Energetics of carbohydrate binding by a 14 kDa S-type mammalian lectin

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The thermodynamics of the binding of derivatives of galactose and lactose to a 14 kDa β-galactoside-binding lectin (L-14) from sheep spleen has been studied in 10 mM phosphate/150 mM NaCl/10 mM β-mercaptoethanol buffer, pH 7.4, and in the temperature range 285–300 K using titration calorimetry. The single-site binding constants of various sugars for the lectin were in the following order: *N*-acetyl-lactosamine thiodigalactoside > 4-methylumbelliferyl lactoside > lactose > 4-methylumbelliferyl α-D-galactoside > methyl-α-galactose > methyl-β-galactose. Reactions were essentially enthalpically driven with the binding enthalpies ranging from −53.8 kJ/mol for thiodigalactoside at 301 K to −2.2 kJ/mol for galactose at 300 K, indicating that hydrogen-bonding and van der Waals interactions provide the major stabilization for these reactions. However, the binding of 4-methylumbelliferryl-α-D-galactose displays relatively favourable entropic contributions, indicating the existence of a non-polar site adjacent to the galactose-binding subsite. From the increments in the enthalpies for the binding of lactose, *N*-acetyl-lactosamine and thiodigalactoside relative to methyl-β-galactose, the contribution of glucose binding in the subsite adjacent to that for galactose shows that glucose makes a major contribution to the stability of L-14–disaccharide complexes. Observation of enthalpy–entropy compensation for the recognition of saccharides such as lactose by L-14 and the absence of it for monosaccharides such as galactose, together with the lack of appreciable changes in the heat capacity (ΔC_p), indicate that reorganization of water plays an important role in these reactions.

INTRODUCTION

The complexity of carbohydrate structures and the specificity of lectin–carbohydrate interactions have implicated them in several important biological functions [1], necessitating extensive *in vitro* studies on the binding processes between them. A wide variety of plant lectins have been studied by physicochemical techniques [2], leading to a better understanding of the molecular basis of the specificities of carbohydrate–lectin interactions.

Since the discovery of the mammalian hepatic lectin by Ashwell and co-workers [3], a large number of lectins from animal sources have been reported. Animal lectins have been grouped into two distinct classes, the S-type and the C-type [4], depending upon the requirement of thiol groups and Ca^{2+} respectively for their activity. The S-type lectins, a group of soluble lectins [5], variously called the S-lac lectins, galaptins, β-galactoside-binding lectins or more recently galectins [6], are classified on the basis of their subunit molecular masses as the (a) 14 kDa, (b) 16–22 kDa, (c) 29–35 kDa and (d) 67 KDa lectins [7].

The most abundant of the S-type lectins, the 14 kDa lectin, also referred to as 'L-14' [8], is found to be ubiquitously distributed in a wide variety of vertebrate tissues. It has been cloned and sequenced, and a high degree of sequence identity exists between the 14 kDa lectins isolated from different sources [8–10]. The X-ray crystal structures of the 14 kDa lectin from bovine spleen [11] and a recombinant human lectin, L-14II [12], have recently been reported.

The biological functions of L-14 still remain elusive. It has been postulated that L-14 might mediate cell–cell and cell–matrix interactions by binding to specific glycoconjugates. In order to participate in adhesion processes, L-14 has to be externalized from the cell. Indeed, developmentally regulated externalization of L-14 has been shown in muscle cells [13]. Furthermore, endogenous L-14 secreted during myoblast differentiation has been shown to promote substrate detachment of these cells by binding to laminin [14]. L-14 has also been shown to mediate *in vitro* adhesion of CHO and F9 cells to immobilized laminin [15]. It has been demonstrated that L-14 binds not only to laminin but also to $\alpha_7\beta_1$ integrin, which is the predominant laminin receptor on differentiating skeletal muscles, and through these interactions plays a role in myoblast differentiation and fusion [16]. A role has also been proposed for L-14 in immune modulation [17,18], and in cell growth regulation [19].

