Fluorescence-polarization studies on binding of 4-methylumbelliferyl β -D-galactopyranoside to *Ricinus communis* (castor-bean) agglutinin

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The binding of *Ricinus communis* (castor-bean) agglutinin 1 to saccharides was studied by equilibrium dialysis and fluorescence polarization by using the fluorescently labelled sugar 4-methylumbelliferyl β -D-galactopyranoside. No appreciable change in ligand fluorescence took place on its binding to *Ricinus* agglutinin 1; on the other hand, the fluorescence of 4-methylumbelliferyl β -D-galactopyranoside was considerably polarized on its binding to the lectin. The association constants obtained by Scatchard analysis of equilibrium-dialysis and fluorescence-polarization data do not differ much from each other, and at 25°C, $K_a = 2.4 (\pm 0.2) \times 10^4 \text{ M}^{-1}$. These values agree reasonably well with that reported in the literature for *Ricinus* agglutinin 1. The number of binding sites obtained by the different experimental procedures is 1.94 ± 0.1 per molecule of 120 000 daltons and is equal to the reported value of 2. The consistency in the values of K_a and number of binding sites indicate the absence of additional subsites on *Ricinus* agglutinin 1 for its specific sugars. In addition, the excellent agreement between the binding parameters obtained by equilibrium dialysis and fluorescence polarization indicate the potential of ligand-fluorescence-polarization measurements in the investigation of lectin–sugar interactions.

Lectins, the carbohydrate-binding and cell-agglutinating proteins, have been utilized as macromolecular probes for delineating the architecture and function of cell-surface glycoconjugates (Lis & Sharon, 1973; Rapin & Burger, 1974; Goldstein & Hayes, 1978). The binding of lectins to the carbohydrate moiety of cell-membrane receptors is a prelude to a wide range of biological effects of lectins on the cell (Novogrodosky & Katchalski, 1971; Cuatrecasas & Tell, 1973; Sharon, 1976). The lectin from castor beans, *Ricinus* agglutinin 1, has found a widespread application in the studies of cell-surface receptors and as an analytical tool in the laboratory, owing to its ability to interact with galactose-containing biopolymers (Nicolson & Blaustein, 1972; Nicolson *et al.*, 1974; Surolia *et al.*, 1975a,b). The interactions of *Ricinus* agglutinin 1 with the cell as well as with the galactose-containing biopolymers are specifically inhibited by galactose, the most effective inhibitors being β -D-galactopyranosyl sugars (Nicolson *et al.*, 1974). Previous studies have shown that this lectin possesses two binding sites per 120 000 daltons for its specific sugars (Podder *et al.*, 1974; van Wauve *et al.*, 1973).

Fluorescently labelled sugars have been used for investigation of the binding parameters of certain lectins such as concanavalin A and wheat (*Triticum*)-

germ agglutinin (Dean & Homer, 1973; Loontjens *et al.*, 1977; Privat *et al.*, 1974). In these lectins, almost total quenching of ligand fluorescence is observed, owing to lectin binding. On the other hand, lectins such as that from *Pisum sativum* (pea), which do not show any appreciable quenching of ligand fluorescence, could not be investigated by utilizing these sugars.

During our investigation of 4-methylumbelliferyl β -D-galactopyranoside binding to *Ricinus* agglutinin 1 by fluorescence spectroscopy, no appreciable quenching or enhancement of ligand fluorescence was observed. Fluorescence polarization of a molecule depends on the molecular geometry and rigidity, and it was anticipated that interaction of 4-methylumbelliferyl β -D-galactopyranoside with the lectin would lead to alterations in polarization of its fluorescence (Weber, 1952; Steiner, 1954; Harber & Bennett, 1962; Kierszenbaum *et al.*, 1969). Therefore changes in fluorescence polarization of the fluorescent sugar on its interaction with *Ricinus* agglutinin 1 appeared to be an ideal approach to characterize this interaction, and, indeed, a marked change in the polarization of ligand fluorescence was observed on binding with *Ricinus* agglutinin 1. The number of binding sites (two per 120 000 daltons) and the association-

constant value ($K_a = 2.9 \times 10^4 \text{ M}^{-1}$) obtained by the fluorescence-polarization measurement compares well with that obtained by equilibrium dialysis and other methods. The internal consistency of the binding parameters obtained by the method described here and the comparison of the results obtained here with those obtained by several other techniques indicate the validity of the approach and its potential for undertaking such studies with lectins, which, even after interaction with the fluorescent sugar, do not alter the ligand fluorescence.

