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Temperature-dependent cooperativity in donor–acceptor substrate binding to the human blood group glycosyltransferases

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Affinities of the human blood group glycosyltransferases, α-(1→3)-*N*-acetylgalactosaminyltransferase (GTA) and α-(1→3)-galactosyltransferase (GTB) for their common acceptor substrate α-L-Fuc*p*-(1→2)-β-D-Gal*p*-O$(CH_2)_7CH_3$ (1), in the absence and presence of bound uridine 5′-diphosphate (UDP) and Mn^{2+} were determined using temperature-controlled electrospray ionization mass spectrometry. The presence of bound UDP and Mn^{2+} in the donor binding site has a marked influence on the thermodynamic parameters for the association of 1 with GTA and GTB. Both the enthalpy and entropy of association (ΔH_a, ΔS_a) decrease significantly. However, the free energy of association (ΔG_a) is unchanged at physiological temperature. The differences in the ΔH_a and ΔS_a values determined in the presence and absence of bound UDP are attributed to structural changes in the glycosyltransferases induced by the simultaneous binding of 1 and UDP.

Keywords: Association constants/electrospray ionization mass spectrometry/human ABO(H) blood group glycosyltransferases/protein-oligosaccharide complexes/stoichiometry

Introduction

Glycosyltransferases represent an enormous family of enzymes that catalyze the synthesis of glycosidic linkages by the stereo- and regiospecific transfer of saccharides from activated sugar-nucleotide donors to specific acceptors (Breton et al. 2006; Schuman et al. 2007). We have identified the highly homologous blood group A and B synthesizing enzymes as models for the study of retaining glycosyltransferase reactions. Remarkably, despite differing in only 4 out of 354 amino acids, these enzymes possess altered donor specificity. Human α-(1→3)-*N*-acetylgalactosaminyltransferase (GTA) and α-(1→3)-galactosyltransferase (GTB) catalyze the transfer of GalNAc or Gal from the sugar-nucleotide donors, uridine 5′-diphosphate-GalNAc (UDP-GalNAc) and uridine 5′-diphosphate-Gal (UDP-Gal), respectively, to α-L-Fuc*p*-(1→2)-β-D-Gal*p*-OR acceptors (where R is a glycoprotein or glycolipid). Although the catalytic mechanisms of GTA and GTB have been probed (Seto et al. 1995; Patenaude et al. 2002), there remain a number of outstanding questions. Notably, an ordered catalytic mechanism (where donor binding precedes acceptor binding), which may imply cooperative substrate binding, has been suggested based on kinetic and NMR data (Kamath et al. 1999; Angulo et al. 2006). Direct evidence for or against cooperative binding, which may provide further insight into the precise catalytic mechanism of these enzymes, is currently lacking. Establishing whether donor and acceptor binding to GTA and GTB operate in a cooperative fashion represents a significant experimental challenge. The substrate affinities are relatively low ($<10^5$ M^{-1}), *vide infra*, which leads to a distribution of protein species in solution at typical concentrations. The situation is further complicated by the presence of a divalent metal ion cofactor, thought to be manganese (Mn^{2+}), which is also required for optimal activity of these enzymes. As a result, direct insight into binding stoichiometry is necessary in order to accurately quantify the substrate affinities.

There are a number of established analytical techniques used to quantify protein–carbohydrate interactions in vitro, each with particular strengths and weaknesses. Isothermal titration microcalorimetry (ITC) is probably the most widely used method and is the only technique that provides a direct measure of the enthalpy of association (ΔH_a) (Bundle and Sigurskjold 1994). However, the ITC method requires large (mg) and often prohibitive quantities of both protein and ligand for each analysis. Other commonly used methods include surface plasmon resonance (SPR), which affords high sensitivity and can be used to evaluate the on–off rate constants (Lundquist and Toone 2002), and frontal affinity chromatography–mass spectrometry (FAC/MS), which is capable of rapidly screening target proteins against libraries of carbohydrate ligands (Schriemer and Hindsgaul 1998; Zhang et al. 1999). However, these methods require the immobilization of either the protein (FAC-MS) or the ligand (SPR). Furthermore, none of the above techniques provide direct information regarding binding stoichiometry and are, therefore, of limited use for studying multi-substrate enzyme systems.

