Thermodynamic analysis of the binding of galactose and poly-*N*-acetyllactosamine derivatives to human galectin-3

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Abstract Galectin-3, with a wide tissue distribution and marked developmental regulation, provides significant insights into the progression of various disease and developmental stages. Recognized by its specificity for galactose, a detailed characterization of its sugar binding ability has been investigated by isothermal titration calorimetry. The results presented here complement well with the earlier studies utilizing hapten inhibition assays. Among the various lactose derivatives studied, A-tetrasaccharide emerged with the highest affinity for binding to galectin-3 combining site. This blood group saccharide exhibited a binding affinity 37-fold higher and a 102 kJ/mol more favorable change in enthalpy over lactose at 280 K indicating the existence of additional subsites for both the α1-3-linked *N*-acetylgalactosamine at the non-reducing end and the α1-2-linked L-fucosyl residue. The thermodynamic parameters evaluated for other ligands substantiate further the carbohydrate recognition domain to be part of an extended binding site. Binding thermodynamics of galectin-3 with the galactose derivatives are essentially enthalpically driven and exhibit compensatory changes in $\Delta H°$ and $T\Delta S$ owing to solvent reorganization. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Galectin-3; Isothermal titration calorimetry; Lactose; Blood group saccharide; Enthalpy–entropy compensation

1. Introduction

A member of the metal ion-independent β-galactoside binding protein family, galectin-3 (M_r 30 000 Da), is a consistent participant in embryonic development, tumor progression and metastasis [1,2]. The galectin family of animal lectins is distinguished by the gal/lac specificity of its carbohydrate recognition domain (CRD), with highly conserved residues between members of the family (galectin-1, -2, -3 and -4) and between the homologs found in various species for any given single member [3,4]. Interestingly, two galectin subtypes have been reported to exhibit carbohydrate specificity unlike other members, namely, galectin-10 (Charcot-Leyden crystal protein) and galectin-11 (Grifin) [5,6]. From a functional point of view, galectins belong either to the proto- or tandem-repeat type of molecular architecture which have, respectively, one and two sugar binding sites of variable binding affinity [7]. Interestingly, galectin-3 possesses a chimera-type of structural organization, which is unique amongst the galectins studied so far. This classification is based on it having one sugar binding domain and another domain with no role in carbohydrate recognition. Although, a recent modeling study has included a stretch of amino acids N-terminus into the galectin-3 CRD [8]. The carbohydrate-independent domain is speculated to interact with biomolecules other than sugars, thus serving as a cross-linker, aiding in cellular targeting and biological functions independent of phosphorylation [9].

Mammalian cell surfaces and the extracellular milieu surrounding them have a rich array of glycoconjugates. Prominent among them are the β-galactosides, in particular the Galβ1-4GlcNAc, Galβ1-3GlcNAc, Galβ1-4Glc and Galβ1-3GalNAc. In vivo, galectin-3, an endogenous human tumor-associated lectin, is a functional molecule that acts as a receptor for ligands encompassing lactose as the basic unit of recognition [10,11]. A differential scanning calorimetric study attested this carbohydrate specificity of galectin-3, wherein only lactose was seen to increase the transition temperature as opposed to the other sugars tested [12]. The set of galectins whose carbohydrate binding specificity has been examined recognize the same structural determinants on lactose and related β-galactosides [13–17]. A multitude of biochemical and biophysical assays suggest a higher order of complexity than the sole requirement of a galactose moiety [18]. This warrants a careful investigation into its binding thermodynamics to delineate the affinity difference in terms of contributions made from the enthalpy ($\Delta H°$) and entropy ($T\Delta S$) for each ligand.

This paper deals with a judiciously selected panel of galactose and poly-*N*-acetyllactosamine derivatives probed by isothermal titration calorimetry (ITC) for their binding propensity to galectin-3. The choice of saccharide is based on studies wherein the only structural constraint on the ligand implicated in binding to the lectin is the presence of terminal non-reducing galactosyl units in β-linkage [14,17,19,20]. This study not only adds to the existing knowledge of the carbohydrate binding specificity of galectin-3, but is also the first report on thermodynamic parameters associated with it.

