Thermodynamic and kinetic studies on saccharide binding to soya-bean agglutinin

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The fluorescence of *N*-dansylgalactosamine [*N*-(5-dimethylaminonaphthalene-1-sulphonyl)galactosamine] was enhanced 11-fold with a 25 nm blue-shift in the emission maximum upon binding to soya-bean agglutinin (SBA). This change was used to determine the association constants and thermodynamic parameters for this interaction. The association constant of $1.51 \times 10^6 \text{ M}^{-1}$ at 20 °C indicated a very strong binding, which is mainly due to a relatively small entropy value, as revealed by the thermodynamic parameters: $\Delta G = -34.7 \text{ kJ} \cdot \text{mol}^{-1}$, $\Delta H = -37.9 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta S = -10.9 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. The specific binding of this sugar to SBA shows that the lectin can accommodate a large hydrophobic substituent on the C-2 of galactose. Binding of non-fluorescent ligands, studied by monitoring the fluorescence changes when they are added to a mixture of SBA and *N*-dansylgalactosamine, indicates that a hydrophobic substituent at the anomeric position increases the affinity of the interaction. The C-6 hydroxy group also stabilizes the binding considerably. Kinetics of binding of *N*-dansylgalactosamine to SBA studied by stopped-flow spectrofluorimetry are consistent with a single-step mechanism and yielded $k_{+1} = 2.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-1} = 0.2 \text{ s}^{-1}$ at 20 °C. The activation parameters indicate an enthalpically controlled association process.

INTRODUCTION

Soya-bean agglutinin (SBA) is a homotetrameric carbohydrate-binding glycoprotein (M_r 120 000) isolated from the seeds of *Glycine max* (Lotan *et al.*, 1974; Goldstein & Hayes, 1978). It binds one sugar molecule per subunit of M_r 30 000 (De Boeck *et al.*, 1983). SBA has attracted much interest with regard to its biological properties. Particularly well studied among these are mitogenicity (Novogrodsky & Katchalski, 1973), preferential agglutination of transformed cells (Sela *et al.*, 1970, 1971; Rapin & Burger, 1974), a specific recognition of nodulating *Rhizobium* strains (Bohool & Schmidt, 1974; Barondes, 1981) and fractionation of different types of cells (Reisner & Sharon, 1980). The primary structure of SBA has been determined (Hemperly & Cunningham, 1983; Vodkin *et al.*, 1983), and the crystallization of this glycoprotein lectin has been reported (Shaanan *et al.*, 1984).

Though a great deal of knowledge has accumulated on the biological properties of SBA, which are manifested by its ability to bind to sugars, studies reporting the specificity and mechanism of sugar binding to this lectin are scarce. Some information on the saccharide specificity is available from haemagglutination-inhibition experiments, but the information obtained by this method is semi-quantitative, and thus there is a need for a thorough study of the saccharide-binding properties of this lectin.

De Boeck *et al.* (1984a) have reported studies on the binding of various saccharides to SBA with the use of equilibrium and kinetic methods. Their results indicate that the binding of simple monosaccharides to this lectin is mainly due to an enthalpic contribution. The strong binding of *N*-dansylgalactosamine (Dns-GalN) is fav-

oured by a positive entropic contribution. Their stopped-flow kinetic studies reveal that the binding of 4-methylumbelliferyl β -*N*-acetyl-D-galactosaminide to SBA follows a single-step mechanism.

In the present paper we report equilibrium and stopped-flow kinetic studies on the binding of Dns-GalN to SBA as monitored by the fluorescence enhancement of this sugar on binding. We have also studied the binding of non-fluorescent ligands by utilizing their ability to displace Dns-GalN from its complex with SBA.

It should be noted that the results obtained by De Boeck *et al.* (1984a) are considerably at variance with those obtained by us. The main differences are in the thermodynamic parameters obtained for the binding of simple saccharides as well as Dns-GalN. In addition, the kinetic rate constant for the binding of sugars to SBA reported by them is at least an order of magnitude slower than that observed by us. Plausible reasons for these discrepancies are discussed in this paper.