Mass spectrometry (MS), combined with electrospray ionization (ES), has emerged as a powerful tool for quantifying the binding stoichiometry and affinity for protein–ligand complexes in solution (Kitova et al. 2001; Daniel et al. 2002; Wang et al. 2003; Heck and van den Heuvel 2004; Loo et al. 2005). The strengths of the ES-MS technique are simplicity (no labeling or immobilization required), speed (measurements can usually be completed within a few seconds), and specificity (the unique ability to provide direct insight into stoichiometry

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and to study multiple binding equilibria simultaneously). When performed using nanoflow electrospray (nanoES), the ES-MS assay affords high sensitivity, normally consuming picomoles or less of protein and ligand per analysis. Additionally, enthalpies (ΔH_a) and entropies (ΔS_a) of association can be estimated from the temperature dependence of the K_a values determined using temperature-controlled ES-MS (Daneshfar et al. 2004).

Here, we describe the results of the first thermodynamic study, performed using the direct ES-MS assay, of the H-disaccharide acceptor analog (α-L-Fuc*p*-(1→2)-β-D-Gal*p*-O$(CH_2)_7CH_3$), **1**, binding to GTA and GTB in the absence and presence of UDP and a metal ion cofactor, Mn^{2+}. Affinities for **1** were measured over a range of solution temperatures and the corresponding thermodynamic parameters (ΔG_a, ΔH_a, and ΔS_a) were established. Importantly, it is shown that occupancy of the donor site dramatically decreases the ΔH_a and ΔS_a parameters for acceptor binding. However, at physiological temperature, ΔG_a is independent of donor site occupancy.

Results and discussion

Effect of UDP binding on the acceptor affinity

The affinities of GTA and GTB for **1** were measured over a range of solution temperatures (10–40°C). Shown in Figures 1A and 2B are the illustrative ES mass spectra measured for solutions of GTA (7 μM) with **1** (30 μM) and GTB (8 μM) with **1** (40 μM), respectively, at 24°C and pH 7. The metal cofactor, Mn^{2+}, was also present in excess (100 μM). The recombinant forms of GTA and GTB used in this study exist exclusively as noncovalently bound homodimers (i.e., GTA_2 and GTB_2) in aqueous solutions at neutral pH (GK Shoemaker et al., in preparation). According to the ES-MS data, at the concentrations investigated, GTA_2 and GTB_2 each bind to 0–2 molecules of **1** (Supplementary Data, Figure S1). Importantly, there is no evidence for the attachment of Mn^{2+} to either enzyme, which suggests that the GTs possess very low affinities for Mn^{2+}, a result that is consistent with related retaining glycosyltransferases (Zhang et al. 2001). The distributions of bound **1**, as determined from the mass spectra, reveal that each homodimer possesses two equivalent binding sites for **1** (Figure S1), with intrinsic affinities (K_a) of $3.2 \pm 0.3 \times 10^4$ M^{-1} (GTA) and $1.7 \pm 0.3 \times 10^4$ M^{-1} (GTB) at 24°C.

To establish whether the presence of bound donor influences the affinities of the GTs for **1**, binding measurements were performed in the presence of UDP. The native donors could not be used because of the rapid changes in donor and acceptor substrate concentrations resulting from an enzymatic reaction (GK Shoemaker et al., in preparation). Shown in Figures 1B and Figures 2B are the illustrative ES mass spectra measured for solutions of GTA (7 μM) with **1** (30 μM) and GTB (8 μM) with **1** (40 μM), respectively, with the addition of UDP (50 μM) and Mn^{2+} (100 μM) at 24 °C and pH 7. Importantly, the distributions of bound **1** change markedly upon the addition of UDP to the solutions. At the concentrations investigated, the active site of each enzyme is fully occupied with both UDP and Mn^{2+}. Under these conditions, the affinities of GTA and GTB for **1** are significantly enhanced, and the (GTA_2 + 2(**1**) + 2(UDP) + 2(Mn^{2+})) and (GTB_2 + 2(**1**) + 2(UDP) + 2(Mn^{2+})) species dominate in solution. Reduction in the concentration of **1** allowed the K_a values to be determined for the sequential addition of **1** to (GTA_2 + 2(UDP) + 2(Mn^{2+})) and (GTB_2 + 2(UDP) + 2(Mn^{2+})) (1C and 2C). The presence of UDP and Mn^{2+} in the active site results in a 5-fold increase in the intrinsic affinity of **1** for GTA ($1.6 \pm 0.3 \times 10^5$ M^{-1}) and a more substantial 9-fold increase for GTB ($1.5 \pm 0.3 \times 10^5$ M^{-1}).