2. Materials and methods

2.1. Saccharides

Lactose, *N*-acetyllactosamine and thiodigalactoside (TDG) were purchased from Sigma. 2′-Fucosyllactose (2′FL), lacto-*N*-biose (LNB), lacto-*N*-tetraose, lacto-*N*-hexaose, linear B-2-trisaccharide and lacto-*N*-triose were obtained from Dextra Laboratories, whereas A-trisaccharide and A-tetrasaccharide were procured from Biocarb.

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2.2. Purification of galectin-3

pGal3, encoding the human galectin-3, a kind gift of Prof. Hakon Leffler, was freshly transformed into BL21(DE3) cells prior to each purification as described earlier [21]. An overnight culture of rGal was grown in LB broth containing 100 μg/ml ampicillin at 37°C. This overnight culture was used as the primary inoculum for 1 l LB which was grown at 37°C until an $OD_{600\ nm}$ of about 0.4–0.6, at which point the culture was induced with 0.4 mM IPTG. After further growth of the culture for 3 h at 37°C, the cells were harvested and the pellet resuspended in ~70 ml MEPBS (75 mM Na/K phosphate, pH 7.2, 2 mM EDTA, 4 mM β-mercaptoethanol (βME)) containing 0.1 mM PMSF. These cells were sonicated using a Braun probe sonicator, and the lysate thus obtained was centrifuged at 100 000 × *g* for 30 min. The clarified bacterial lysate thus obtained was applied to a lactosyl-Sepharose affinity matrix. The column was washed with 10 bed volumes of MEPBS and the protein eluted with 0.15 M lactose. The fractions containing the pure lectin were pooled, concentrated and extensively dialyzed against MEPBS.

2.3. ITC

Isothermal titration microcalorimetry was performed using an OMEGA Microcalorimeter from Microcal Inc. (Northampton, MA, USA). In individual titrations, injections of 5–8 μl of carbohydrate solution were added from the computer-controlled 250 μl syringe at an interval of 3 min into the galectin solution dissolved in the same buffer as the saccharide, while stirring at 400 rpm. Control experiments performed by identical injections of saccharide into the cell containing buffer alone showed insignificant heats of dilution. The experimental data were fitted to a theoretical titration curve using the software provided by Microcal. All experiments were performed with c values $1 < c < 200$ in the present study where $c = K_b \times M_t(0)$, $M_t(0)$ is the initial macromolecule concentration and K_b is the binding constant [2]. The thermodynamic parameters were calculated from the basic equations of thermodynamics:

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_b$$

where ΔG, ΔH and ΔS are the changes in free energy, enthalpy and entropy of binding, respectively. T is the absolute temperature and $R = 8.314$ J/mol K.

3. Results and discussion

A typical titration calorimetry curve for the binding of A-tetrasaccharide to galectin-3 is shown in Fig. 1A. The results show a monotonic decrease in the exothermic heat of binding with successive injection until saturation is reached. Also shown is the fit of the incremental heats of binding per mol of added ligand, which closely follows the injection number (Fig. 1B). The thermodynamic parameters and their relative affinities for the binding of various sugars to galectin-3 in MEPBS at pH 7.2 thus obtained are documented in Table 1. The values for $\Delta H°$ and the binding constants for the various sugars range from −20.07 to −126.01 kJ/mol and from 1160 to 43 100 M^{-1}.