As can be seen, the above-mentioned functions of L-14 involve multicomponent systems where specific interactions occur between L-14, cell-surface glycoconjugates and matrix glycoproteins such as laminin. Hence it becomes imperative to study the specificity of interactions between L-14 and each of its implicated ligands. Previous studies on L-14 reported the relative inhibitory values of various sugars based on the amount of ligand required to inhibit L-14 (50 or 100%) binding to rabbit erythrocytes [20] or immobilized asialofetuin [21,22] or lactose [23]. However, to understand better the putative functions of L-14, a more quantitative study is warranted. As a first step towards this objective we have carried out a detailed investigation on the carbohydrate-binding properties of L-14 isolated from sheep spleen with derivatives of galactose and lactose, using titration calorimetry. The thermodynamics of the L-14–sugar binding interaction in terms of the binding constants (K_b), changes in the free energy (ΔG_b), the binding enthalpy (ΔH_b) and the binding entropy (ΔS_b) are reported here.

MATERIALS AND METHODS

Materials

The sugars galactose, methyl-α-galactose, methyl-β-galactose, 4-methylumbelliferyl α-D-galactoside (MumbαGal), lactose, *N*-

Abbreviations used: L-14, 14 kDa β-galactoside-binding lectin; β-ME, β-mercaptoethanol; PMSF, phenylmethanesulphonyl fluoride; thiodiGal, thiodigalactoside; LacNAc, *N*-acetyl-lactosamine; MumbLac, 4-methylumbelliferyl lactoside; MumbαGal, 4-methylumbelliferyl α-D-galactoside; MumbβGal, 4-methylumbelliferyl β-D-galactoside; MeαGal, Methyl α-galactoside; MeβGal, methyl β-galactoside.

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acetyl-lactosamine (LacNAc), 4-methylumbelliferyl β-D-lactoside (MumbLac) and thiodigalactoside (thiodiGal) were obtained from Sigma. All other chemicals used were of reagent grade and obtained locally.

Preparation of L-14

Sheep spleen was obtained at the local slaughterhouse and stored at −20 °C until use. L-14 was isolated from sheep spleen essentially as described previously [24] with a few modifications. Spleen was cut into small pieces and homogenized in 10 vol. of ice-cold acetone containing 0.01 M β-mercaptoethanol (β-ME) and 0.25 mM phenylmethanesulphonyl fluoride (PMSF). The homogenate was filtered on a Buchner funnel and the acetone-dried powder dissolved at 4 ml/g in PBSM [10 mM phosphate buffer (pH 7.4)/0.15 mM NaCl/0.01 M β-ME] containing 0.1 M lactose and was left overnight with stirring. All the reactions were carried out at 4 °C. The homogenate was centrifuged at 12200 *g* for 30 min, the supernatant dialysed extensively against PBSM, loaded on to a lactamyl-Sepharose affinity column (column volume 15 ml), prepared as reported previously [25]. The column was washed with PBSM until the absorbance at 280 nm was less than 0.05, and the protein eluted with the same buffer containing 0.1 M lactose. The protein was dialysed against PBSM to remove lactose and stored at −20 °C until use. The yield of the protein was ~ 5 mg/100 g of tissue. The purity of the protein was checked by SDS/PAGE, where a single band corresponding to a molecular mass of 14 kDa was obtained. The minimum concentration of the protein required for the haemagglutination of rabbit erythrocytes was found to be 2 μg/ml. The protein concentration was determined by using a specific absorption coefficient ($A_{280}^{1\%,1\,cm}$) of 5.5, as reported previously [24].

Preparation of sugar solutions

Solutions of sugars were prepared by weight in PBSM. Concentrations of 4-methylumbelliferyl-sugars were estimated by using a molar absorption coefficient of $1.36 \times 10^4\ M^{-1} \cdot cm^{-1}$ at 318 nm.

