

Evaluation of the stoichiometry and energetics of carbohydrate binding to *Ricinus communis* agglutinin: a calorimetric study

Shalini SHARMA*, Satish BHARADWAJ†, Avadhesh SUROLIA†¹ and Sunil Kumar PODDER*

*Department of Biochemistry and †Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

High-sensitivity isothermal titration calorimetry has been used to investigate the thermodynamics of binding of *Ricinus communis* agglutinin to galactose, lactose and their derivatives in the temperature range 280.5–298 K. The present study unequivocally establishes the carbohydrate-binding stoichiometry of the tetrameric agglutinin from castor bean as two, i.e. the (As–sB)₂-type tetramer of the agglutinin has two equivalent sites that are non-interacting and independent. The site binding constants range from $2.2 \times 10^3 \text{ M}^{-1}$ at 282 K for galactose to $4.84 \times 10^4 \text{ M}^{-1}$ at 281 K for *N*-acetyl-lactosamine. The binding enthalpies range from $-21.9 \text{ kJ} \cdot \text{mol}^{-1}$ at 293 K for 4-methylumbelliferyl- β -galactoside to $-50.2 \text{ kJ} \cdot \text{mol}^{-1}$ at 292.9 K for thiodigalactoside.

The observation of limited entropy–enthalpy compensation for binding of the sugars to the lectin indicates that reorganization of water molecules plays an important role in binding. As the slope of the compensation plot is greater than unity, the reactions are largely enthalpically driven. These studies show that the stronger binding of *N*-acetyl-lactosamine than lactose is due to a favourable interaction between the acetamido group of the reducing-end *N*-acetylglucosamine of the former and the corresponding loci in the agglutinin molecule. Preferential binding of methyl- β -galactoside over methyl- α -galactoside also indicates the apolar nature of the interaction with the methyl group of the former sugar.

INTRODUCTION

Ricin and *Ricinus communis* agglutinin (RCA), two galactose-specific lectins, are present in the seeds of *Ricinus communis*, the castor bean plant [1]. Ricin, a potent inhibitor of protein synthesis in eukaryotic cells, is a 60 kDa disulphide-linked As–sB-type heterodimeric protein, the A-chain of which is an RNA *N*-glycosidase while the B-chain is a galactose-specific lectin [1,2]. RCA, on the other hand, is a 120 kDa tetramer consisting of two As–sB-type dimers, which associate non-covalently. The A-chain of RCA isolated from the seeds [3] and its recombinant counterpart [4] inhibit protein synthesis in cell-free systems. Although the native agglutinin exhibits a strong haemagglutinating activity in comparison with ricin and also binds to other eukaryotic cells, it does not inhibit cellular protein synthesis [1,5]. The A- and B-chains of RCA have 93 and 84% identity respectively with the corresponding chains of ricin [6]. In spite of this similarity, the differences seem to be enough to accommodate the disparity in not only the oligomeric pattern, but also the sugar-binding properties of ricin and RCA, and therefore studies on structure–function relationships of these proteins are of great interest.

X-ray crystallography [7] and other biophysical studies [8,9] have shown that the B-chain of ricin has a minimum of two sugar-binding sites. It has two domains and each of these domains has four subdomains, λ , α , β and γ [7]. The two sugar-binding sites lie in subdomains 1α and 2γ in domain 1 and domain 2 respectively. Each sugar-binding pocket has a conserved aromatic residue (Trp-37 and Tyr-248 in subdomains 1α and 2γ respectively) and a tripeptide (Asp-Val-Arg). Although no crystallographic data are available for RCA, on the basis of sequence identity with the B-chain of ricin, the B-chain of RCA can also be divided into two domains, each consisting of four subdomains. In the case of RCA, the aromatic residue in subdomain 2γ has been replaced by a histidine and it has been

suggested that this change leads to a loss of lectin activity in this subdomain [6].

Studies on binding of simple sugars to the agglutinin using equilibrium dialysis [10–12] and fluorescence polarization [13] have shown that each molecule of tetrameric RCA possesses two identical and independent sugar-binding sites. In contrast, using the same techniques, Houston and Dooley [14] concluded that each B-chain of RCA has two identical and independent sugar-binding sites, i.e. there are a total of four sugar-binding sites on the tetrameric agglutinin. More recently, Lord and co-workers [15] have addressed this problem using site-directed mutagenesis, which led them to conclude that there are no more than two binding sites on each molecule of the agglutinin.