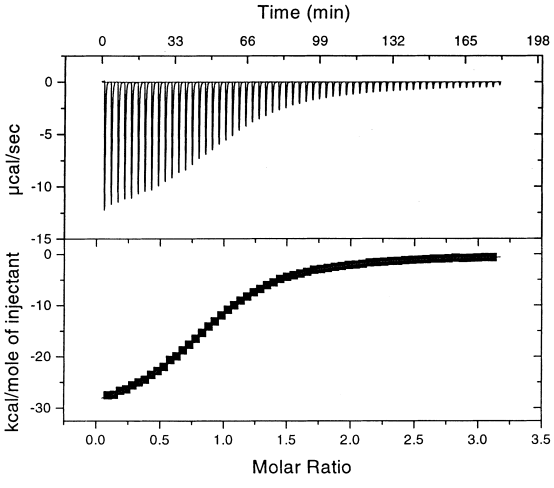

Fig. 1. Calorimetric titration of galectin-3 (0.3 mM) with A-tetrasaccharide (2.4 mM) at 280 K. Top: data obtained for 50 automatic injections, each 5.2 μl aliquots of A-tetrasaccharide; bottom: the integrated curve shows experimental points (■) and the best fit (–). The buffer used was 75 mM MEPBS (75 mM Na/K phosphate, 2 mM EDTA and 4 mM βME), pH 7.2.

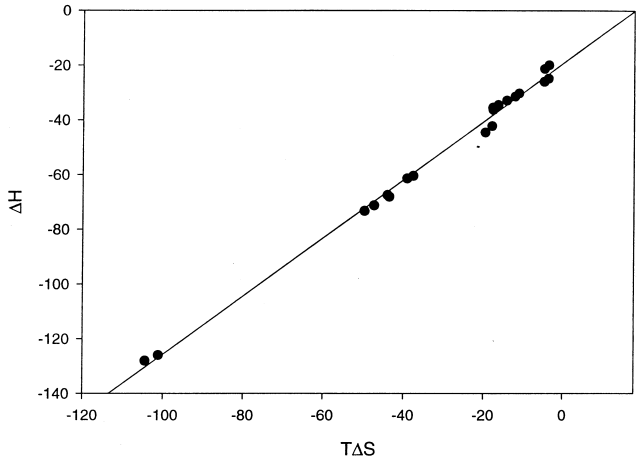

Fig. 2. Enthalpy–entropy compensation plot. Plot of $-\Delta H°$ vs. $-T\Delta S_b$ for the binding of the galactose and poly-*N*-acetyllactosamine sugar derivatives to galectin-3. The plot shows a linear relationship with a slope of 18.26 and a correlation coefficient of 0.996.

This study provides an insight into the galectin-3 CRD from a thermodynamic point of view. The binding interaction of galectin-3 with the galactose and poly-*N*-acetyllactosamine derivatives is essentially enthalpically driven and exhibits compensatory changes in $\Delta H°$ and $T\Delta S$, as shown in Fig. 2, which appears to stem from the solvent reorganization upon ligand binding [22]. This in turn implies that favorable changes observed during the binding process as a consequence of removal of water molecules from the combining site are offset by the compensatory changes in enthalpy due to water-mediated hydrogen bonds and van der Waals interactions. The linearity of the enthalpy–entropy plot suggests a similar mode of recognition by the galectin-3 binding site for all the ligands studied.

Earlier studies have established the necessity of an axial configuration of the hydroxyl group at the C4 position of the sugar for galectin-3 to participate in the binding reaction [14,17]. Thus, the topography of the combining site can be visualized to accommodate specifically a terminal non-reducing galactose. The three-dimensional crystal structure of the CRD of galectin-3 complexed with lactose and LacNAc at 2.1 Å resolution provides, additionally, the basis for interpreting the data in molecular terms [23]. The main residues that contact lactose and LacNAc in galectin-3 are Glu165, Arg186, Glu184, Arg162, Asn160, Asn174, Arg144 and His158 [23].

From the thermodynamic parameters obtained, lactose is seen to bind with a binding affinity close to 1160 M^{-1}. Extension at the non-reducing end of the central disaccharide with Fucα1-2, as in 2′FL, marginally increases the binding constant over lactose, which is mostly due to favorable change

Table 1
Thermodynamic parameters for the binding interaction of galectin-3 with galactose and poly-*N*-acetyllactosamine derivatives

