MICROCALORIMETRIC MEASUREMENTS OF RICIN–SACCHARIDE BINDING

Christian ZENTZ, Jean-Pierre FRÉNOY and Roland BOURRILLON
Laboratoire de Biochimie, Centre de Recherches sur les Protéines, Faculté de Médecine, Lariboisière-Saint-Louis, 45, Rue des Saints-Pères, 75006 Paris, France

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1. Introduction

Lectins, proteins essentially extracted from seeds of leguminous plants, are known for their ability to agglutinate erythrocytes, neoplasic cells and to modify cell metabolism [1–3]. These various biological effects require the prior interaction of lectins with glycoproteins and oligosaccharides of the cell surface.

Until now, no calorimetric measurements of lectin–saccharide interaction have been undertaken although this technique has proved to be useful in complement with other types of studies, to describe the different thermodynamic parameters of interacting systems [4–6].

Ricin (RCA II, RCA_{60}), one of the two lectins of *Ricinus sanguineus,* binds specifically galactose and carbohydrates containing a terminal non reducing galactose residue [7–9]. This specificity was determined by studying inhibition of lectin-induced agglutination of erythrocytes with sugars.

In the present study, thermodynamic quantities (ΔG, ΔH, ΔS) obtained from the reaction of ricin with its specific ligands, galactose and lactose (4-*O*-β-D-galactopyranosyl-D-glucopyranose), are reported.

2. Materials and methods

Galactose and lactose were purchased from Sigma and used without further purification.

Ricin was prepared from *Ricinus sanguineus,* according to the procedure used for *Ricinus communis* by Nicolson and Blaustein [8]. The lectin was homogeneous as studied by polyacrylamide gel electrophoresis, at pH 7.5, immunoelectrophoresis and analytical ultracentrifugation; its activity was checked by hemagglutination tests. Ricin concentration was determined by absorbance measurements at 280 nm using A_{280} 12.

Calorimetric measurements were carried out at 25 ± 0.1°C using an LKB batch microcalorimeter 10 700. The two compartments in the reaction vessel were filled, respectively, with 2 ml of ricin solution (5.88×10^{-5} or 1.75×10^{-4} M) and 4 ml of sugar solution; the reference vessel was filled with the same quantity of lactose or galactose as the reaction vessel.

Two series of experiments were performed, one with galactose and one with lactose. Concentration ranges of carbohydrates were from 5.5×10^{-4} M to 7.1×10^{-2} M and from 0.9×10^{-4} M to 5.8×10^{-2} M, respectively.

In order to avoid any artefact due to heat of chemical modifications (mutarotation, for example) or group ionization in the calorimeter, several precautions were taken : before experiments, ricin solutions were dialyzed for 48 h against 0.025 M sodium phosphate buffer, pH 6.9, containing 0.15 M NaCl and the saccharide solutions were prepared, in the same buffer 5–7 h before the calorimetric experiments. Heat of dilution of ricin has been measured in a blank cell and found negligible comparatively to heats of binding.

Analytical ultracentrifugations were carried out in a Spinco model E ultracentrifuge equipped with Schlieren optic, at 20°C and 23 150 rev/min. The protein samples were at the same concentration as at the end of microcalorimetry experiments and in the same buffer. The partial specific volume of ricin was assumed to be 0.715 ml/g as calculated according to Cohn and Edsall [10] and Gibbons [11] from its aminoacid and carbohydrate composition.

3. Results

3.1. *Thermochemistry of ricin–galactose binding*

Results from the calorimetric measurements of ricin–galactose interaction are reported in fig.1. The heat evolution (expressed as kcal/mol ricin) corrected for heats of dilution is plotted versus the total sugar concentration in the final calorimetric solution. It can be seen that the reaction is exothermic. In the calorimetric titration curve, the experimental heat of reaction, Q, is directly related to the amount of lectin–sugar [LS] complex formed according to the equilibrium:

$$L + S \rightleftharpoons LS \qquad (1)$$

In the case of a 1:1 complex, the thermodynamic changes accompanying process can be expressed as:

$$\frac{1}{Q} = \frac{1}{Q_m} + \frac{1}{Q_m \times K_a} \times \frac{1}{[S]} \qquad (2)$$

where Q_m is the corrected heat quantity liberated when all the lectin present in the reaction vessel is saturated by galactose, and K_a the association constant of system.

[S] is the concentration of free sugar in the final solution. $[S_t]$ and $[L_t]$ are the total concentrations of sugar and lectin respectively.

$$[S] = [S_t] - [L_t] \frac{Q}{Q_m} \qquad (3)$$

The treatment of experimental data in fig.1, according to eq. (2), is shown in fig.2. It is seen that an excellent straight line is obtained, which, together with the results of equilibrium dialysis measurements performed on ricin from *Ricinus communis* [9], strongly suggests formation of a simple 1:1 complex.