Materials and methods

Ricinus communis agglutinin 1 was prepared from locally available castor beans as previously described (Appukuttan *et al.*, 1977). The protein was found to be homogeneous on polyacrylamide-gel electrophoresis performed as described by Davis (1964). The concentrations of the protein solutions were determined spectrophotometrically at pH 7.2 by using an $A_{280}^{1\% \cdot 1\text{cm}}$ value of 14, and were expressed on the basis of an equivalent weight equal to 60 000, corresponding to two binding sites per molecule of 120 000 daltons.

Fluorescent ligand

4-Methylumbelliferyl β -D-galactopyranoside was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The concentrations of 4-methylumbelliferyl β -D-galactopyranoside solutions were determined by its ϵ_{318} , which corresponds to a value of $1.36 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Loontjens *et al.*, 1977). All the solutions of the fluorescent sugar were checked for the presence of 7-hydroxy-4-methylcoumarin, and were found to be free of it, since no fluorescence at 440 nm was found. Analytical-Reagent-grade lactose was obtained from BDH, Poole, Dorset, U.K., and all other chemicals used were of Analytical grade.

Fluorescence spectra

The fluorescence spectra were obtained with a Perkin-Elmer MPF-44A ratio-recording spectrofluorimeter with slit openings corresponding to 5 nm of both monochromators. The excitation of fluorescent sugar was at 318 nm and the uncorrected emission spectrum was recorded above 340 nm. The samples were in $1 \text{ cm} \times 1 \text{ cm} \times 4.5 \text{ cm}$ cuvettes mounted on thermostatically controlled holders. The fluorescence spectra of the sugar were recorded in the absence of protein and after addition of several portions of $192 \mu\text{M}$ -*Ricinus* agglutinin 1. Experiments were carried out in 0.02 M-phosphate buffer, pH 7.2, containing 0.1 M-NaCl at 25°C.

Fluorescence-polarization measurement

Fluorescence-polarization measurements were made on the above spectrofluorimeter equipped with

the polarizing accessory. Excitation and emission wavelengths were 318 and 375 nm respectively. The changes in the polarization of a $2.2 \mu\text{M}$ -4-methylumbelliferyl β -D-galactopyranoside solutions were recorded when it was titrated against increasing amounts of a $192 \mu\text{M}$ solution of *Ricinus* agglutinin 1. In another set of experiments, the concentration of *Ricinus* agglutinin 1 was fixed ($20 \mu\text{M}$) and the polarization of various concentrations of 4-methylumbelliferyl β -D-galactopyranoside was recorded. The experiments were carried out in 0.02 M-phosphate buffer, pH 7.2, with 0.1 M-NaCl at 25°C.

Equilibrium dialysis

For equilibrium dialysis, cells having a 1 ml capacity for each compartment were used. One compartment of each cell had a constant amount of lectin (6 mg/ml) and the other compartment had various amounts of 4-methylumbelliferyl β -D-galactopyranoside. After equilibrating for 24 h at 25°C, a portion was taken from the protein-free half-cell and read for the A_{318} of 4-methylumbelliferyl β -D-galactopyranoside. The data were analysed by using the Scatchard (1949) relationship.