As almost all the reactions are accompanied by heat changes, calorimetry provides a direct method for characterizing protein–ligand interactions. In the case of batch calorimetry, incorporation of several corrections are mandatory in order to ascertain the heats of reactions and to rule out extraneous heat effects [16]. Isothermal titration calorimetry (ITC) has the potential to simultaneously and accurately determine the stoichiometry, the binding constant and the binding enthalpy of protein–ligand interactions and it has been used in the present study to re-examine the stoichiometry as well as to characterize the energetics of the binding of mono- and di-saccharides to RCA.

MATERIALS AND METHODS

Seeds of *Ricinus communis* were purchased from the National Seed Corporation, Hebbal, Bangalore, India. The sugars lactose, *N*-acetyl-lactosamine (LacNAc), thiodigalactoside (ThiodiGal), galactose, methyl- α -galactopyranoside (Me α Gal), methyl-

Abbreviations used: RCA, *Ricinus communis* agglutinin; ThiodiGal, thiodigalactopyranoside; LacNAc, *N*-acetyl-lactosamine; Mumb β GalNAc, 4-methylumbelliferyl- β -2-acetamido-2-deoxygalactopyranoside; Mumb α Gal, 4-methylumbelliferyl- α -D-galactopyranoside; Mumb β Gal, 4-methylumbelliferyl- β -D-galactopyranoside; Me α Gal, methyl- α -galactopyranoside; Me β Gal, methyl- β -galactopyranoside; ITC, isothermal titration calorimetry.

¹ To whom correspondence should be addressed.

β -galactopyranoside (Me β Gal), 4-methylumbelliferyl- α -D-galactopyranoside (Mumb α Gal) and 4-methylumbelliferyl- β -D-galactopyranoside (Mumb β Gal) were from Sigma Chemical Company. All other chemicals were of reagent grade and obtained locally.

RCA was purified from the seeds of the castor bean plant using lactamyl-Sepharose affinity chromatography and gel filtration on Sephadex G-100 as described previously [5]. Protein concentration was determined using an $A^{1\%}$ of 15.7 at 280 nm. Molar concentration of RCA was calculated using a molecular mass of 120 kDa.

All the sugar solutions were prepared by weight in PBS. The concentrations of 4-methylumbelliferyl sugar solutions were determined using a molar absorption coefficient of $1.36 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 318 nm.

Titration calorimetric measurements

The calorimetric titrations were performed using a Microcal Omega titration calorimeter as described elsewhere [17,18]. Aliquots of sugar solutions were injected into the protein solution in the 1.34 ml sample cell. Concentrations of the protein used for the titrations were such that the C value ($C = \text{binding constant} \times \text{protein concentration}$) was typically in the range 3.7–10 and the sugar concentrations were in 8–12-fold molar excess over the binding sites. The total heat, Q_t , is related to the total ligand concentration, $[L]_t$, via the following equation [17]:

$$Q_t = 2n[P]_t \Delta H_b V \{1 + [L]_t/n[P]_t + 1/nK_b[P]_t\}^{-1} - \{[(1 + [L]_t/n[P]_t) V + 1/nK_b[P]_t]^2 - 4[L]_t/n[P]_t\}^{1/2} / 2 \quad (1)$$

where n is the number of binding sites, $[P]_t$ is the total protein concentration, V is the cell volume, K_b is the binding constant and ΔH_b is the binding enthalpy. The expression for the heat released in the i th injection is given by the equation [18]:

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

where dV_i is the volume of the titrant injected into the protein solution. The thermodynamic parameters, ΔG_b and ΔS_b , were calculated using the basic equations:

$$\Delta G_b^\circ = \Delta H_b - T\Delta S_b \quad (3)$$

$$\Delta G_b^\circ = -nRT \ln \{K_b\} \quad (4)$$

where n is the number of binding sites in the agglutinin, T is the temperature and $R = 8.3151 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$.

