) of highly purified unglycosylated unliganded Siglec-7

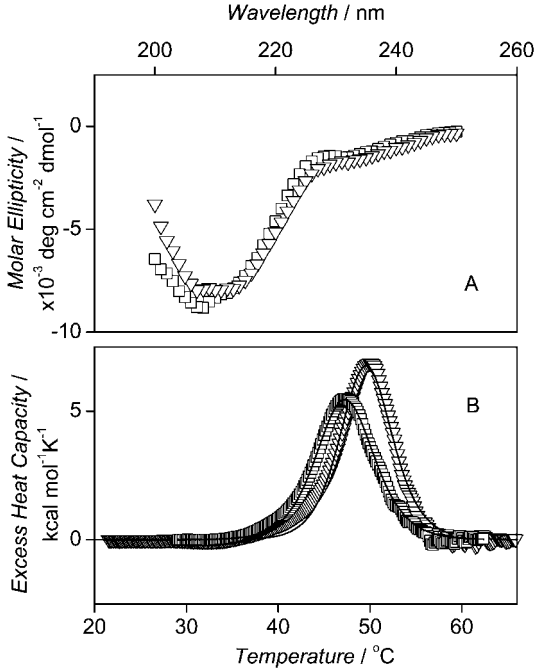

Figure 1. *A) Circular dichroism spectra of 0.100 mM Siglec-7 in the far-UV range, in 25 mM 2-morpholinoethanesulfonic acid (MES; pH 5.5) at 22°C, without (□) and with 1 mM (▽) disialyllactose, recorded at 4 s intervals at a scan rate of 50 nm min^{-1} and a band with of 1 nm. B) Typical DSC endotherms showing the apparent excess heat capacity for the thermal unfolding of Siglec-7 in 25 mM MES (pH 5.5) at 60 K h^{-1}, without (□) and with 1.5 mM (▽) disialyllactose; Siglec-7 concentrations were 0.170 and 0.189 mM, respectively. The buffer–buffer baseline-subtracted and protein concentration-normalized raw data, whose pre- and post-transition baselines were connected by the progress baseline option of ORIGIN and subsequently subtracted, are represented by open symbols. The nonlinear least-squares fits of the data utilizing a two-state transition model are shown as solid lines.*

and one in which it was saturated with the saccharide disialyllactose (Table 1), did not show drastic secondary-structural perturbation (β-sheet ≈ 40% and α-helix ≈ 11%). This is corroborated by a close three-dimensional similarity (rmsd 1.7 Å for all Cα atoms) of the former in the crystalline state,[9b] albeit with a low primary-sequence identity with glycosylated Siglec-1 bound to 3′-sialyllactose.[11] High-sensitivity differential scanning calorimetry (DSC) showed scan-rate and protein-concentration-independent two-state thermal transition of Siglec-7 occurring without oligomeric change (Figure 1 B). This is consistent with the migration of Siglec-7 as a monomer in size-exclusion fast-protein liquid chromatography and by matrix-assisted laser desorption ionization mass spectrometry (see Supporting Information). Insight into two thermodynamic domains that subsist within the Siglec-7 monomer with cooperativity index,[12] $\Delta H_v/\Delta H_c \approx 2$ (Table 2), was gathered from the 1.45 Å-resolution crystal structure of the identical Siglec-7;[9b] this indi-

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Table 1. *List of saccharide compounds used in this study.*

Saccharide	Structural formula
sialic acid	Neu5Ac
sialic acid methoxy ester	methyl-*O*-Neu5Ac
lactose	β-D-Gal-(1→4)-D-Glc
Thomsen–Friedenreich antigen	β-D-Gal-(1→3)-D-GalNAc
disialyllactose	α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc
3′-sialyllactose	α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc
6′-sialyllactose	α-Neu5Ac-(2→6)-β-D-Gal-(1→4)-D-Glc
3′-sialyllactosamine	α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-GlcNAc
3′-sialylfucosyllactose	α-Neu5Ac-(2→3)-β-D-Gal-(1→4) [α-L-Fuc-(1→3)]-D-Glc
3′-sialyl Lewis[a]	α-Neu5Ac-(2→3)-β-D-Gal-(1→3) [α-L-Fuc-(1→4)]-D-GlcNAc
3′-sialyl Lewis[x]	α-Neu5Ac-(2→3)-β-D-Gal-(1→4) [α-L-Fuc-(1→3)]-D-GlcNAc