Results

A measurement of absorptivity of 4-methylumbelliferyl β -D-galactopyranoside allowed a direct determination of the parameters of its binding to *Ricinus* agglutinin 1 by equilibrium dialysis. The data plotted by using the Scatchard (1949) relationship are shown in Fig. 1. The value of K_a thus obtained corresponds to $2.6 \times 10^4 \text{ M}^{-1}$, with the number of binding sites (n) equal to 1.94 per 120 000 daltons. These values are fairly consistent with the values reported in literature, where other sugars were used for binding studies (van Wauve *et al.*, 1973). The relative fluorescence of 4-methylumbelliferyl β -D-galactopyranoside decreased as a function of solvent polarity, as determined by increasing concentration of *p*-dioxan; a 95% decrease was observed when the dioxan concentration was 100%.

The fluorescence of 4-methylumbelliferyl β -D-galactopyranoside even in the presence of large concentrations of *Ricinus* agglutinin 1 shows an enhancement of 2.5% on binding to this lectin, indicating that there is virtually no change in the polar environment of the umbelliferyl moiety of the sugar when it is bound to the lectin from that of free sugar. In this respect it differs from concanavalin A and wheat-germ agglutinin, where a nearly complete quenching of the fluorescence of umbelliferyl glycosides of their specific sugar is observed (Loontjens *et al.*, 1977; van Landschoot *et al.*, 1977).

The changes in fluorescence polarization of a $2.2 \mu\text{M}$ solution of 4-methylumbelliferyl β -D-galacto-

Fig. 1. Scatchard plot for the binding of 4-methylumbelliferyl β -D-galactopyranoside to *Ricinus agglutinin 1*, determined by equilibrium dialysis at 25°C

The binding was monitored at 318 nm for 4-methylumbelliferyl β -D-galactopyranoside from the protein-free compartment. R (on the ordinate) is the ratio of bound ligand to total protein and m is the free 4-methylumbelliferyl β -D-galactopyranoside concentration at equilibrium. From the slope, K_a is calculated as $[2.60 \pm 0.0298 \text{ (S.E.M., } n=6)] \times 10^4 \text{ M}^{-1}$ with $[1.94 \pm 0.048 \text{ (S.E.M., } n=6)]$ binding sites. The straight line is drawn according to the regression equations ($n=6$, $r=0.999$), where n is the number of points and r is the regression coefficient.

pyranoside on titration with $192 \mu\text{M}$ -*Ricinus agglutinin 1* at 25°C are presented in Fig. 2. An increase in polarization of ligand fluorescence with increasing concentration of *Ricinus agglutinin 1* is observed. The polarization of the sugar approaches a saturation value at $20 \mu\text{M}$ -lectin. No change in the fluorescence polarization of 4-methylumbelliferyl β -D-galactopyranoside was observed when *Ricinus agglutinin 1* preincubated with 0.1 M-lactose was added to a solution of the fluorescent sugar, demonstrating that the changes in the polarization of fluorescent sugar are only due to the sugar-specific binding of the lectin to this ligand (Fig. 2). Moreover, a complete reversal of the fluorescence-polarization changes of 4-methylumbelliferyl β -D-galactopyranoside was observed when 0.1 M-lactose was added in a solution of the sugar containing *Ricinus agglutinin 1*.

Polarization, p , of a molecule is expressed quantitatively in terms of intensities, I , polarized either parallel ($\parallel$) or perpendicular ($\perp$) to the incident electric field:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (1)$$

For randomly oriented molecules in a rigid medium, the maximum value of p observed with linearly polarized light is 0.5; if the molecules are subject to rotary Brownian motion, the molecular rotation taking place between the time of absorption

Fig. 2. Changes in fluorescence polarization of 4-methylumbelliferyl β -D-galactopyranoside at different concentrations of *Ricinus agglutinin 1*

O, A $2.2 \mu\text{M}$ solution of 4-methylumbelliferyl β -D-galactopyranoside was titrated with portions of $192 \mu\text{M}$ *Ricinus agglutinin 1*. ●, Titration curve of $2.2 \mu\text{M}$ -4-methylumbelliferyl β -D-galactopyranoside with $192 \mu\text{M}$ *Ricinus agglutinin 1* preincubated with 0.1 M-lactose.

and emission may be expected to result in values of p lying between 0.5 and zero. The extent of this rotation is a function of molecular dimensions and structure, solvent and temperature. Low-molecular-weight compounds will give rise to a depolarized fluorescence, and thus, for 4-methylumbelliferyl β -D-galactopyranoside, the p value is 0.07 at 25°C.