An increase in the acceptor affinity in the presence of a donor has been previously observed in ITC studies of a closely related bovine α-(1→3)-galactosyltransferase binding to its *N*-acetyllactosamine acceptor (Boix et al. 2002). In this case, it was shown that acceptor binding was undetectable in the absence of a native donor. It should be noted, however, that due to limitations of the ITC technique for studying weak interactions ($K_a \leq 10^4$ M^{-1}), it was not possible to establish the binding stoichiometry from these experiments.

Temperature dependence of donor–acceptor binding cooperativity

Affinity measurements were performed at varying temperatures and the intrinsic K_a values are reported in the form of van 't Hoff plots, Figure 3. Importantly, the van 't Hoff plots (which exhibit excellent linearity) determined in the absence and presence of UDP are markedly different for both GTs. In the absence of UDP, the ΔH_a and ΔS_a values for acceptor binding are −8.5 kcal mol^{-1} and −8.0 cal mol^{-1} K^{-1}, respectively, for GTA and −5.8 kcal mol^{-1} and −0.3 cal mol^{-1} K^{-1}, respectively, for GTB (Table I). At higher temperatures, the donor sites are no longer fully occupied. For example, at 37°C, distributions of bound UDP (and Mn^{2+}) and **1** are observed (Figure S2). The determination of K_a is not possible under these conditions because the ions corresponding to the attachment of UDP to the GTs are not fully resolved from those corresponding to the attachment of **1**. Consequently, the K_a values shown in Figure 3 are restricted to temperatures where full donor site occupancy was achieved. In the presence of bound UDP, the corresponding ΔH_a and ΔS_a values are: −37 kcal mol^{-1} and −99 cal mol^{-1} K^{-1}, respectively, for GTA and −36 kcal mol^{-1} and −96 cal mol^{-1} K^{-1}, respectively, for GTB. The similarities in the ΔH_a and ΔS_a values for the GTs suggest that the nature of the binding interactions with **1** is identical for GTA and GTB when UDP is bound. Importantly, for each GT, the van 't Hoff plots for **1** binding in the absence and presence of UDP intersect at ∼37°C, indicating that the binding of **1** is independent of UDP at this temperature. These results may suggest that native donor–acceptor substrate binding to the GTs is also noncooperative at physiological temperature. Taken on their own, the present findings imply that the GTs bind to their donor and acceptor substrates in a random fashion. This contrasts with an ordered mechanism of binding, which is supported by kinetic data (Kamath et al. 1999). The apparent discrepancy between the thermodynamic and kinetic data may be due to acceptor binding in a noncatalytically competent mode, or that UDP, and not the native donor, was used in the binding measurements. Acceptor affinity measurements performed in the presence of inert donor analogs are now needed to assess whether the galactose moiety of the native donor substrate influences acceptor binding.

Analysis of crystallographic data obtained for free GTB and GTB bound with **1** and UDP (Patenaude et al. 2002; Alfaro et al. 2008) suggests a possible explanation for the changes in ΔH_a and ΔS_a values measured for **1** binding with the GTs in the absence or presence of UDP. Specifically, in the presence of UDP, the binding of **1** results in a conformational change in GTB