Table 2. *Thermodynamic quantities from DSC for the thermal unfolding of siglec-7 at a scan rate of 60 °C h^{-1}.*

[Siglec-7] [mM]	Saccharide	[Saccharide] [mM]	T_p [°C]	T_m [°C]	ΔH°_c [kcal mol^{-1}]	ΔH°_v [kcal mol^{-1}]	$\Delta H^\circ_v/\Delta H^\circ_c$
0.170	–		47.35	47.31 ± 0.02	46.0 ± 0.2	96.6 ± 0.6	2.10
0.189	disialyllactose	1.5	49.98	49.61 ± 0.03	51.4 ± 0.4	109.0 ± 1.0	2.12
0.117	6′-sialyllactose	0.2	47.38	47.36 ± 0.01	50.1 ± 0.2	101.0 ± 0.5	2.02

Model-independent parameter: the temperature at which the unfolding transition peak is at maximum, T_p, was obtained from raw DSC data. Model-dependent parameter: the melting temperature at half the peak area, T_m, was obtained by DSC data analyses as described. An ideal cooperative, two-state transition system composed of a single entity displays $\Delta H_c = \Delta H_v$, while for an entity composed of two unfolding units, the calorimetric enthalpy (ΔH_c) estimated from the area under the transition, per unit is one-half of the predicted van't Hoff enthalpy (ΔH_v) for unfolding of the whole entity.[12]

cated that glycosylation has no significant effect on their overall conformation.

Siglecs share a wide cleft amid their two β-sheets ABED and A′GFCC′, which form the β-sandwich.[6e, 11, 9b] Specifically, an intrasheet disulfide bridge linking Cys46 and Cys106 replaces the immunoglobulin intersheet one, whilst the standard immunoglobulin cysteine on the F strand is replaced by Phe123 in Siglec-7, such that the distance between the Cα positions of Cys46 and Phe123 is 9.6 Å, as compared to the characteristic 6–8 Å separation of immunoglobulin-like domains. Thus Siglec-7 bestows on each of its β-sheets, a disposition of an independent, thermally identical single DSC unfolding transition. Concomitantly, since the two β-sheets dwell within the same polypeptide chain, a character of simultaneous unfolding with same T_m is conferred on both of them with a higher degree of cooperativity than a putative single sheet; this explains the twofold higher magnitude of ΔH_v than ΔH_c. The lack of sialooligosaccharide-dependent alteration in the Siglec-7 thermal-transition cooperativity index, notwithstanding increases in T_p, T_m, ΔH_v, and ΔH_c (Table 2), attests to a conservation of identity of the constituent domains and combining-site architecture prior and subsequent to sialooligosaccharide binding. The thermodynamic evidence complements and extends the structural proposal,[11] of the evolution of a Siglec N-terminal V-set domain from a canonical immunoglobulin domain. Were it not for an exposure of sialic acid-binding residues by β-sandwich widening, their burial in the hydrophobic core would have disallowed sialooligosaccharide recognition. Sialooligosaccharide binding preferentially to the folded state of Siglec-7 was succinctly underscored by increases in the T_p, T_m, ΔH_v, and ΔH_c (Table 2) of thermal transition.

The pioneering work of Lemieux suggested that even with precisely positioned hydroxyl groups and small hydrophobic insignia in the saccharide, its resemblance to a water cluster confounds how considerable energetic costs are overcome before it is accepted by a receptor in a 55 000-fold molar excess of water molecules.[13] Isothermal titration calorimetry (ITC)[14] was a method of choice that permitted the measurement of the quantitative thermodynamic parameters for carbohydrate recognition by Siglec-7. Analyses of exothermic heat change associated with its specific reaction with cognate disialyllactose at 277 K (Figure 2A, B) and 298 K (Figure 2C), or 3′-sialyllactose, 6′-sialyllactose, 3′-sialyllactosamine, at 277 K returned stoichiometry close to one; this indicated that each Siglec-7 V-set domain contained a single saccharide binding site. Moderate free-energy changes, 4–6 kcal mol^{-1}, comparable to typical lectin–saccharide reactions, led us to test the promiscuity of its combining site at 100-fold molar excess of saccharide. Here, no reaction of lactose or the tumor-associated Thomsen–Friedenreich antigenic disaccharide (Figure 2D) was observed (Table 3). The specific binding nature of Siglec in solution points to the remarkable selectivity of Siglec-7 for sialooligosaccharide at modest affinities.