If p , p_B and p_F represent the polarization of the sample, bound sugar and free sugar respectively, the molar fraction bound sugar (F_B) is given by:

$$F_B = \frac{p - p_F}{p_B - p_F} \quad (2)$$

from which the molar fraction of free sugar (F_F) can be calculated as:

$$F_F = 1 - F_B = \frac{p_B - p}{p_B - p_F} \quad (3)$$

This will yield:

$$\frac{F_B}{F_F} = \frac{p - p_F}{p_B - p} \quad (4)$$

provided the quantum yields of bound and free sugar are the same. If not,

$$\frac{F_B}{F_F} = \frac{Q_F}{Q_B} \frac{p - p_F}{p_B - p} \quad (5)$$

where Q_F and Q_B are the quantum yields of free and bound sugar respectively. The Scatchard equation can be written as:

$$\frac{F_B}{F_F} [P_r] = n/K_D - \frac{1}{K_D} \frac{F_B [F]}{[P_r]} \quad (6)$$

Where $[F]$ and $[P_r]$ are the concentrations of sugar and protein respectively, n is the number of sugar molecules bound per molecule of protein and K_D is the dissociation constant. Thus a plot of $(F_B/F_F)/[P_r]$ against $(F_B[F])/[P_r]$ yields a straight line with intercepts of n/K_D and n on the y - and x -axes respectively. Of the parameters employed, p_F and Q_F are the values for free sugar, whereas p_B and Q_B have to be determined by keeping a fixed concentration of sugar and varying the protein concentration. The values of polarization and quantum yield when the probe is completely bound to the lectin will give p_B and Q_B respectively. In one case where complete binding cannot be achieved experimentally, p_B and Q_B are obtained by an extrapolation as described for antigen-antibody interaction by Dandliker *et al.* (1978). If equilibrium values of p plotted against concentration (M) are extrapolated to zero concentration, where (M) is the total molar concentration of the ligand in both free and bound forms, p approaches a limit, p' ; values of p' for different lectin concentrations plotted against $(p' - p_F)$ divided by lectin concentration in any arbitrary convenient units give p_B as the intercept of a straight line (Dandliker *et al.*, 1978).

$$p' = p_B - Q_F \frac{(p' - p_F)}{Q_B K_{F, \max.}} \quad (7)$$

This procedure makes it unnecessary to know absolute values of $K_{F, \max.}$ beforehand. In cases where $Q_F/Q_B = 1$, eqn. (7) can further be simplified to:

$$p' = p_B - \frac{(p' - p_F)}{K_{F, \max.}} \quad (8)$$

A similar relationship to eqn. (7) can be used to determine Q_B :

$$Q' = Q_B + \frac{Q_F - Q'}{K_{F, \max.}} \quad (9)$$

Since, for *Ricinus* agglutinin 1, Q_F/Q_B is 1, and p_B can be experimentally determined, as shown by the preceding data, eqn. (4) can be used to generate the Scatchard plot. The changes in fluorescence

polarization of *Ricinus* agglutinin 1 ($20 \mu M$) on titration against various concentrations of 4-methylumbelliferyl β -D-galactopyranoside were subjected to Scatchard analysis according to eqn. (4), and the results are shown in Fig. 3. At pH 7.2, the value of K_a was $2.97 \times 10^4 M^{-1}$ and the number of binding sites (n) was 1.6 per 120000 daltons. The value of the number of binding sites obtained by fluorescence-polarization measurements is slightly different from that obtained by equilibrium dialysis. This apparent variance between the two values could be due to the different experimental procedures involved in deriving the parameters for the Scatchard plots. Assuming that the stoichiometry for equilibrium dialysis is applicable for the Scatchard data obtained by fluorescence polarization, the K_a ($2.97 \times 10^4 M^{-1}$) value is in agreement with those obtained by equilibrium dialysis and reported in literature (Podder *et al.*, 1974, 1978; Olsnes *et al.*, 1974). The values of K_a by this method are in close agreement with that obtained by equilibrium dialysis and those obtained kinetically (Podder *et al.*, 1978). From the fluorescence-polarization data, the value of K can be obtained by a modified form of the relationship given previously (Chipman *et al.*, 1967);