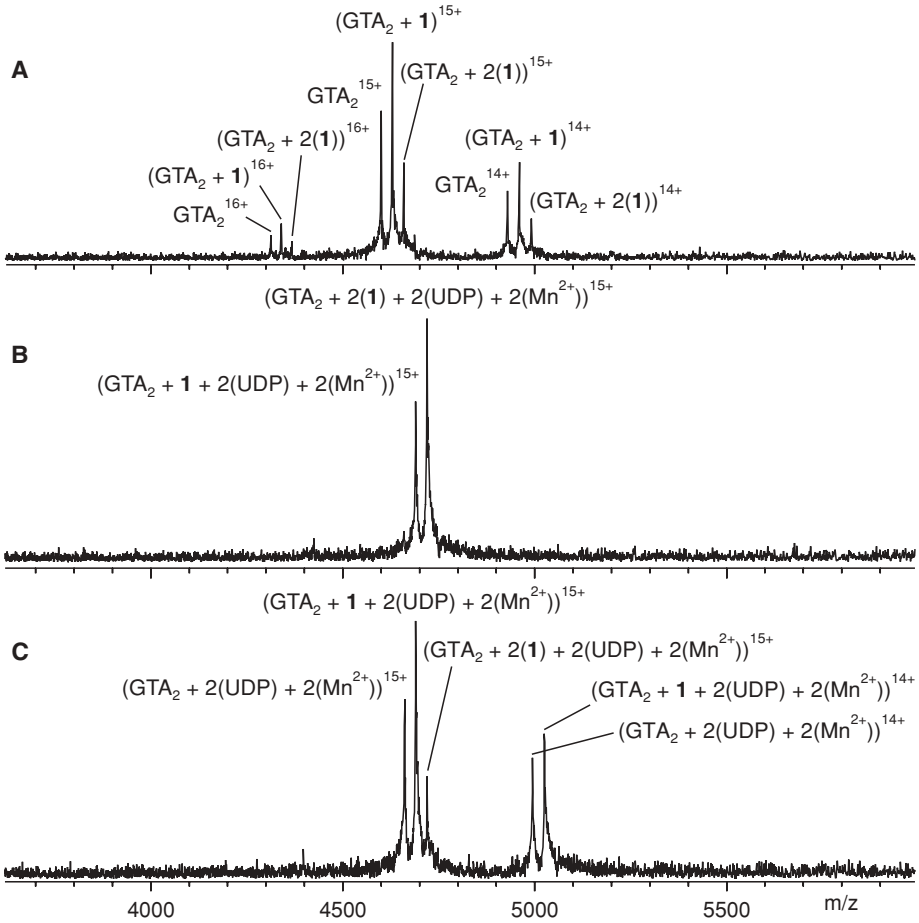

Fig. 1. Illustrative nanoES mass spectra of solutions (pH 7, 24°C) containing 7 μM GTA_2 and 100 μM Mn^{2+}. (**A**) 30 μM **1**, (**B**) 30 μM **1** and 50 μM UDP, or (**C**) 10 μM **1** and 50 μM UDP.

in which two disordered loop regions (residues 175–195 and 359–367) adopt a more ordered structure. A similar conformational change is not observed upon the binding of **1** or UDP separately. In the absence of other effects, loop ordering in GTB upon the binding of **1** is consistent with the reduction in the ΔS_a value. Similarly, the more favorable ΔH_a term is consistent with the formation of new or stronger intramolecular interactions which accompanies the conformational change in GTB. While other structural studies on glycosyltransferases have shown substrate-induced conformational changes upon donor substrate binding (Qasba et al. 2005), this is the first example where the effects of the conformational changes on the association thermodynamic parameters are quantified.

Conclusions

Using the temperature-controlled ES-MS assay, we have measured the affinities of GTA and GTB for the disaccharide acceptor **1** in the presence and absence of UDP. At physiological temperature, the binding of **1** is independent of UDP, while positive cooperativity is observed at lower temperatures. It is proposed that structural changes in the GTs, upon the binding of **1** in the presence of UDP, are primarily responsible for the decrease in ΔH_a and ΔS_a values. The present study, the first demonstration of the direct ES-MS assay for quantifying the association thermodynamic parameters for a multi-substrate enzyme system, highlights the benefits of having direct insight into binding stoichiometry when determining the thermodynamic parameters for multi-component complexes.

Materials and methods

Sample preparation

The ES-MS measurements were carried out using recombinant, soluble fragments (amino acids 63–354) of the human blood group glycosyltransferases, GTA (monomer MW 34 519 Da), and GTB (monomer MW 34 483 Da), consisting of the full C-terminal and catalytic domains as well as a truncated N-terminal domain (Seto et al. 1995). The enzymes were expressed in *Escherichia coli* and purified using procedures described previously (Seto et al. 1995; Marcus et al. 2003). The recombinant single chain variable fragment (scFv) of the monoclonal antibody Se155-4 (MW 26 539 Da) was also produced using established protocols (Zdanov et al. 1994). The scFv was used as a reference protein in the binding measurements to account for possible nonspecific contributions to the mass spectra from the nonspecific attachment of **1** to the GTs during the ES process. All protein solutions were exchanged into an aqueous 50 mM ammonium acetate (pH 7) solution except for scFv,