MATERIALS AND METHODS

Materials

N-acetylglucosamine, methyl α -galactoside, methyl β -galactoside, 2-deoxygalactose, fucose, galactosamine, galactose, L-arabinose, melibiose and 5-dimethylaminonaphthalene-1-sulphonic acid were products of Sigma Chemical Co., St. Louis, MO, U.S.A. Lactose was from BDH Chemicals, Poole, Dorset, U.K. (All the sugars used in this study are D-sugars unless otherwise specified.) Soya-bean seeds (cultivar KH Sb 2) were obtained from the University of Agricultural Sciences, Bangalore, India. All other reagents were of analytical grade.

Abbreviations used: SBA, soya-bean agglutinin; Dns-GalN, *N*-dansylgalactosamine [*N*-(5-dimethylaminonaphthalene-1-sulphonyl)galactosamine].

Preparation of SBA

SBA was prepared by affinity chromatography as reported by Gordon *et al.* (1972), except that the affinity column used was of lactosaminyl-aminoethyl-P-300 (Reinhold *et al.*, 1977). The lectin eluted with lactose was homogeneous by polyacrylamide-gel electrophoresis in the presence and in the absence of SDS.

Preparation of Methyl α -N-acetylgalactosaminide

Methyl α -N-acetylgalactosaminide was prepared by the method of Sarkar & Kabat (1979).

Preparation of Dns-GalN

Dns-GalN was prepared by the method of Lartey & Derechin (1979). The product, positive for both carbohydrate and fluorescent moiety, was purified by t.l.c. on silica gel (15% methanol in dichloromethane, R_F 0.25 on 1 mm plates). M.p. (100–103 °C) and i.r. ($\bar{\nu}$ = 1140 and 1300 cm^{-1} , sulphonamide stretching) agreed well with reported values (Roberts, 1983).

Fluorescence measurements

Fluorescence measurements were made on a Union Giken FS 501 fluorescence polarizer equipped with photon-counting photomultipliers. Samples were excited at 330 nm with a 7 nm slit, and emission was monitored by means of a band-pass metal interference filter (λ_1 = 6.5 nm) centred at 520 nm along with a 510 nm cut-off filter. Temperature was maintained constant (± 0.1 °C) by means of Lauda constant-temperature bath. Titrations were performed under constant stirring by the addition of small defined portions of the lectin to a 3.0 μM solution of Dns-GalN in the cuvette. Measurements were made after an interval of 2 min. The fluorimeter is controlled by a microprocessor that allows averaging of several measurements. Typically the average of ten measurements was taken and the standard deviation was less than 0.5%. From the titration data the association constant, K_a , was determined by the method of Chipman *et al.* (1967). Binding of non-fluorescent inhibitory saccharides to SBA was studied essentially as described by Khan *et al.* (1981).

In some of the experiments fluorescence measurements were made on a Perkin-Elmer MPF 44A spectrofluorimeter with 5 nm slits on both the monochromators.

Kinetic studies

Stopped-flow fluorescence studies were performed on a Union Giken RA 401 stopped-flow spectrometer. Excitation was at 330 nm and emission was collected above 460 nm with the use of a cut-off filter at right-angles to the exciting beam. The dead time of the instrument was determined to be 0.5 ms. The sample reservoirs and cell compartment were maintained at a constant temperature (± 0.1 °C) by means of a constant-temperature bath.

The rate constant for a first-order approximation, k_{app} , was determined from the slopes of linear plots obtained by plotting $\ln(\Delta F_t - \Delta F_e)$ versus time when SBA was mixed with Dns-GalN, where F_t and F_e are fluorescence intensities at a particular time t and after the system has reached equilibrium respectively. In these experiments the concentration of the sugar was fixed at 3.75 μM (after mixing) and that of the protein was varied from 32 μM to 400 μM after mixing. The dissociation of Dns-GalN from SBA–Dns-GalN complex was monitored

by mixing a solution containing 17.5 μM -SBA and 15 μM -Dns-GalN in phosphate-buffered saline (0.15 M-NaCl/20 mM-phosphate buffer, pH 7.4) with a 10 mM solution of *N*-acetylgalactosamine in the same buffer.