Sugar	Structure	T (K)	K_b (M^{-1})	Rel. aff.	ΔH° (kJ/mol)	ΔG (kJ/mol)	TΔS (kJ/mol/K)	ΔS (J/mol)
Lac	Galβ1-4Glc	279.6	1160.0	1.00	-20.07	-16.40	-3.60	-12.89
		298.2	802.0	0.69	-21.22	-16.58	-4.67	-15.65
2'FL	Fucα1-2Galβ1-4Glc	280.2	3710.0	3.20	-30.27	-19.15	-11.15	-39.79
		298.1	2420.0	2.09	-31.37	-19.31	-12.09	-40.54
TDG	Galβ1-S-1βGal	280.7	1930.0	1.66	-35.33	-17.65	-17.71	-63.08
		298.3	1780.0	1.53	-36.19	-18.56	-17.62	-59.08
LNB	Galβ1-3GlcNAc	281.0	2880.0	2.48	-32.87	-18.61	-14.26	-50.75
		298.2	1500.0	1.29	-34.49	-18.13	-16.37	-54.88
LacNAc	Galβ1-4GlcNAc	280.8	8645.0	7.45	-36.59	-21.16	-3.74	-13.30
		298.0	5238.0	4.52	-35.89	-21.22	-4.77	-16.01
LNTet	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	279.7	18100.0	15.60	-60.44	-22.80	-37.64	-134.59
		298.0	8040.0	6.93	-61.43	-22.28	-39.15	-131.38
LNhex	Galβ1-4GlcNAcβ1 6 Galβ1-4Glc 3 Galβ1-3GlcNAcβ1	279.4	34309.0	29.58	-42.19	-24.26	-17.94	-64.21
		298.2	23277.0	20.07	-44.53	-24.93	-19.60	-65.73
B-Tri	Galα1-3Galβ1-4GlcNAc	280.2	22928.0	19.77	-67.44	-23.39	-44.06	-157.23
		298.3	18672.0	16.10	-68.04	-24.39	-43.65	-146.33
A-Tri	GalNAcα1-3Gal 2 Fucα1	280.2	28200.0	24.31	-71.26	-23.87	-47.39	-169.14
		298.2	13391.0	11.54	-73.29	-23.56	-49.73	-166.78
A-tetra	GalNAcα1-3Galβ1-4Glc 2 Fucα1	280.4	43100.0	37.16	-126.01	-24.88	-101.14	-360.69
		298.2	13830.0	11.92	-128.06	-23.64	-104.42	-350.18

in enthalpy of −30 kJ/mol. This is consistent with the formation of a water-mediated hydrogen bond between C4-OH of its fucosyl residue and Glu165 of the galectin [20]. TDG, on the other hand, with a sulfur linkage, exhibits 1.5-fold higher affinity than lactose. The higher affinity supports earlier studies by Leffler and Barondes and Lee et al., wherein *S*-galactoside was shown to lodge itself in the galectin-3 CRD more securely than the *O*-galactoside [9,10]. Moreover, the second galactose moiety in TDG appears to be suitably positioned to form additional H-bonds with Arg162 and Glu184 [24]. The increase in enthalpy (∼10–15 kJ/mol) for 2′FL and TDG over lactose is therefore in accordance with the more favorable binding of these ligands as a consequence of the additional hydrogen bonds. In LNB, GlcNAc attached to galactose via a β1-3 linkage allows better interaction of the N2 with Glu165 as well as O6 of GlcNAc with Glu184 via a water molecule. Though in this molecule the interaction of Arg162 and Glu184 with O3 of GlcNAc is lost, both these residues are able to interact with O4 of GlcNAc. These two new H-bonds could account for the 2.5-fold higher affinity of galectin-3 for LNB compared to lactose. On the other hand, LacNAc exhibits 7-fold higher affinity than lactose and 14 kJ/mol of favorable enthalpy change, as compared to lactose, due to the acetamide group on C2 in GlcNAc that establishes favorable contacts with Glu165 via a water molecule, in addition to the hydrogen bonds established by the Arg162 and Glu184 with O3 of GlcNAc. This suggests the β1-4 linkage at this position to be more conducive for binding, which can be rationalized on the basis that the acetamide group is oriented away from Glu165 in LNB, thus compromising the binding.