Lack of sialic acid or its methoxy ester binding by Siglec-7 (Table 3) was unanticipated, since nearly all direct interactions between Siglec-1 and 3′-sialyllactose in the crystal structure of the complex occurred through the terminal sialic acid group.[11] Taken together, sialic acid and a linked saccharide in concert influence recognition by Siglec-7, the minimum glycotope being a disaccharide containing Neu5Ac at its nonreducing end. Given this and that the glycan pool from Siglec-7 contains sialylated and core-fucosylated bi-, tri-, and tetra-antennary oligosaccharides,[6f] it is tempting to speculate that the type 2 glycosynapse,[2] supports *cis* event(s) amongst two or more receptor molecules on the same NK cell surface, such as homophilic Siglec-7–Siglec-7 and/or heterophilic Siglec-7–sialoglycoconjugate. It is plausible that *trans*-orientated interaction(s) occur between Siglec-7 on NK and sialoglycoconjugate on the target-cell surfaces, as well as soluble endogenous sialoglycoconjugate–Siglec-7 ones. Enzyme-linked immunosorbent assay of biotinylated polyacrylamide-conjugated sialooligosaccharide with Siglec-7-Fc chimeras immobilized by protein A,[6c] and fluorescently tagged whole-cell binding assays with a COS cell

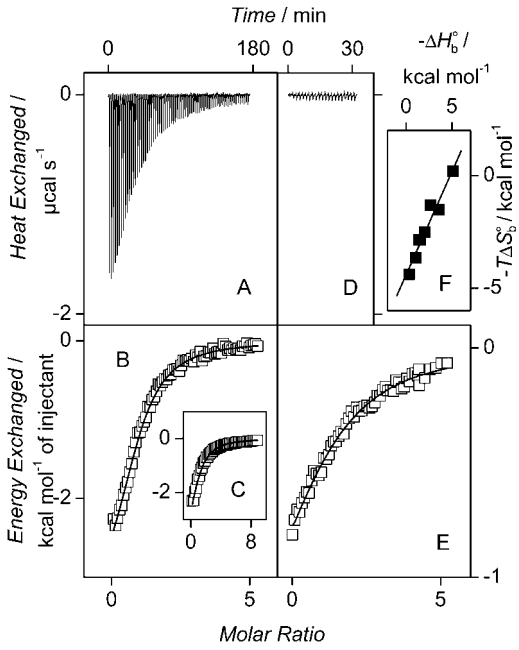

Figure 2. *Titration calorimetric analyses of saccharide recognition by Siglec-7. Raw data obtained from A) 70 automatic injections of 1.5 μL aliquots of 15 mM disialyllactose solution, or D) 19 injections of 15 μL aliquots of 23 mM Thomsen–Friedenreich antigen solution into 0.232 mM Siglec-7 solution in 25 mM MES (pH 5.5 ± 0.1) at 277 K. The heats in D) are indistinguishable from heats of dilution. Nonlinear least-squares fits by ORIGIN (B, C, E, solid lines) of the incremental heat per mole of added ligand (□) for the titration follow closely the molar ratio of the disialyllactose over Siglec-7 at B) 277 K and C) 298 K and E) 3′-sialyl Lewis^x at 277 K. The close fit of the data to the identical-site model shows that the disialyllactose binds to each Siglec-7 molecule independently. The thermodynamic parameters at 298 K for the binding of disialyllactose to Siglec-7 are $K_b = (4.23 \pm 0.29) \times 10^3\ M^{-1}$ and $-\Delta H^\circ_b = 5.13 \pm 0.37$ kcal mol^{-1}. Since Siglec-7 is monomeric in solution at pH 5.5 (see Supporting Information), an identical-site model utilizing a concentration of the protein monomer was the simplest binding model found to provide the best fit to the ITC data. F) Enthalpy–entropy compensation plot of $-\Delta H^\circ_b$ as a function of $-T\Delta S^\circ_b$ for the binding of sialooligosaccharides to Siglec-7. The straight line obtained by linear-regression analysis with ORIGIN has a slope of 1.03 ± 0.10 (r = 0.973).*