Concentration determination

Protein concentrations were determined spectrophotometrically by using an $A_{280}^{1\% \cdot 1\text{cm}}$ value of 12.8 for SBA (Lotan *et al.*, 1974) and expressed as protomer concentrations. Concentrations of Dns-GalN were determined from its ϵ_{330} value of 4800 $\text{M}^{-1} \cdot \text{cm}^{-1}$ (De Boeck *et al.*, 1984b). Concentrations of *p*-nitrophenyl α -N-acetylgalactosaminide were determined from its ϵ_{305} value of 10000 $\text{M}^{-1} \cdot \text{cm}^{-1}$. All other concentrations were determined by weight measurements.

RESULTS

Fluorescence measurements

The fluorescence of Dns-GalN was enhanced in a striking manner on binding to SBA (Fig. 1). For totally bound sugar an 11-fold enhancement in the fluorescence was observed with a concomitant blue-shift of the emission maximum by 25 nm. Addition of an inhibitory

Fig. 1. Fluorescence spectra of Dns-GalN in the presence and in the absence of SBA

The samples were placed in a 1 cm $\times$ 1 cm $\times$ 4.5 cm cuvette at 25 °C and excited at 330 nm with 5 nm slits on both monochromators in a Perkin-Elmer MPF 44A spectrofluorimeter. The fluorescence (curve A) of the ligand (3.5 μM) is enhanced dramatically (curve B) on addition of SBA (33 μM final concentration). Under these conditions 98% of Dns-GalN is bound to the protein. Curve C is the control for the buffer alone.

Fig. 2. Titration of Dns-GalN with SBA

The fluorescence intensity is plotted as a function of the added protein. A $3.3 \mu\text{M}$ solution of Dns-GalN in phosphate-buffered saline at 20°C was titrated with a $333 \mu\text{M}$ stock of SBA in the same buffer. The inset gives a graphical representation for the determination of the association constant. For details see the Results section.

saccharide such as *N*-acetylgalactosamine totally reversed this effect.

A plot of $1/F$ versus $1/[P]$, where F is the fluorescence of the sample at a protein concentration of $[P]$, yielded a straight line. From the intercept of the plot, the

fluorescence intensity, F_∞ , at infinite protein concentration was obtained. When $\log[P]_f$, where $[P]_f$ is the free protein concentration, is plotted against $\log[(F_c - F_0)/(F_\infty - F_c)]$, the abscissa intercept yielded the K_a values for the SBA–Dns-GalN interaction. F_0 and F_∞ are the fluorescence intensities of free Dns-GalN and Dns-GalN bound to SBA respectively. F_c is the fluorescence intensity at any point of titration. Such a plot for the interaction of Dns-GalN and SBA at 20°C is shown in Fig. 2. The value of association constant determined from this plot is $1.5 \times 10^6 \text{ M}^{-1}$. The thermodynamic parameters for this interaction, determined from Van't Hoff plots (Fig. 4) of the temperature-dependent K_a values (Table 1), are listed in Table 2.

The binding of non-fluorescent ligands to SBA was studied by monitoring the decrease in fluorescence due to the release of Dns-GalN bound to SBA on addition of the inhibiting sugar. The fluorescence data were analysed by using the following reaction scheme:

where P, S and D refer to the lectin, inhibiting ligand and the fluorescent ligand. PS, PD and PSD are the most likely lectin–sugar complexes, where PSD is the complex when both S and D are bound to P at the same site. Since the binding of Dns-GalN to SBA was totally and specifically reversed by the addition of non-fluorescent ligands such as *N*-acetylgalactosamine, $[PSD] = 0$ and $K_{SD} = K_{DS} = 0$. The above reaction scheme is then characterized by the following equation:

$$\left(\frac{[P]_t}{[PD]} - 1 \right) [D]_f = \frac{K_S}{K_D} [S]_f + \frac{1}{K_D}$$

where $[P]_t$ is the total concentration of P, $[PD]$ is the concentration of protein–fluorescent-ligand complex,