Among the poly-*N*-acetyllactosamine derivatives studied for their binding thermodynamics, lacto-*N*-tetraose exhibit 16-fold higher affinity at 280 K, with a favorable change in enthalpy ($\sim$40 kJ/mol) driving the binding reaction. The 16-fold higher binding propensity of the tetrasaccharide provides evidence for the existence of determinants, on the protein surface adjacent to the primary binding site, that regulate the ability to recognize two additional monosaccharide units. The next higher order structure investigated was the branched oligosaccharide, lacto-*N*-hexaose. It exhibits a binding constant 30-fold higher than lactose ($K_b = 34\,309\ M^{-1}$ at 279.4 K), and a mere 2-fold increase over tetraose. The increase in binding affinity is accompanied by a significant change in enthalpy, i.e. −42 kJ/mol. A closer look at the thermodynamic parameters suggests the binding of the branched saccharides to be entropically driven, rather than enthalpically as observed for the other ligands. Binding of the lacto-*N*-tetraose is accompanied by a change in enthalpy and entropy of −60 kJ/mol and −134.6 J/mol, respectively. Lacto-*N*-hexaose, on the other hand, exhibits a change of the order of −42 kJ/mol and −64.2 J/mol, respectively. Structure of the branched hexasaccharide suggests the Galβ1-4Glc to be recognized by the primary binding site and that substitution of the Gal at its α1-3 arm is accommodated in an extended subsite. The substitution at the α1-6 arm appears not to participate for binding to the protein surface. On the other hand, the possibility of galectin-3 monomers to oligomerize upon binding is ruled by the stoichiometry = 1 obtained from the ITC studies. This is in contrast to the binding characteristics obtained with the biantennary mannooligosaccharides to ConA which exhibit $n = 0.5$ [24]. Since this branched oligosaccharide interacts with the lectin in a univalent fashion, its enhanced binding propensity reflects the favorable influence of the α1-6 branch on the orientation of the binding portion of the saccharide on the β1-3 branch. This is consistent with the entropically driven nature of the reaction as well.

Galectin-3 was also tested out for its propensity to combine with the human blood group saccharides. They are seen to be good binders with affinities of 19-, 24- and 37-fold for linear B-trisaccharide, A-trisaccharide and A-tetrasaccharide, respectively, compared to lactose at 280 K. A-tetrasaccharide emerged as the best binder with the most favorable change in enthalpy, i.e. 102–116 kJ/mol. A comparison of the binding enthalpies of A-tetrasaccharide and A-trisaccharide with 2′FL underscores the dominant energetic contribution of the α1-3-linked terminal GalNAc of a tetrasaccharide for the binding process. Our results corroborate the observations from the docking experiments, wherein the A-tetrasaccharide shows increased binding by virtue of the additional hydrogen bonds that it is able to establish [20]. The A-tetrasaccharide is speculated to be tethered to the extended CRD through a water-mediated hydrogen bond between Lys176 and the N2, O7 atoms of GalNAc, as well as a hydrogen bond between the GalNAc and Arg144. The α1-2-linked fucose, on the other hand, establishes a hydrogen bond only with a single residue visible with Glu165. Thermodynamic parameters reported here support that the strong binding of A-tetrasaccharide to the galectin is largely due to additional hydrogen bonds.

Most galectin CRDs examined in detail thus far exhibit similar affinities for lactose and LacNAc and have highly conserved amino acid residues in the primary carbohydrate binding site involved in binding to the β-galactoside. Galectin-3 differs in its binding specificity as exemplified by its propensity for binding to the blood group saccharides, which suggests an extended binding site [23,25]. These fine differences in carbohydrate specificity imply that each galectin may interact with a discrete spectrum of glycoconjugate receptor meting out the desired downstream effects [8]. Increase in the enthalpy for binding of A reactive tri- and tetrasaccharides confirms the observation that the combining site of galectin-3 is extended. Nevertheless, the extended site is quite specific because sugars such as lacto-*N*-triose are very poor binders.