Calorimetric titration measurements

The calorimetric titrations were performed with a Microcal Omega titration calorimeter as described in detail by Wiseman et al. [26] and Yang [27]. Microlitre amounts of the ligand solution were added by means of a rotating stirrer–syringe to the protein solution contained in a 1.38 ml cell. The additions were 3 min apart to allow the exothermic peak which appeared after each addition to return to the baseline. The total heat, Q_t, was then fitted via a non-linear least-squares minimization method to the total ligand concentration, X_t, using the following equation:

$$Q_t = nM_t\Delta H_b V\{1 + X_t/nM_t + 1/nK_bM_t - [(1 + X_t/nM_t + 1/nK_bM_t)^2 - 4X_t/nM_t]^{\frac{1}{2}}\}/2 \quad (1)$$

where n is the number of sites, M_t is the total protein monomer concentration, V is the cell volume, K_b is the binding constant and ΔH_b is the binding enthalpy. The expression for the i^{th} injection, $\Delta Q(i)$, is then given by [27]:

$$\Delta Q(i) = \Delta Q(i) + dV_i/2V_i/2V\,[Q(i) + Q(i-1)] - Q(i-1) \quad (2)$$

where dV_i is the volume of titrant added to the solution. The thermodynamic parameters, ΔG_b^0 and ΔS_b are calculated from the basic eqns. (3) and (4):

$$\Delta G_b^0 = -RT\ln K_b \quad (3)$$

$$\Delta S_b = \Delta H_b - \Delta G_b^0/T \quad (4)$$

RESULTS AND DISCUSSION

The results of a typical titration calorimetry experiment with the addition of 3.6 μl aliquots of LacNAc to 156 μM of the protein solution are shown in Figure 1. The results exhibit a monotonic decrease in the exothermic heat of binding with each successive injection until saturation is achieved. It may be noted that the amount of heat released decreases gradually, allowing an accurate determination of the thermodynamic data. A plot of incremental heat evolved as a function of injection number after subtracting the heat of dilution. For LacNAc these data are shown in Figure 2. Best fits of eqn. (1) for similar plots of various sugars provide the K_b, ΔH_b and n values that are presented in Table 1 along with the calculated values for ΔS_b. Monomer concentrations of the lectin were used in the fitting procedures, and the stoichiometries from the fits for these titrations were close to one for disaccharides. In contrast with monosaccharides, larger S.D. values are observed. This is probably due to an uncertainty in fitting the data at K_b values of less than 100 M^{-1}. For monosaccharides, impractically high concentrations of the protein (20–50 mM) in the cell are required to obtain more precise values of these parameters. Since we are dealing with a simple symmetric binding reaction, we were able to obtain more accurate values of ΔH_b for the monosaccharides by titrating a large excess of these sugars in the cell (500 mM) with three or four successive 50 μl additions of L-14 (160 μM) [28]. Under these conditions C, a unitless constant

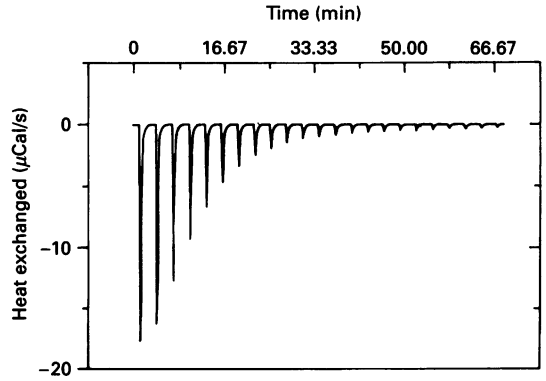

Figure 1 Calorimetric titration of 3.6 μl aliquots of 16.1 mM LacNAc into 156 μM of L-14 at 298.6 K (note: 1 μCal ≡ 4.184 μJ)

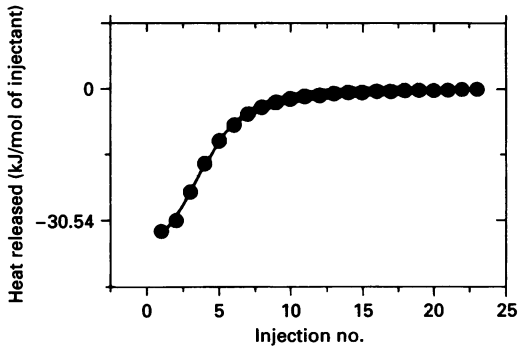

Figure 2 Plot of total heat released as a function of injection number for the titration shown in Figure 1

The continuous line is the result of the best least-square fit of the data to eqn. (1).