Table 1. Equilibrium association constants for the binding of various saccharides to SBA

Abbreviation: Nph, *p*-nitrophenyl

Sugar	$K_a (\text{M}^{-1})$			
	Temp. . . . 20°C	25°C	30°C	40°C
Dns-GalN	1.51×10^6 (1.2×10^6)†	5.9×10^5 *	9.1×10^5 (1.1×10^6)†	5.6×10^5 (5.1×10^5)†
GalNAc	9.2×10^3		5.3×10^3	3.8×10^3
Me α -GalNAc	1.3×10^4	2.43×10^4 *	7.4×10^3	5.1×10^3
Me α -Gal	7.0×10^2	9.8×10^2 *	4.9×10^2	3.6×10^2
Me β -Gal	5.5×10^2			
Galactose	2.3×10^2			
Lactose	3.5×10^2			
LacNAc	4.4×10^2			
Melibiose	5.5×10^2			
Nph α -GalNAc	1.1×10^5			
dGal	0.4×10^2			
Fucose	0.6×10^2			
L-Arabinose	0.5×10^2			

* Values from De Boeck *et al.* (1984a).

† Values in parentheses are equilibrium constants derived from kinetic data.

Table 2. Thermodynamic parameters for the binding of various saccharides to SBA at 20 °C

Sugar	K_a (M^{-1})	$-\Delta G$ ($kJ \cdot mol^{-1}$)	$-\Delta H$ ($kJ \cdot mol^{-1}$)	$-\Delta S$ ($J \cdot mol^{-1} \cdot K^{-1}$)
Dns-GalN	1.5×10^6	34.7	37.9	10.9
	$1.2 \times 10^{6*}$	34.5	38.3*	13.3*
	$(5.9 \times 10^5)^\dagger$	$(32.9)^\dagger$	$(15.0)^\dagger$	$(-60.0)^\dagger$
GalNAc	9.2×10^3	22.2	33.5	38.6
Me α -GalNAc	1.3×10^4	23.1	37.5	49.1
	$(2.43 \times 10^4)^\dagger$	$(25.0)^\dagger$	$(45.0)^\dagger$	$(67.0)^\dagger$
Me α -Gal	6.96×10^2	16.0	25.6	32.8
	$(9.8 \times 10^2)^\dagger$	$(17.1)^\dagger$	$(38.0)^\dagger$	$(69.0)^\dagger$

* Values obtained from kinetic data.

† Values in parentheses, determined at 25 °C, are taken from De Boeck *et al.* (1984a).

and $[D]_f$ and $[S]_f$ are the concentrations of free D and S respectively.

Now, let F_0 be the fluorescence intensity of free D, which on addition of the protein changes to F_p . Addition of S decreases the value to F_s . If $\Delta F = (F_s - F_0)$ and $\Delta F' = (F_p - F_s)$, then the concentration of the free inhibiting ligand, $[S]_f$, is obtained from the expression:

$$[S]_f = [S]_t - \frac{\Delta F'}{\Delta F_\infty} \cdot [P]_t$$

where $[S]_t$ refers to the total inhibitory ligand concentration and ΔF_∞ is the fluorescence change at infinite concentration of the protein. The concentration of the free fluorescent ligand $[D]_f$, can be obtained from the expression:

$$[D]_f = [D]_t - \left(\frac{\Delta F}{\Delta F_\infty} \cdot [D]_t \right)$$

where $[D]_t$ is the total fluorescent sugar concentration and the term in parentheses in the right-hand side of the equation corresponds to the concentration of PD complex.

A plot of $[S]_f$ versus $\{([P]_t/[PD]) - 1\} \cdot [D]_f$ yields a straight line, from the ordinate intercept of which the K_a value for the binding of fluorescent sugar can be obtained. A representative plot for the binding of *N*-acetylgalactosamine to SBA is shown in Fig. 3. The K_a value for the inhibiting sugar is obtained by multiplying the slope with the intercept. K_a values thus obtained for various saccharides are listed in Table 1. By using this method the binding of methyl α -galactoside, methyl α -*N*-acetylgalactosamide and *N*-acetylgalactosamine to SBA was studied at 20 °C, 30 °C and 40 °C and the thermodynamic parameters were obtained from Van't Hoff plots (Fig. 4). These Van't Hoff plots are linear in the temperature range 20–40 °C, and the values of ΔH and ΔS obtained from these plots are presented in Table 2.