RESULTS AND DISCUSSION

A typical ITC profile for the binding of lactose to RCA is shown in Figure 1. A monotonic decrease in the heat evolved when increasing amounts of the ligand is added suggests that RCA displays only one type of binding site. The data for total heat released on each injection was found to give the best least-squares fit to eqn. (1). The values of K_b , ΔH_b and n for all the sugars studied were obtained from similar plots and eqns. (3) and (4) and are given in Table 1. The results clearly show that each molecule of tetrameric RCA has two equivalent and non-interacting binding sites. The ΔH_b values for binding of these sugars range from $-21.9 \text{ kJ} \cdot \text{mol}^{-1}$ at 293 K for Mumb β Gal to $-50.2 \text{ kJ} \cdot \text{mol}^{-1}$ at 292.9 K for ThiodiGal. The enthalpy of binding of the various sugars does not vary significantly with temperature, indicating that there is no change in the ΔC_p on their binding to RCA. Figure 2 shows the plot of ΔH_b as a function of $T\Delta S$, which is linear, indicating the occurrence of limited entropy–enthalpy compensation for RCA–sugar inter-

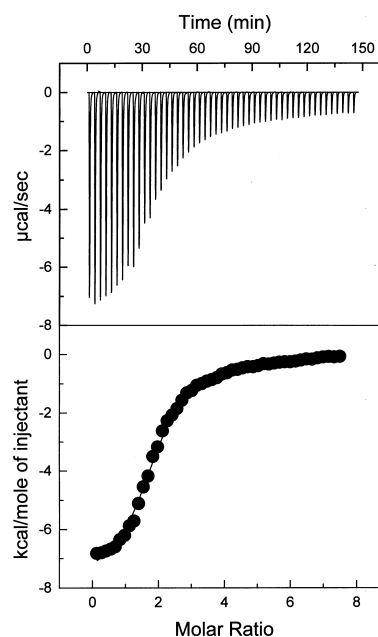


Figure 1 Calorimetric titration of RCA (0.146 mM) with lactose (4 mM) at 283 K

Top, data obtained for 49 injections ($6.7 \mu\text{l}$ each) into RCA solution; bottom, plot of total energy exchanged (as kcal/mol of injectant) as a function of molar ratio of the ligand to the agglutinin. The solid line is the best least-square fit of the data to eqn. (1). The inflection point of the curve is 2.2.

actions. The reactions are largely enthalpically driven, as the slope of the compensation plot is greater than unity (slope = 1.11). The insignificant change in heat capacities, together with the observation of enthalpy–entropy compensation, suggests that rearrangement of water molecules plays an important role in the binding of sugars to RCA [19–22].

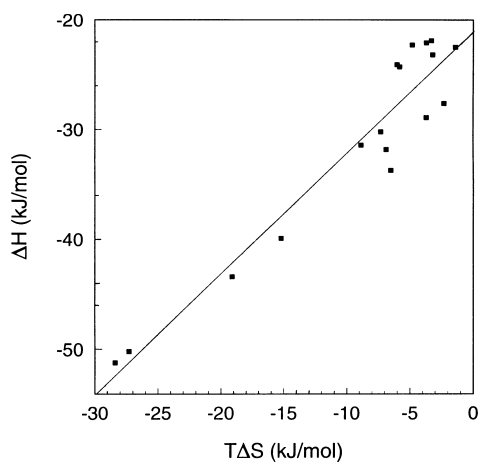
The binding constants for different sugars range from $2.2 \times 10^3 \text{ M}^{-1}$ at 282 K for galactose to $4.84 \times 10^4 \text{ M}^{-1}$ at 281 K for LacNac. The order of binding affinity is LacNac > lactose > ThiodiGal > Mumb β Gal > Me β Gal > Mumb β GalNac > Me α Gal > Mumb α Gal > galactose. The association constant values for lactose, galactose and their derivatives are in reasonable agreement with those reported previously [12,23]. Lactose and LacNac are the most complementary ligands for RCA. In addition, ITC data show that the enthalpy of binding, ΔH_b , for lactose, LacNac and ThiodiGal is higher than the value observed for the corresponding monosaccharide, Me β Gal, by 11.3, 4.4 and $28.7 \text{ kJ} \cdot \text{mol}^{-1}$ respectively. This indicates that the second hexapyranoside of these saccharides binds to a site adjacent to the galactose-binding site. Although ThiodiGal differs from lactose in several respects including the linkage (the glycosidic atom and the reducing sugar are β (1 \rightarrow 1)-linked, sulphur and galactose respectively in the case of lactose and β (1 \rightarrow 4)-linked, oxygen and glucose respectively, in the case of ThiodiGal), their overall topographies are strikingly similar. This explains the preferential binding of ThiodiGal over Me β Gal. The binding of LacNac is slightly entropically favoured compared with that of lactose. This means that the acetamido group of the reducing-end sugar GlcNac in LacNac is involved in non-polar interaction in the combining site of RCA. Similarly to LacNac, the methyl group of Me β Gal is involved in a more favourable interaction than the methyl group of Me α Gal in the binding pocket of RCA.