Table 1 Thermodynamic parameters for the binding of carbohydrates to L-14

Sugar	Temp. (K)	n	$10^{-3} \times K_b$ (M^{-1})	$-\Delta H_b$ (kJ/mol)	$-\Delta G_b$ (kJ/mol)	$-\Delta S_b$ (J/mol per K)	$10^{-3} \times K_b$* (M^{-1})
ThiodiGal†	301.3	0.96	11.3	54	23	103	5.8[a]
	294.3	1.03	31.1	48	25	78	4.4[b]
LacNAc†	298.6	1.04	31.9	42	26	54	18.9[a]
	288.6	0.98	46.4	44	26	62	5.6[b]
MumbLac†	298.3	0.96	14.4	36	24	40	
	290	1	17.3	43	24	66	
Lactose†	300	1.01	5.6	37	22	50	1.8[a]
	285.5	0.96	12.6	36	22	49	1.1[b]
MumbαGal†	292.3	1.1	4	10	20	−34	
	285.6	1.1	6.9	9	21	−42	
Galactose†	300	1.2	0.05	2.3	10	24	0.03[a]
MeαGal†	300	1.1	0.05	3	10	24	0.02[a]
MeβGal†	300	0.86	0.04	2	9	24	0.01[a]
Galactose‡	300		–	2.4, 2.6, 2.3	–	–	
MeαGal‡	300		–	2.9, 2.7, 2.5	–	–	
MeβGal‡	300		–	2.1, 2.3, 1.8	–	–	

*Apparent K_b values calculated from 50% inhibition values reported in literature: [a][22]; [b][21].
†ΔH determined by single injections of L-14 (163 μM) to a large excess of monosaccharides (500 mM) so that the C value ($K_b S_t$) is about 20–30.
‡The errors in the determination of K_b, n and ΔH_b were 4.7, 2.7 and 3.1% respectively for the disaccharides. For the monosaccharides they were 90, 60 and 60% respectively.

defined as $K_b S_t(0)$, where $S_t(0)$ is the initial ligand concentration [26], could be achieved in the range of 20–30. Under these conditions, greater than 95% of the L-14 is expected to be complexed. Hence the measured ΔH_b values are more reliable. Values of ΔH_b thus obtained after corrections for the heats of dilution of L-14 for each of the three successive injections of L-14 to monosaccharides are also listed in Table 1. It may be noted that glucose (500 mM) taken as a negative control did not give any significant ΔH_b value under identical conditions.

Best least-square fits of the data using several L-14 concentrations greater than $1/K_b$ yielded stoichiometries close to 1 for each of the disaccharides when the equivalent weight of 14000 was used for the protein. Values of K_b thus determined are consistent with the semiquantitative estimates available in the literature, where the concentrations required for 50% inhibition of L-14 binding to rabbit erythrocytes or immobilized asialofetuin or lactose [20–23] have been reported (Table 1). These results also show that there is no interaction between the two sites of L-14 dimer and that they are equivalent. Crystallographic data are also consistent with these stoichiometries.

An examination of these data reveals that the binding reactions for L-14 are enthalpically driven. The enthalpies for the binding of various sugar ligands excepting thiodiGal and MumbLac do not vary appreciably over the temperature range 285–300 K, indicating very little if any change in heat capacities (ΔC_p) accompany these reactions. Low values of ΔC_p have been observed in all of the plant lectin–sugar interactions studied thus far [29–31]. This small or insignificant ΔC_p value may constitute a general feature of lectin–sugar interactions. The enthalpically driven nature of these reactions suggests that van der Waals interactions and hydrogen-bonding principally stabilize L-14–carbohydrate complexes. This observation is consistent with the X-ray-crystallographic analysis of L-14–lactose and L-14–LacNAc complexes, where several hydrogen bonds between the