At a constant pressure and volume, the heat evolution, Q, is a direct measure of the enthalpy of reaction and ΔH is obtained from the intercept $1/Q_m$ in fig.2, after which, the equilibrium constant is calculated from the slope of the line, $1/(Q_m \times K_a)$.

The derived thermodynamic values, ΔG and ΔS, are reported in table 1. They correspond to a calculated association constant, K_a 3.9×10^3 M^{-1}.

The same thermodynamic values are found for the two different ricin concentrations (1.96×10^{-5} M and 5.85×10^{-5} M) used in the experiments.

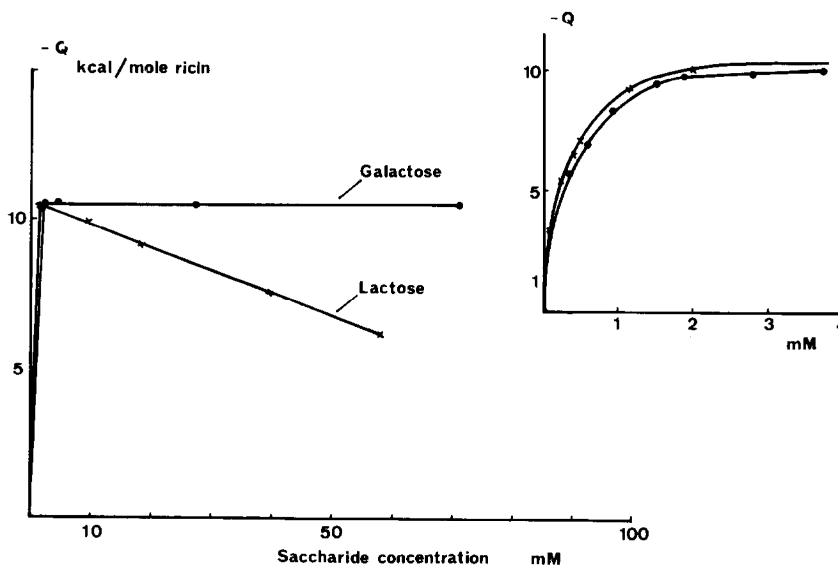

Fig.1. Experimental heats of binding of galactose and lactose to ricin as a function of final saccharide concentration. Experiments were performed at 25°C, pH 6.9 and a final concentration 1.96×10^{-5} M (1.33 mg/ml). Inset: the first phases of the titration curves plotted with an expanded scale.

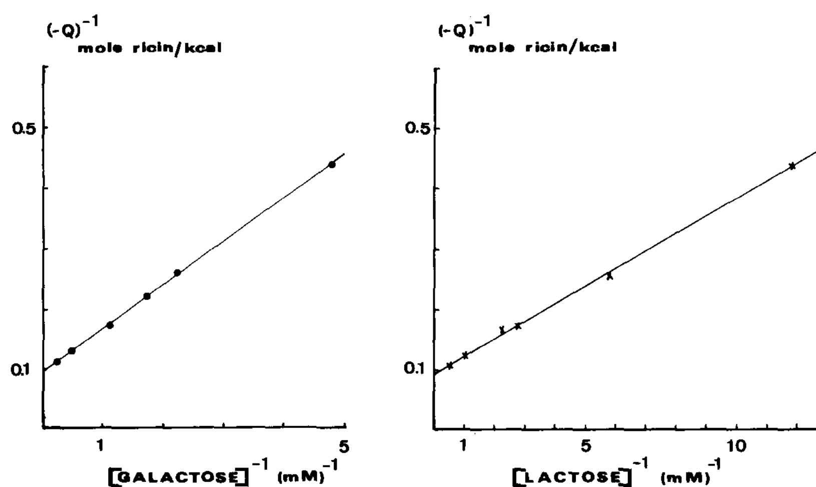

Fig.2. Double reciprocal plots of the curves represented in fig.1. The experimental data have been treated by the least square method.

3.2. *Thermochemistry of ricin–lactose binding*

In the second series of experiment, the ricin–lactose binding has been studied. The calorimetric titration curve reported in fig.1 shows two parts.

For lactose concentrations lower than 2×10^{-3} M, the first ascending part of the curve in fig.1 is a typical saturation curve, and the thermodynamic quantities of the reaction can be calculated. They are summarized in fig.2 and table 1. As for galactose, the 'double-reciprocal' plot in fig.2 suggests the existence of a 1:1 complex.

At lactose concentration higher than 2×10^{-3} M, the second part of the calorimetric titration curve is descending and does not correspond to a plateau as expected when the saturation of the protein by ligand is reached. Moreover, this second part of the curve questions the interpretation of the thermodynamic quantities of the interaction reported in table 1.