line derived from the kidney of the African green old-world monkey, *Cercopithicus aethiops*[6d] could not detect Siglec-7's ability to recognize the fucosylated tumor cell antigens 3′-sialyl Lewis^x or 3′-sialyl Lewis^a. However methods that require ligand alteration sometimes camouflage authentic binding,[6g] especially so as Siglec-7 is constitutively masked on the NK cell surface.[6h] Lack of masking in our receptor preparation in conjunction with ITC that permits the direct detection of universal signature of heats of binding in solution, not only enabled us to uncover the ability of Siglec-7 to bind 3′-sialyl Lewis^x (Figure 2E), 3′-sialyl Lewis^a, and the fucosylated tetrasaccharide 3′-sialylfucosyllactose, but also provide their energetic bases (Table 3). Siglec-7's reaction with 3′-sialyllactose or 3′-sialyllactosamine is entropically enhanced, despite enthalpic losses, by the presence of an α1–3-linked fucoside on the underlying Glc or GlcNAc that forms the 3′-sialylfucosyllactose or 3′-sialyl Lewis^x epitope (Table 3). Siglec-7's recognition of 3′-sialylfucosyllactose is also entropically enhanced by the presence of an acetamido group at the 2′ position of the glucosyl moiety that forms the 3′-sialyl Lewis^x epitope (Table 3).

The results thus evoke a novel property for a Siglec competition with Selectin-mediated recognition process and unveil an unexpected new target for tumor therapy, that is, a specific inhibition of the 3′-sialyl Lewis$^{x/a}$–Siglec-7 complex. The results (Figure 2E, Table 3) impinge on the ability of masked Siglec-7, with its sialooligosaccharide site loaded with 3′-sialyl Lewis$^{x/a}$-like epitope(s), to bind α2–6-,[6c] or α2–8-linked[6d] sialooligosaccharide efficiently. A nonrequirement of unmasking of Siglec-7 for it to bind α2–6- or α2–8-linked sialooligosaccharide in *trans*, implies an extended site on the receptor for avid multivalent sialoglycoconjugate–Siglec-7 engagement in vivo and highlights the nontrivial nature of tumor-cell escape from NK surveillance.

Prior to this study, the quantitative preference, if at all, of Siglec-7 for 3′-sialyllactose or 6′-sialyllactose was unclear. As compared to Siglec-7's reaction with 3′-sialyllactose ($K_{b=}$ 1470 M^{-1}), preferential recognition of 6′-sialyllactose ($K_{b=}$ 4130 M^{-1}) is entropic in origin, while that of disialyllactose ($K_{b=}$ 10170 M^{-1}) stems from a more favorable enthalpic contribution to the binding free-energy change at 277 K (Table 3). The basic nature of the Siglec-7 combining site,[6e,9b] might explain the sevenfold higher affinity for the more negatively charged disialyl moiety that resides on a number of glycoconjugates, such as the gangliosides GT1b and GD3.[6h,i] Siglec-7 recognizes 3′-sialyllactosamine with a similar affinity as it does 3′-sialyllactose, despite a loss in enthalpy change of 1.29 kcal mol^{-1} due to a relatively more favorable entropic component of 1.54 kcal mol^{-1}. Siglec-7 also recognizes the 3′-sialyl Lewis^a and 3′-sialyl Lewis^x epitopes with similar free-energy changes, despite different enthalpy and entropy. A linear enthalpy–entropy-compensation (EEC) relationship be-

Table 3. *Thermodynamic quantities for binding of saccharides to siglec-7 at 277 K.*[a]

Saccharide	K_b[b] [$\times 10^3$ M^{-1}]	$-\Delta G^\circ_b$ [kcal mol^{-1}]	$-\Delta H^\circ_b$ [kcal mol^{-1}]	$T\Delta S^\circ_b$ [kcal mol^{-1}]
sialic acid	NB[c]	NB	NB	NB
sialic acid methoxy ester	NB	NB	NB	NB
lactose	NB	NB	NB	NB
Thomsen–Friedenreich antigen	NB	NB	NB	NB
disialyllactose	10.17 ± 0.49	5.08	3.56 ± 0.10	1.52
3′-sialyllactose	1.47 ± 0.14	4.01	2.69 ± 0.48	1.32
6′-sialyllactose	4.13 ± 1.87	4.59	2.06 ± 0.75	2.53
3′-sialyllactosamine	2.25 ± 0.28	4.26	1.40 ± 0.19	2.86
3′-sialylfucosyllactose	3.25 ± 0.35	4.46	1.58 ± 0.15	2.88
3′-sialyl Lewis^a	5.62 ± 1.38	4.76	0.37 ± 0.05	4.39
3′-sialyl Lewis^x	5.43 ± 0.58	4.74	1.09 ± 0.07	3.65