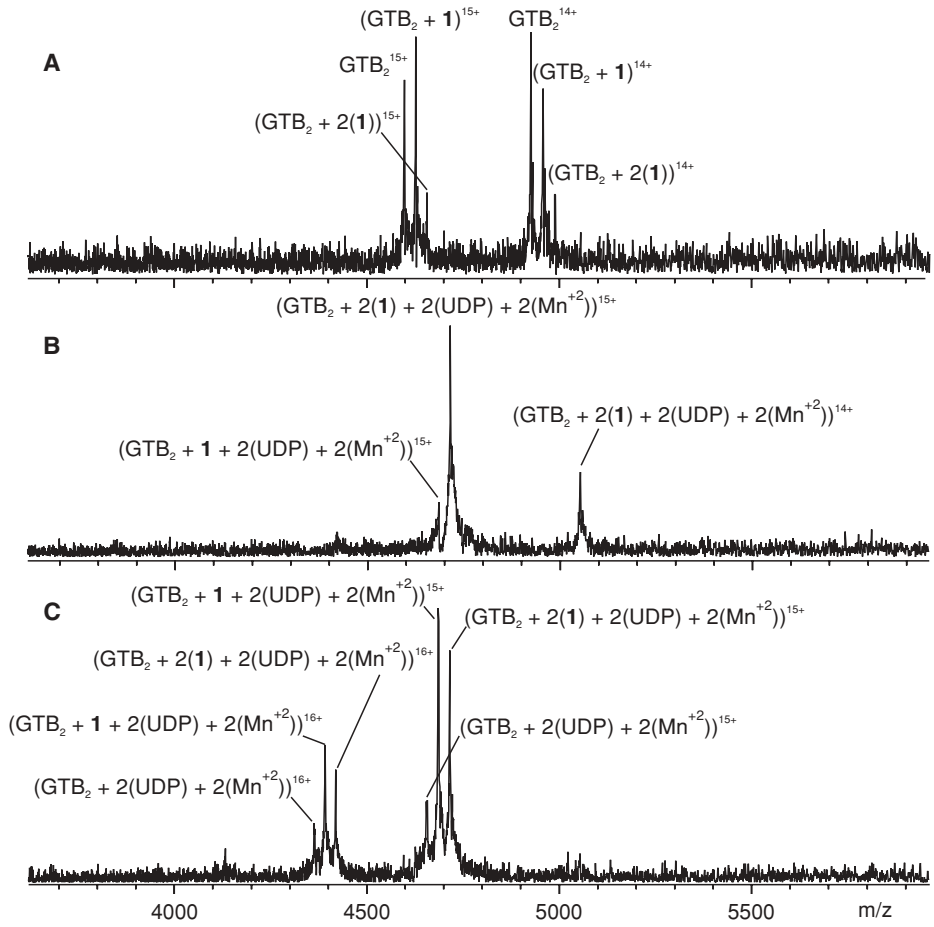

Fig. 2. Illustrative nanoES mass spectra of solutions (pH 7, 24°C) containing 8 μM GTB_2 and 100 μM Mn^{2+}. (**A**) 40 μM **1**, (**B**) 40 μM **1** and 50 μM UDP, or (**C**) 20 μM **1** and 50 μM UDP.

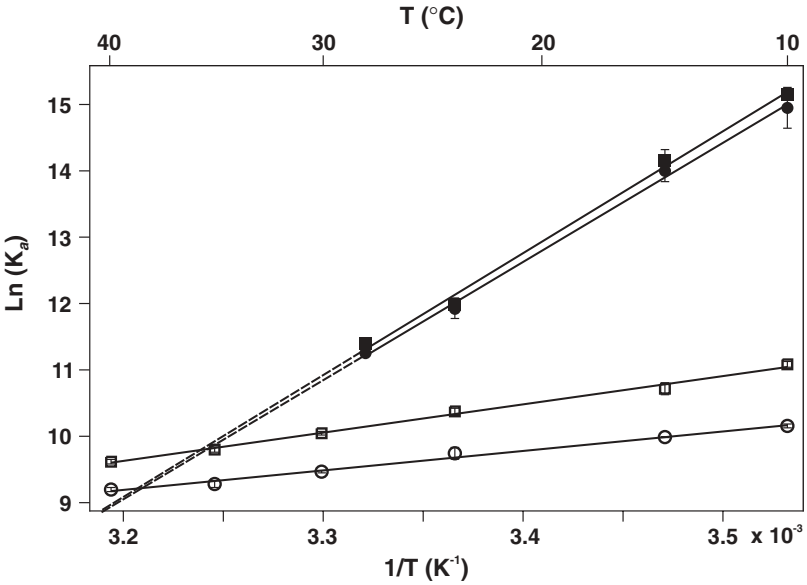

Fig. 3. Van 't Hoff plots for the association of **1** with GTA_2 (□), (GTA_2 + 2UDP + $2Mn^{2+}$) (■), GTB_2 (○), and (GTB_2 + 2UDP + $2Mn^{2+}$) (●).