Ricin from *Ricinus communis* is composed of two different subunits with similar molecular weights [9,12], the same subunit structure has been found

Table 1
Thermodynamic values for the binding of oligosaccharides to ricin

Sugar	K_a (M^{-1})	ΔG (kcal. mol^{-1})	ΔH (kcal. mol^{-1})	ΔS (cal. mol^{-1}. deg^{-1})	Experimental method (ref.)
Galactose	3900	− 4.9	− 10.5 ± 0.7	− 18.6	Calorimetry pH 6.9, 25°C This work
Lactose	6500	− 5.2	−10.4 ± 0.8	− 17.3	Calorimetry pH 6.9, 25°C This work
	15 000	− 5.5			Equilibrium dialysis pH 7.1, 20°C Olsnes et al. [9]
	3900	− 5.07			Equilibrium dialysis pH 7.7, 37°C Olsnes et al. [13]

for the protein of *Ricinus sanguineus* (J. Font, unpublished results). An explanation for the abnormal behaviour of heats of interaction could be the dissociation or polymerisation of the ricin molecule in presence of high concentrations of lactose.

In order to test this hypothesis, molecular weight determinations of ricin were carried out in absence or presence of lactose. The concentration of ricin was the same as at the end of microcalorimetry experiments (1.96×10^{-5} M = 1.33 mg/ml) and the concentrations of saccharide corresponded to the maximum (2×10^{-3} M) and descending (5.8×10^{-2} M) part of the calorimetric titration curve.

It appears that the polymerisation state of ricin is unmodified whatever lactose concentration is used. In each case, the molecular weight found was 65 000 ± 3000 using a partial specific volume, $\bar{v} = 0.715$.

4. Discussion

The present report is the first calorimetric study of a lectin–saccharide interaction.

Binding constants calculated in the present work indicate a higher affinity for the binding of lactose than of galactose to ricin. These data are in agreement with semi-quantitative results obtained by inhibition of ricin-induced agglutination of erythrocytes with sugars (J. Font, unpublished results).

Taking into account the different conditions of experiments, there is also a good agreement between the values found by microcalorimetry and the results obtained by equilibrium dialysis for the ricin–lactose system: the existence of one binding site with an association constant, 1.5×10^4 M^{-1} [9].

It is clear from table 1 that galactose and lactose contribute by the same large enthalpy value to the Gibbs free energy term which, from a thermodynamic point of view, is responsible for specificity. Considering the negative entropy changes of the association, hydrophobic interactions probably do not play a dominant role in the complex formation.

The heat of ricin–glucose interaction is very low ($\leqslant -1$ kcal/mol) and difficult to measure in our experimental conditions but is not negligible, suggesting a low specificity of binding of glucose on ricin molecule. Moreover, when a mixture of lactose 2×10^{-3} M (corresponding to the end point of the ricin–lactose titration curve) and glucose 1.8×10^{-1} M is allowed to react with ricin in the calorimeter, an exothermic reaction with low ΔQ is observed (one third of ricin–lactose ΔQ) indicating that excess glucose interferes with ricin–lactose binding.

Of considerable interest is the second descending part of the ricin–lactose titration curve.

A possible explanation was the superimposition of a second chemical reaction to the ricin–sugar binding itself. But the hypothesis of a modification in the polymerisation state of the ricin molecules in presence of various lactose concentrations can be ruled out since there is no change of the molecular weight in presence of sugar. On the other hand, the fact that the same thermodynamic values are determined whatever ricin concentrations are used in the calorimetric experiments, supports the idea that there is no concentration-dependent aggregation of ricin.

Since for galactose, only a single kind of interaction with ricin is observed, in the studied concentration range of ligand, the abnormal behaviour of the ricin–lactose interaction has to be explained by the structural differences between the two ligands; lactose is a disaccharide with a glucose bound to galactose.

In the present state of the work, two hypothesis can be drawn to explain the shape of the ricin–lactose titration curve and the influence of excess glucose on ricin–lactose binding. The two hypotheses are related to the presence of one or several binding sites, for glucose alone or the glucose moiety of lactose, on ricin molecule:

(1) In addition to the galactose binding site, ricin molecule contains a second site with low affinity for glucose and the observed titration curve corresponds to negative cooperativity exhibited by ricin toward lactose, through its glucose moiety. The existence of such a second sugar binding site does not preclude the possibility of an extended site for lactose, and is supported by the fact that ricin does agglutinate red cells, an unexpected result for a one site protein.

(2) Ricin molecule contains a unique sugar binding site as revealed by equilibrium dialysis experiments [9]. This site has to be an extended site, larger than the size of a monosaccharide, to accomodate

galactose and glucose, as already suggested by Nicolson et al. from results of inhibition studies [8,12]. The affinity for galactose would be higher than the affinity for glucose. In presence of high lactose concentrations ($> 2 \times 10^{-3}$ M), a second type of interaction, similar to inhibition by excess substrate, would appear between ricin and lactose through its glucose moiety, altering the original interaction and giving a less exothermic total energetic evaluation.

Extended binding sites have already been proposed for concanavalin A [14,15], wheat germ agglutinin [16,17] and for the lectins of potato [18] and peanut [19].

Experiments are in progress to elucidate the mechanism of sugar binding on ricin, they include a systematic study of the interference of glucose on ricin–lactose and ricin–galactose binding.

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