The results discussed so far prompt us to reiterate the existence of four subsites in the CRD of galectin-3 as described by Knibbs et al. [17]. As per their nomenclature, subsites 3 and 4 appear to be the primary binding site and extensions at the non-reducing terminal of the disaccharide are accommodated in subsites 1 and 2 [14]. Moreover, since the branched hexasaccharide exhibits only 2-fold higher affinity over lacto-*N*-tetraose, it substantiates further the existence of four indentations on the protein surface to bind to its complementary ligands, each accommodating a monosaccharide. The 2-fold higher affinity of hexasaccharide due to the greater flexibility of the structure perhaps allows a more favorable orientation in the combining site. This investigation allows us to conclude that the minimal epitope recognized by galectin-3 is a tetrasaccharide with defined orientations of the monosaccharide units.

References

[1] Abbott, W.M., Hounsell, E.F. and Feizi, T. (1988) Biochem. J. 252, 283–287.
[2] Barondes, S.H. et al. (1994) Cell 76, 597–598.
[3] Andersen, R.L. and Wang, J.L. (1992) Trends Glycosci. Glycotechnol. 4, 43–52.
[4] Hirabayashi, J. (1993) Glycobiology 3, 297–304.
[5] Swaminathan, G.J., Leonidas, D.D., Savage, M.P., Ackerman, S.J. and Acharya, K.R. (1999) Biochemistry 38, 13837–13843.
[6] Ogden, A.T., Nunes, I., Ko, K., Wu, S., Hines, C.S., Wang, A.F., Hegde, R.S. and Lang, R.A. (1998) J. Biol. Chem. 273, 28889–28896.
[7] Barondes, S.H., Cooper, D.N.W., Gitt, M.A. and Leffler, H. (1994) J. Biol. Chem. 269, 20807–20810.
[8] Barboni, E.A., Bawumia, S., Henrick, K. and Hughes, R.C. (2000) Glycobiology 10, 1201–1208.
[9] Kasai, K. and Hirabayashi, J. (1996) J. Biochem. 119, 1–8.
[10] Vasta, G.R., Quesenberry, M., Ahmed, H. and O'Leary, N. (1999) Dev. Comp. Immunol. 23, 401–420.
[11] Ahmed, H. and Vasta, G.R. (1994) Glycobiology 4, 545–548.
[12] Agrwal, N., Sun, Q., Wang, S.Y. and Wang, J.L. (1993) J. Biol. Chem. 268, 14932–14939.
[13] Lee, R.T., Ichikawa, Y., Allen, H.J. and Lee, Y.C. (1990) J. Biol. Chem. 265, 7864–7871.
[14] Leffler, H. and Barondes, S.H. (1986) J. Biol. Chem. 261, 10119–10126.
[15] Oda, Y., Herrmann, J., Gitt, M.A., Turck, C.W., Burlingame, A.L., Barondes, S.H. and Leffler, H. (1993) J. Biol. Chem. 268, 5929–5939.
[16] Sato, S. and Hughes, R.C. (1992) J. Biol. Chem. 267, 6983–6990.
[17] Knibbs, R.N., Agrwal, N., Wang, J.L. and Goldstein, I.J. (1993) J. Biol. Chem. 263, 14940–14947.
[18] Feizi, T., Solomon, J.C., Yuen, C.T., Jeng, K.C., Frigeri, L.G., Hsu, D.K. and Liu, F.T. (1994) Biochemistry 33, 6342–6349.
[19] Sparrow, C.P., Leffler, H. and Barondes, S.H. (1987) J. Biol. Chem. 262, 7383–7390.
[20] Henrick, K., Bawumia, S., Barboni, E.A., Mehul, B. and Hughes, R.C. (1998) Glycobiology 8, 45–57.

[21] Massa, S.M., Cooper, D.N., Leffler, H. and Barondes, S.H. (1993) Biochemistry 32, 260–267.
[22] Rani, P.G., Bachhawat, K., Misquith, S. and Surolia, A. (1999) J. Biol. Chem. 274, 29694–29698.
[23] Seetharaman, J., Kanigsberg, A., Slaaby, R., Leffler, H., Barondes, S.H. and Rini, J.M. (1998) J. Biol. Chem. 273, 13047–13052.
[24] Dam, T.K., Roy, R., Das, S.K., Oscarson, S. and Brewer, C.F. (2000) J. Biol. Chem. 275, 14223–14230.
[25] Leffler, H. (1997) Trends Glycosci. Glycotechnol. 9, 9–19.