Table I. Thermodynamic parameters (ΔH_a and ΔS_a) for the association of **1** with GTA or GTB in the presence and absence of bound UDP (and Mn^{2+}) in aqueous solutions at pH 7[a,b,c]

Enzyme	ΔH_a (kcal mol^{-1})	ΔS_a (cal mol^{-1} K^{-1})
GTA	-8.5 ± 0.3	-8.0 ± 0.9
GTA + UDP + Mn^{2+}	-37 ± 6	-99 ± 2
GTB	-5.8 ± 0.1	-0.3 ± 0.4
GTB + UDP + Mn^{2+}	-36 ± 2	-96 ± 5

[a]All solutions contained 100 μM $Mn(C_2H_4O_2)_2$.
[b]The reported ΔH_a and ΔS_a represent intrinsic values, in which the statistical factors arising from the two equivalent binding sites for **1** have been accounted for.
[c]The reported errors are one standard deviation.

which was exchanged directly into Milli-Q water, using an Amicon microconcentrator with a molecular weight cutoff of 10 kDa. The GTB, GTA, and scFv concentrations were determined by lyophilizing a known volume of the protein solution and measuring the corresponding mass of the protein. The synthetic ligand, **1** (α-L-Fuc*p*-(1→2)-β-D-Gal*p*-O$(CH_2)_7CH_3$), was prepared as described (Lowary and Hindsgaul 1993) and UDP was purchased from Sigma-Aldrich, Canada. Ligand stock solutions were prepared by dissolving the ligand into Milli-Q water; these were stored at −20°C. The ES solutions were prepared from the stock solutions and used for MS analysis immediately after preparation.

Mass spectrometry

All experimental measurements were performed using an Apex II 9.4 T FT-ICR/MS instrument (Bruker, Billerica, MA) equipped with a temperature-controlled nanoES ion source (Daneshfar et al. 2004). NanoES tips, with an outer diameter of ∼5 μm, were pulled from borosilicate tubes (1 mm o.d., 0.68 mm i.d.) using a P-2000 micropipette puller (Sutter Instruments, Novato, CA). A platinum wire inserted into one end of the nanoES tip was used to establish an electrical contact with the nanoES solution. A potential of +600 to +800 V was applied to the platinum wire in the nanoES tip in order to spray the solution. The tip was positioned 1–2 mm from a stainless steel sampling capillary using a microelectrode holder. The typical solution flow rate was ∼20 nL min^{-1}. Charged droplets and solvated ions emitted by the nanoES tip were introduced into the vacuum chamber of the mass spectrometer through a heated stainless steel sampling capillary (0.43 mm i.d.) maintained at an external temperature of 66°C. The gaseous ions sampled by the capillary (+48 V) were transmitted through a skimmer (+4 V) and accumulated for 5 s in an rf hexapole (+600 Vp-p). The ions were subsequently ejected from the hexapole and injected at −2700 V into the bore of the superconducting magnet, decelerated, and introduced into the ion cell. The typical base pressure for the instrument was $\sim 5 \times 10^{-10}$ mbar. Data acquisition was performed using the XMASS software (version 5.0). The time-domain signals, consisting of the sum of 50 transients containing 128 K data points per transient, were subjected to one zero-fill prior to Fourier transformation.