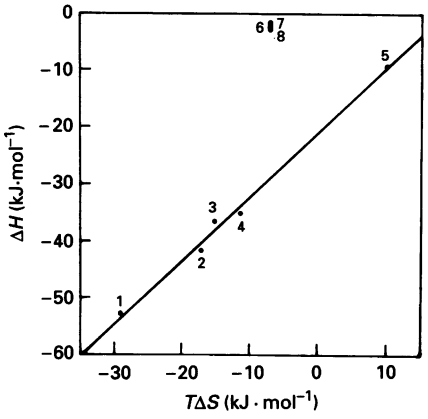

Figure 3 Plot of ΔH_b versus $T\Delta S$ at 300 K for L-14 saccharide complexes

The values at 300 K for thiodiGal, LacNAc, MumbLac and MumbαGal have been calculated from the experimental values reported in Table 2. [ThiodiGal, 1; LacNAc, 2; lactose, 3; MumbLac, 4; MumbαGal, 5; galactose, 6; MeβGal, 7; MeαGal, 8.] Galactose, methyl-α-galactose and methyl-β-galactose do not conform to the pattern displayed by the other sugars. The straight line is the best least-squares fit of the data for the rest of the sugars to a straight line.

hydroxyl groups of these sugars and the corresponding loci in the combining site of the protein have been observed [11,12].

The data for the thermodynamics of the binding reaction shown in Table 1 indicate that the changes in enthalpy and entropy upon binding are compensatory. This compensatory effect is displayed in Figure 3, where ΔH_b values have been plotted as a function of $T\Delta S$ at 300 K. The slope of the plot is close to 1. Enthalpy–entropy compensation has been observed in

Table 2 Summmary of the differences in the thermodynamic parameters between methyl-β-galactose and the β-linked disaccharides at 300 K

Sugar	$-\Delta H$ (kJ/mol)	$-\Delta\Delta H$ (kJ/mol)	$-\Delta G$ (kJ/mol)	$-\Delta\Delta G$ (kJ/mol)	$-\Delta S$ (J/mol per K)	$-\Delta\Delta S$ (J/mol per K)
Meβ Gal	2	0	9	0	24	0
Lactose	37	35	22	13	50	26
LacNAc	42	40	26	17	57	33
ThiodiGal	53	51	24	15	97	73

several protein–ligand interactions where experimental conditions are the same and the ligand structure is altered [32–34] and have been rationalized by implicating a unique role of water in mediating protein–carbohydrate recognitions [35]. It may be noted that galactose, methyl-β-galactose and methyl-α-galactose do not fit into this pattern.

Galactose is an extremely poor ligand: at 300 K its K_b is 45 M^{-1}. This is in agreement with the inhibition studies where the affinity for galactose was found to be ~ 130-fold lower than that for lactose [23]. The poor affinity of galactose is related to very low binding enthalpy coupled with moderately large unfavourable entropy. Methyl-α-galactose exhibits slightly higher affinities over galactose, while methyl-β-galactose is a slightly poorer ligand. In contrast with these insignificant effects of the introduction of methyl group in either of these configurations, the introduction of the 4-methylumbelliferyl group in the α-configuration as in 4-methylumbelliferyl α-D-galactoside (Mumb-α-Gal) dramatically increases the affinity. This large increase in the affinity (~ 50 fold) for Mumb-α-Gal relative to galactose is largely due to a favourable change in entropy for its binding. This favourable ΔS_b indicates the release of highly structured water molecules during the binding process. Thus the strong binding of Mumb-α-Gal to the lectin reflects hydrophobic interactions between this aglycone and the combining site of L-14. This also suggests the existence of a non-polar site contiguous to the galactose-binding site that could accommodate the methylumbelliferyl group in the α configuration. It is possible that it has favourable stacking interactions with tryptophan-68 in the combining region of L-14 [11]. The methylumbelliferyl group in β configuration, as in 4-methylumbelliferyl β-D-galactoside (Meumb-β-Gal) does not appear to exert any such favourable effect on binding, even at its maximum concentration (0.5 mM); addition of 50 μl aliquots produced immeasurable exothermic heats of binding. The increase in enthalpy for the binding of lactose over that observed for methyl-β-galactose amounts to 34 kJ/mol (Table 2), indicating that the reducing-end hexapyranosyl group of this disaccharide is bound in a subsite adjacent to the galactose-binding site. Likewise LacNAc and thiodiGal also exhibit a favourable enthalpic change of 39 and 51 kJ/mol over that observed for binding to methyl-β-galactose, indicating that both of the hexapyranose residues of these β-linked disaccharides also interact simultaneously with the combining site of L-14. This is in accordance with the crystal structure, where both the monosaccharides of these β-linked sugars are seen to interact with L-14 [11,12]. LacNAc is about three times better a ligand as compared with lactose and this is perhaps related to additional van der Waals interactions between its acetamido group at the C-2 carbon atom and the side chains of arginine-73 and glutamic acid-71 [11].