Table 1 Thermodynamic parameters for binding of carbohydrates to each of the As-sB dimers of RCAResults are means \pm S.E.M. for 4 determinations.

Sugar	Temperature (K)	n^*	$10^{-3} \times K_b$ (M^{-1})	$-\Delta H_b$ ($kJ \cdot mol^{-1}$)	$-\Delta G_b$ ($kJ \cdot mol^{-1}$)	$-\Delta S_b$ ($J \cdot mol^{-1} K^{-1}$)	$-T\Delta S$ ($kJ \cdot mol^{-1}$)
LacNAc	281.0	1.05 ± 0.08	48.4 ± 2.7	28.9 ± 0.3	25.2 ± 0.1	13.3 ± 1.6	3.7
	292.6	1.21 ± 0.09	32.9 ± 1.3	27.6 ± 0.2	25.3 ± 0.1	7.9 ± 1.1	2.3
Lactose	283.0	1.1 ± 0.07	40.0 ± 4.7	31.8 ± 1.0	24.9 ± 0.2	24.3 ± 4.6	6.9
	284.0†	1.1 ± 0.08	37.2 ± 1.0	33.7 ± 0.2	24.9 ± 0.1	31.2 ± 0.8	6.5
	292.1	0.9 ± 0.04	28.6 ± 4.0	31.4 ± 1.1	25.3 ± 0.3	22.4 ± 4.9	8.9
ThiodiGal	280.5	1.2 ± 0.05	17.0 ± 0.5	51.2 ± 0.3	22.7 ± 0.1	101.4 ± 1.2	28.4
	292.9	1.1 ± 0.09	12.4 ± 0.6	50.2 ± 0.6	22.9 ± 0.1	92.9 ± 2.3	27.2
Mumb α Gal	281.7	1.0 ± 0.06	2.7 ± 0.2	24.3 ± 1.7	18.5 ± 0.2	20.5 ± 6.7	5.8
	293.0	0.96 ± 0.09	2.0 ± 0.1	21.9 ± 2.0	18.6 ± 0.2	11.3 ± 7.5	3.3
Mumb β Gal	282.7	1.2 ± 0.05	37.3 ± 2.4	39.9 ± 0.8	24.7 ± 0.1	53.7 ± 3.5	15.2
	298.1	1.13 ± 0.09	18.2 ± 1.6	43.4 ± 2.9	24.3 ± 0.3	64.2 ± 10.6	19.1
Me α Gal	281.1	1.02 ± 0.08	2.6 ± 0.2	22.1 ± 4.7	18.0 ± 0.1	13.0 ± 17.5	3.7
	296.9	0.94 ± 0.08	1.2 ± 0.1	22.3 ± 5.4	18.4 ± 0.2	16.1 ± 19.1	4.8
Me β Gal	282.7	1.12 ± 0.06	7.7 ± 0.3	22.5 ± 0.6	21.0 ± 0.1	5.1 ± 2.5	1.4
	292.7	0.95 ± 0.04	3.8 ± 0.4	23.2 ± 3.2	20.0 ± 0.2	10.9 ± 11.8	3.2
Mumb β GalNAc	279.6	0.93 ± 0.06	19.0 ± 1.4	30.2 ± 0.7	22.9 ± 0.2	26.2 ± 3.1	7.3
Galactose	282	1.06 ± 0.1	2.2 ± 0.1	24.1 ± 1.4	18.0 ± 0.1	21.3 ± 5.3	6.0

* The stoichiometries for the binding of sugars to the agglutinin tetramer are twice these values.