[a] The stoichiometry (*n*) values ranged from 0.95 to 1.04 with uncertainties from 2% to 5%. [b] K_b refers to equilibrium association constant. [c] No binding.

tween the enthalpy change (ΔH°_{b}) and entropy change ($T\Delta S^{\circ}_{b}$), with a slope close to unity (Figure 2F), indicating nearly complete compensation, suggests that the group of analogous species interact by the same mechanism; this implicates solvent reorganization[15a–c] in sialooligosaccharide–Siglec-7 reactions. A displacement of solvent molecules from hydrophobic regions of the Siglec-7 and sialooligosaccharide, might provide a favorable entropic contribution to complex formation, as substantiated by the negative heat-capacity change (ΔC_p),[16] a hallmark of hydrophobic forces. Disialyllactose–Siglec-7 reactions (Figure 2A–C) displayed a temperature-dependent (275 K to 298 K) linear increase in exothermicity that corresponded to $\Delta C_p = -86 \pm 15$ cal mol^{-1} K^{-1}, while the concomitant reduction in equilibrium association constant was unrelated to any destabilization effect, as confirmed by DSC (Figure 1B).

For the tested sialooligosaccharide recognition by Siglec-7, favorable contributions to ΔG°_{b} emanate from both enthalpic and entropic sources (Table 3), unlike most carbohydrate–protein reactions.[4] The enthalpically supported role of hydrogen bonding[17] in contributing favorably to ΔG°_{b}, and entropic assistance by apolar forces, such as stacking and C-H/π interactions,[18] may be rationalized by examining the putative binding site of Siglec-7, which harbors a number of hydrophobic (Tyr26, Trp132) and basic (Arg23, Arg120, Lys135) residues.[9b] In addition to the conserved Arg124, which forms a salt bridge with the carboxylate group of sialic acid, in the 3′-sialyllactose–Siglec-1 complex,[11] hydrophobic contacts are formed between the glycerol side chain of sialic acid and Trp106 (equivalent to Trp132 in Siglec-7), and between the acetamido methyl group of sialic acid and Trp2 (Tyr26 in Siglec-7). Whilst the energetic contribution of stacking involving the hydrophobic face of the saccharide and the aromatic side chains in the lectin binding site is not unequivocal, its role in sialooligosaccharide recognition by Siglec is likely.

Biological receptor systems appear to solve the carbohydrate-recognition problem by evolving two distinct energetic alternatives. The first broad class, which includes lectins and periplasmic sugar binding proteins, takes advantage of the fact that saccharides present a complex, divergent array of polar (mostly hydroxy) functional groups capable of stereospecifically[19] donating and accepting hydrogen bonds. These proteins predominantly recognize carbohydrates in an enthalpically favorable fashion that is offset by unfavorable entropy. The second thermodynamic class of receptors, as exemplified by Siglec-7 and antibodies, perceive the polyamphiphilicity[13] of saccharides, thereby utilizing both enthalpic and entropic forces to bind them. Optimizing the balance of hydrogen bonding and hydrophobic interactions rather than maximizing either of them appears to be effective for carbohydrate receptors with the desired specificity. Knowledge of the thermodynamic repertoire of carbohydrate–protein reactions[4] (see also Table 3) extending to a range greater than previously believed, provides a framework for future strategies that should not ignore hydrophobicity in receptor design.

Siglec-7 binds fucosylated sialyl antigens (Figure 2E, Table 3), as presented by mucin-type adhesion structures organized with signal transducers in cholesterol-rich lipid microdomain, the type 2 glycosynapse.[2] The extracellular portion of Siglec-7 comprises one N-terminal V-set immunoglobulin-like domain that binds sialylated glycoconjugates and two C2-set immunoglobulin-like domains. The cytoplasmic tail of Siglec-7 contains an immunoreceptor tyrosine-inhibition motif that undergoes tyrosine phosphorylation upon receptor engagement by its ligand; this results in strong inhibition of NK cell cytotoxicity.[6] One may now envisage identifying and dissecting the energetics of the multicomponent reactions of Siglecs of *cis* and/or *trans*, homophilic and/or heterophilic type(s) at the glycosynapse[2] utilizing the approach presented herein.

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Keywords: calorimetry · carbohydrates · molecular recognition · receptors · sialic acids

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