$$\log \left(\frac{p - p_F}{p_B - p} \right) = \log K + \log \left[(P_0) - \frac{L_0(p - p_S)}{p} \right] \quad (10)$$

where p , p_F and p_B are the polarizations of the sample, free sugar and bound sugar at infinite

Fig. 3. Scatchard plot for the binding of 4-methylumbelliferyl β -D-galactopyranoside to *Ricinus* agglutinin 1, determined by fluorescence polarization at $25^\circ C$. *Ricinus* agglutinin 1 ($20 \mu M$) was titrated with various concentrations of 4-methylumbelliferyl β -D-galactopyranoside. From the slope, $K_a = [2.97 \pm 0.0361 \text{ (S.E.M., } n = 5)] \times 10^4 M^{-1}$ with $1.6 \pm 0.05 \text{ (S.E.M., } n = 5)$ binding sites per 120000 daltons. The straight line is drawn according to the regression equations ($n = 7$, $r = 0.987$).

protein concentration. P_0 is the total protein concentration and L_0 is the total ligand concentration. A plot of $\log [(p - p_F)/(p_B - p)]$ against $\log (P_F)$, where

$$P_F = \left[(P_0) - \frac{L_0(p - p_F)}{p} \right]$$

assuming a 1:1 stoichiometry for ligand-protein interaction, for 60 000-dalton *Ricinus* agglutinin 1, gives a straight line (Fig. 4). The slope of the plot is unity, showing that the assumed stoichiometry is correct. The intercept on the x -axis gives the value of K_a , which is $4.2 \times 10^4 \text{ M}^{-1}$, and is fairly close to that obtained here by equilibrium dialysis and by the temperature-jump-relaxation kinetic data of Podder *et al.* (1978).

Discussion

4-Methylumbelliferyl glycosides are increasingly being used as ligands for the study of protein-carbohydrate interactions. The high absorption coefficient of methylumbelliferyl glycoside is

Fig. 4. Sample plot of fluorescence-polarization data for *Ricinus* agglutinin 1 with various concentrations of 4-methylumbelliferyl β -D-galactopyranoside at pH 7.2 and 25°C

p , p_B and p_F are the relative polarizations of sugar sample, bound sugar and free sugar. See the text for experimental details. The straight line is drawn according to the regression equations ($n = 6$, $r = 0.92$). K_a is calculated as $[4.2 \pm 0.105 \text{ (S.E.M., } N = 5)] \times 10^4 \text{ M}^{-1}$ and the number of binding sites as $0.89 \pm 0.035 \text{ (S.E.M., } N = 5)$ per 60 000 daltons.

convenient in the spectrophotometric monitoring of equilibrium-dialysis experiments. The fluorescence of methylumbelliferyl glycosides is markedly influenced on binding with several proteins, a consequence of a change in the polarity of the umbelliferyl group of the glycosides on binding to these proteins (Dean & Homer, 1973; Loontjens *et al.*, 1977). The fluorescence of 4-methylumbelliferyl chito-oligosaccharides is enhanced on binding to lysozyme (Delmotte *et al.*, 1975); on the other hand, it is quenched on binding to the lectin from wheat-germ (Privat *et al.*, 1974; van Landschoot *et al.*, 1977).