† Experiment carried out with RCA solution preincubated at 25 °C for 30 h.

**Figure 2** Plot of ΔH_b versus $T\Delta S$ for RCA-saccharide complexes

The slope of the fitted line is 1.11.

This is consistent with the threefold higher K_b observed for Me β Gal as compared with those of Me α Gal and galactose (Table 1). Mumb β Gal is a better ligand than Mumb α Gal, as the bulky 4-methylumbelliferyl group is accommodated in a hydrophobic pocket in the combining site of the lectin. Lack of such an interaction for this group in α -configuration apparently accounts for the poor binding of 4-Mumb α Gal compared with its β counterpart.

The ITC data on the binding of Mumb β Gal and the other sugars studied at two temperatures clearly show that each native (As-sB) $_2$ -type tetrameric RCA molecule has two identical and independent sugar-binding sites (Figure 3). In other words, each

B-chain in a 120 kDa RCA binds to only one mono- or disaccharide. Earlier studies using batch calorimetry indicated the presence of more than one binding site on RCA for lactose with different thermal stabilities [23]. In order to establish our results unequivocally and to detect the presence of additional sites, if any, binding experiments were also carried out with very high concentrations of lactose. These experiments did not show any change in either the stoichiometry or the binding affinity (Table 1). Preincubation of RCA at 277 and 298 K for 24 h before calorimetric measurements also did not alter the binding parameters (Table 1).

Mutational analysis of the B-chain of recombinant RCA has shown that on modification of amino acids in only one sugar-binding site in subdomain 1 α (corresponding to ricin B-chain domain), the B-chain had very weak lectin activity, so much so that it was only retarded on an immobilized lactose column [15]. Lectins are known to be notorious for their retardation on unrelated polysaccharide matrices. For example, ricin and the winged bean basic lectin (both galactose/GalNAc binding) are retarded on Sephadex matrices [22,23]. A second sugar-binding site on the B-chain of RCA, should it exist at all, would have very poor lactose-binding affinity. The inability to detect any additional binding sites on RCA even with very high concentrations of lactose (30 mM) indicates that such a site would have an affinity considerably lower than $100 M^{-1}$ and therefore is unlikely to be detected by most other physicochemical techniques. The quaternary structure of RCA is not known, but it has been suggested previously that the second putative sugar-binding site in the 2 γ subdomain of RCA B-chain may be inactive because of the substitution of Tyr-248 by histidine, or be cryptic because of oligomerization [7]. Thus it is difficult to explain how the intact molecule of RCA could have four sugar-binding sites as claimed by Houston and Dooley [14]. Their assertion of temperature-dependent loss of binding sites on RCA is also not supported, as the stoichiometry of binding of RCA is retained, even after