Determination of K_a values using the direct ES-MS assay

The GTA_2 and GTB_2 homodimers (≡GT_2) possess two acceptor (**1**) binding sites. The relevant reactions and equilibrium expressions are given below:

$$GT_2 + \mathbf{1} \rightleftharpoons (GT_2 + \mathbf{1})K_{a,1} = \frac{[GT_2 + \mathbf{1}]}{[GT_2][\mathbf{1}]} \quad (1a)$$

$$(GT_2 + \mathbf{1}) + \mathbf{1} \rightleftharpoons (GT_2 + 2(\mathbf{1}))K_{a,2} = \frac{[GT_2 + 2(\mathbf{1})]}{[GT_2 + \mathbf{1}][\mathbf{1}]} \quad (1b)$$

The equilibrium concentrations $[GT_2]$, $[GT_2 + \mathbf{1}]$, and $[GT_2 + 2(\mathbf{1})]$ can be determined from the relative abundance of the corresponding ions observed in the mass spectrum and the equations of mass balance given below:

$$[GT_2]_o = [GT_2] + [GT_2 + \mathbf{1}] + [GT_2 + 2(\mathbf{1})] \quad (2a)$$

$$[\mathbf{1}]_o = [\mathbf{1}] + [GT_2 + \mathbf{1}] + 2[GT_2 + 2(\mathbf{1})] \quad (2b)$$

After substituting the equilibrium concentrations with the corresponding ion intensity ratios, the K_a values can be calculated from Eqs. (3) and (4):

$$K_{a,1} = \frac{R_1}{[\mathbf{1}]_o - \frac{(R_1+2R_2)[GT_2]_o}{1+R_1+R_2}} \quad (3a)$$

$$K_{a,2} = \frac{R_2}{R_1\left([\mathbf{1}]_o - \frac{(R_1+2R_2)][GT_2]_o}{1+R_1+R_2}\right)} \quad (3b)$$

where the ratios R_1 (= $[GT_2 + \mathbf{1}]/[GT_2]$) and R_2 (= $[GT_2 + 2(\mathbf{1})]/[GT_2 + \mathbf{1}]$) are calculated using Eq. (4):

$$R_1 = \frac{\sum_n I(GT_2 + 1)^{n+}/n}{\sum_n I(GT_2)^{n+}/n} \quad (4a)$$

$$R_2 = \frac{\sum_n I(GT_2 + 2(\mathbf{1}))^{n+}/n}{\sum_n I(GT_2 + \mathbf{1})^{n+}/n} \quad (4b)$$

The values of $K_{a,1}$ and $K_{a,2}$ measured for GTA and GTB binding to **1** will differ due to the presence of two equivalent acceptor binding sites (Shoemaker et al. 2007). The values are related by statistical factors related to the number of equivalent free and occupied binding sites. The intrinsic K_a (the affinity of each binding site) can be determined for each and any of the ligand binding reactions

$$K_a = \frac{K_{a,1}}{2} \quad (5a)$$

$$K_a = 2 \times K_{a,2} \quad (5b)$$

Any nonspecific binding between GT_2 and **1** during the ES process will influence the intensities measured for the GT_2^{n+}, $(GT_2 + \mathbf{1})^{n+}$, and $(GT_2 + 2(\mathbf{1}))^{n+}$ ions (Wang et al. 2003). Recently, a method to quantitatively account for the contribution of nonspecific complexes to the nanoES mass spectra was developed in our laboratory (Sun et al. 2006). Briefly, this method involves the addition of a reference protein (P_{ref}), which exhibits no specific affinity for the ligands of interest, to the ES solution. The distribution of ligands bound nonspecifically to P_{ref} can be used to correct the measured intensities of the P^{n+}

and specific PL^{n+} ions for the occurrence of nonspecific ligand binding. A complete description of the correction process is given elsewhere (Sun et al. 2006).

The enthalpies and entropies of association (ΔH_a and ΔS_a) of GTA and GTB binding with **1** were estimated from the temperature dependence of the intrinsic K_a, which was modeled using the linear form of the van't Hoff equation:

$$\ln K_a = -\frac{\Delta H_a}{R_g T} + \frac{\Delta S_a}{R_g} \quad (6)$$

where R_g is the ideal gas constant and T is the temperature.

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Conflict of interest statement

There are no conflicts of interest.

Abbreviations

ES-MS, electrospray ionization mass spectrometry; FT-ICR, Fourier-transform ion cyclotron resonance; GTA, α-(1→3)-*N*-acetylgalactosaminyltransferase; GTB, α-(1→3)-galactosyltransferase; ITC, isothermal titration microcalorimetry; nanoES, nanoflow electrospray ionization; scFv, single chain variable fragment; SPR, surface plasmon resonance; UDP, uridine 5′-diphosphate.

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