We did not find any quenching or appreciable enhancement of fluorescence on binding to the *Ricinus* agglutinin 1. This indicated that there was practically no change in the polarity of environment of the sugar on binding to *Ricinus* agglutinin 1. The less intense change in the fluorescence emission of this ligand as reported here precluded a detailed investigation of its binding to *Ricinus* lectin as well as for the lectin from *Pisum sativum* (Dean & Homer, 1973; Loontjens *et al.*, 1977). Alterations in the polarization of 4-methylumbelliferyl β -D-galactopyranoside of fluorescence were observed, presumably due to a change in its molecular geometry, volume and rigidity on its interaction with the lectin. The observed changes in the polarization of ligand fluorescence that occur on binding of 4-methylumbelliferyl β -D-galactopyranoside to *Ricinus* agglutinin 1 offer distinct advantages for binding studies over the use of small changes in the ligand fluorescence, since they provide a direct and rapid measure of the bound/free ratio. The small amount of material required for these investigations is an added advantage. These considerations led us to quantify the equilibrium parameters of the interaction of *Ricinus* agglutinin 1 with 4-methylumbelliferyl β -D-galactopyranoside by fluorescence polarization.

The alterations in the polarization of ligand fluorescence were found to be due to sugar-specific interaction of 4-methylumbelliferyl β -D-galactopyranoside with this lectin. This is unequivocally demonstrated by a lack of ligand-fluorescence alterations by the lectin bound to lactose as well as by the depolarization of the fluorescence of the ligand bound to the lectin by lactose.

The results of equilibrium dialysis demonstrate that *Ricinus* agglutinin 1 possesses two binding sites for the fluorescent sugar per 120 000 daltons, without any noticeable interaction between them. The linearity of Scatchard plots for equilibrium dialysis at fractional saturation from 0.3 to 1.0, and no deviation at high saturation, exclude the existence of any additional affinity sites. This is in agreement with previous results obtained with lactose (Podder *et al.*, 1974) and with p -nitrophenyl β -D-galacto-

pyranoside (van Wauve *et al.*, 1973). The Scatchard plots of the fluorescence-polarization data obtained by titration of lectin with various concentrations of 4-methylumbelliferyl β -D-galactopyranoside yields a linear plot with the number of binding sites equal to two per molecule of the lectin. The absence of interaction between the two sites is discernible from the linearity of the Scatchard plot. This is further substantiated by the results of the titration of 4-methylumbelliferyl β -D-galactopyranoside with various concentrations of lectin (Fig. 4), which shows a slope with a value of one for the lectin with equivalent weight of 60000.

The association-constant (K_a) values of the binding of the fluorescent galactoside to the *Ricinus* lectin obtained from equilibrium dialysis ($2.6 \times 10^4 \text{ M}^{-1}$) and fluorescence polarization according to Scatchard analysis ($2.97 \times 10^4 \text{ M}^{-1}$) and eqn. (10) ($4.2 \times 10^4 \text{ M}^{-1}$) at 25°C are quite close to each other. The values for the association constant for the binding of *p*-nitrophenyl β -D-galactopyranoside with the *Ricinus* agglutinin 1 is reported to be $1.65 \times 10^4 \text{ M}^{-1}$ by equilibrium dialysis (van Wauve *et al.*, 1973), and $1.5 \times 10^4 \text{ M}^{-1}$ by a ratio of the forward rate constant to the dissociation rate constant of the interaction of this glycoside with the lectin in a relaxation-kinetics experiment (Podder *et al.*, 1978).

The internal consistency in the number of binding sites and the K_a values for *Ricinus* agglutinin 1 as obtained by equilibrium dialysis and fluorescence polarization as well as with the values reported in the literature by other methods demonstrate the validity of our approach.

In summary, the present work demonstrates that the fluorescence-polarization measurements can be used for the determination of (a) the number of binding sites, (b) the degree of heterogeneity of the site and (c) K_a values for the binding of lectins to their specific sugars. Fluorescent polarization will prove a valuable tool in elucidating the mode of interaction of sugars, especially fluorescently labelled sugars, with several other lectins that do not markedly influence the emission of ligand fluorescence.

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