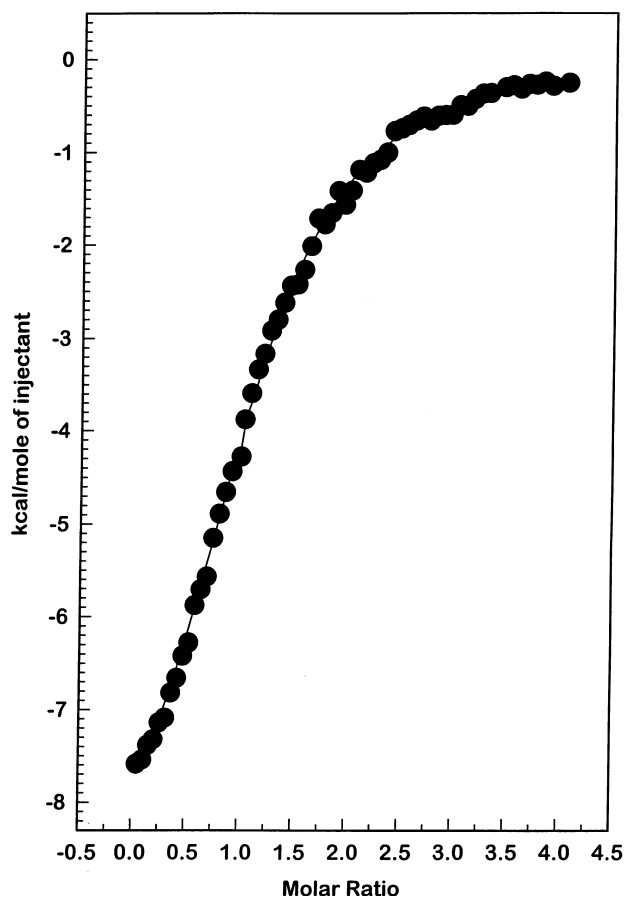


Figure 3 Calorimetric titration of RCA (0.6 mM) with Mumb β Gal (0.69 mM) at 282.7 K

Plot of total energy exchanged (as kcal/mol of injectant) as a function of molar ratio of ligand to the agglutinin. The solid line is the best least-square fit of the data to eqn. (1). The inflection point for the curve is 1.8.

preincubation at elevated temperature for an extended period of time (Table 1).

This work was supported by grants from the Council for Scientific and Industrial Research (CSIR), India to S.K.P. and from the Department of Science and Technology, Government of India to A.S.

REFERENCES

- Olsnes, S. and Phil, A. (1982) in *Molecular Action of Toxins and Viruses* (Cohen, P. and van Heyningen, S., eds.), pp. 51–106, Elsevier, New York
- Endo, Y. and Tsurugi, K. (1987) *J. Biol. Chem.* **262**, 8128–8130
- Olsnes, S., Fernandez-Puentes, C., Carrasco, L. and Vazquez, D. (1975) *Eur. J. Biochem.* **60**, 281–288
- O'Hare, M., Roberts, L. M. and Lord, J. M. (1992) *FEBS Lett.* **299**, 209–212
- Hegde, R. and Podder, S. K. (1992) *Eur. J. Biochem.* **204**, 155–165
- Roberts, L. M., Lamb, F. I., Pappin, D. J. C. and Lord, J. M. (1985) *J. Biol. Chem.* **260**, 15682–15686
- Rutenber, E. and Robertus, J. D. (1991) *Proteins* **10**, 260–269
- Zentz, C., Frenoy, J.-P. and Bourrillon, R. (1978) *Biochim. Biophys. Acta* **536**, 18–26
- Hatakeyama, T., Ohba, H., Yamasaki, N. and Funatsu, G. (1989) *J. Biochem. (Tokyo)* **105**, 444–448
- Podder, S. K., Surolia, A. and Bachhawat, B. K. (1974) *Eur. J. Biochem.* **44**, 151–160
- Olsnes, S., Saltvedt, E. and Phil, A. (1974) *J. Biol. Chem.* **249**, 803–810
- van Wauve, J. P., Loontjens, F. G. and de Bruyne, C. K. (1973) *Biochim. Biophys. Acta* **313**, 99–105
- Khan, M. I., Mathew, M. K., Balaran, P. and Surolia, A. (1980) *Biochem. J.* **191**, 395–400
- Houston, L. L. and Dooley, T. P. (1982) *J. Biol. Chem.* **257**, 4147–4151
- Sphyris, N., Lord, J. M., Wales, R. and Roberts, L. M. (1995) *J. Biol. Chem.* **270**, 20292–20297
- Fisher, H. F. and Singh, N. (1995) *Methods Enzymol.* **259**, 194–221
- Wiseman, T., Williston, S., Brandts, J. F. and Lin, L. N. (1989) *Anal. Biochem.* **179**, 131–137
- Yang, C. P. (1990) *Omega Data in Origin*, p. 66, Microcal Inc., Northampton, MA
- Williams, B. A., Chervanek, M. C. and Toone, E. J. (1992) *J. Biol. Chem.* **267**, 22907–22911
- Lemieux, R. U., Spohr, U., Acharya, S. and Surolia, A. (1994) *Can. J. Chem.* **72**, 158–163
- Puri, K. D. and Surolia, A. (1994) *Pure Appl. Chem.* **66**, 497–502
- Gupta, D., Cho, M., Cummings, R. D. and Brewer, C. F. (1996) *Biochemistry* **35**, 15236–15243
- Zentz, C., Frenoy, J.-P. and Bourrillon, R. (1979) *Biochimie* **61**, 1–6