ThiodiGal has an ~ 2-fold higher affinity for L-14 than lactose. It has been shown to be conformationally akin to lactose [21]. Hence its stronger binding to L-14 could be due to the favourable contribution of the thioglycosidic linkage to the binding process. Inhibition studies have shown that a galactose residue at the reducing end does not favour the binding to the lectin [22]. Yet thiodiGal containing a galactose residue at its reducing end is able to bind to L-14 [21]. This could be explained on the basis of recent structural analyses of L14–lactose complex by Lobsanov et al. [12], who have shown that C-3 oxygen for reducing-end glucose donates a hydrogen bond to glutamic acid-68 of L-14. Disaccharides of galactose (such as Galβ1-3GalNAc) bind weakly with L-14 because of an axially oriented hydroxy group at the corresponding position, i.e. the C-4 carbon. Thiodigalactoside has an equatorially oriented hydroxy group at the corresponding locus (i.e. C-2) like the C-3 hydroxy group of the reducing-end glucose of lactose, which is perhaps advantageous for the interaction with glutamic acid-68 of the lectin. Addition of the methylumbelliferyl group in the β configuration at the reducing end of lactose favours its association to L-14 by about 2-fold over lactose, primarily due to a favourable entropic component for the binding process. This again implies the existence of a non-polar region adjacent to glucose where a hydrophobic umbelliferyl group could be accommodated.

On the basis of the above results the following model for the topography of the combining site of L-14 is proposed. The monosaccharide specificity of L-14 is determined by galactose. Hence it must occupy the primary subsite. However, the free energy and enthalpy changes for the binding of galactose are dismally low. On the other hand, contribution of a glucopyranose residue at the reducing end assigned here to interact with the secondary subsite of the lectin is an order of magnitude greater than that for galactose ($\Delta\Delta H$) (Table 2). An examination of the structures of L-14–lactose and L-14–LacNAc reveals that hydroxy groups from galactose participate in a larger number of contacts, including hydrogen-bonding with the protein surfaces, as compared with those observed for glucose, yet its affinity for galactose itself is low. It is possible that the energetic contribution of lactose is more due to the fact that the presence of glucose at the reducing end of the galactose generates a certain type of interaction with water which is crucial for binding to L-14. Consequently the net observed thermodynamic attribute of the binding of sugars to L-14 is controlled by the behaviour of water around the interacting surfaces of both proteins and carbohydrates, rather than specific interactions between protein and carbohydrates alone. This is consistent with the fact that galactose etc. deviate remarkably from lactose etc. in terms of enthalpy–entropy compensation. The role of water near two complementary surfaces in determining the thermodynamic properties of protein–carbohydrate interactions has been highlighted elegantly by Lemieux and his group during the recent years [35]. Our observations of enthalpy–entropy compensation and the low values of ΔC_p in the binding of L-14 with carbohydrates coupled with the fact that dehydration of sugars in a aqueous solution, if not impossible, is an energetically costly process suggests that reorganization of water plays an important role in this recognition.

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