Kinetic studies

The large change in fluorescence of Dns-GalN when it binds to SBA provided a convenient means to monitor the kinetics of binding. A representative stopped-flow fluorescence trace is given in Fig. 5. Under pseudo-first-order conditions, the kinetic data could be interpreted in terms of a single-step reaction according to the expression:

Fig. 3. Competitive binding of *N*-acetylgalactosamine to SBA in the presence of Dns-GalN

A mixture of 3.3 μM -Dns-GalN and 1.7 μM -SBA in phosphate-buffered saline (2.5 ml) was titrated with a 105 mM solution of *N*-acetylgalactosamine in the same buffer. The fluorescence intensity after appropriate correction for dilution was plotted as a function of the amount of *N*-acetylgalactosamine added. The inset shows the determination of the association constants for Dns-GalN and *N*-acetylgalactosamine. From the ordinate intercept the K_a value of Dns-GalN was determined to be $1.43 \times 10^6 M^{-1}$, and the slope yielded a value of $9.2 \times 10^3 M^{-1}$ for *N*-acetylgalactosamine.

where P and D refer to the lectin (SBA) and Dns-GalN respectively. The values of k_{+1} and k_{-1} were obtained from the slope and intercept of linear plots of k_{app} versus $[P]$ (Fig. 6). The value of the dissociation rate constant, k_{-1} , was also obtained by a direct method, by monitoring the dissociation of PD complex on mixing with a 10 mM solution of *N*-acetylgalactosamine in the stopped-flow instrument. Fig. 7 shows a representative stopped-flow

fluorescence trace of the dissociation reaction. Increasing the concentration of *N*-acetylgalactosamine to 20 mM did not affect the first-order disappearance of the fluorescence.

Fig. 4. Van't Hoff plots for the association of various saccharides with SBA

Symbols: ○, methyl α -galactoside; ●, *N*-acetylgalactosamine; △, methyl α -*N*-acetylgalactosaminide; □, Dns-GalN (equilibrium titrations); ■, Dns-GalN (kinetically determined K_a values).

Fig. 5. Stopped-flow fluorescence trace for the binding of Dns-GalN to SBA

Dns-GalN (7.5 μ M) and SBA (86 μ M) at 20 °C were allowed to mix in the stopped-flow instrument. The samples were excited at 330 nm and emission was collected above 460 nm with the use of a cut-off filter. The spectrum represents the average of six measurements. Inset: when $\ln(\Delta F_\infty - \Delta F_t)$ was plotted as a function of time (t), the plot yielded a straight line, suggesting that the binding reaction involves a single step. The slope of the plot yielded a k_{app} value of 6.5 s⁻¹.

The values of k_{+1} and k_{-1} determined by the graphical method are 2.1×10^5 M⁻¹·s⁻¹ and 0.2 s⁻¹ respectively at 20 °C. Values of k_{+1} and k_{-1} determined at several temperatures are listed in Table 3. From the value of k_{+1} and k_{-1} determined at each temperature, the K_a values were calculated. These values are in good agreement with

Fig. 6. Determination of the rate constant for the association of SBA and Dns-GalN

k_{app} values determined at 20 °C were plotted as a function of the protein concentration after mixing, $[P]$. The concentration of the sugar was fixed at 3.75 μ M after mixing. The slope of the line yielded a k_{+1} value of 2.1×10^5 M⁻¹·s⁻¹. Measurement of the rate constant with two different preparations of the protein yielded an average value of 2.4×10^5 M⁻¹·s⁻¹.

Fig. 7. Determination of the rate constant for the dissociation of SBA-Dns-GalN complex by *N*-acetylgalactosamine, by the stopped-flow method

The Figure represents a stopped-flow fluorescence trace (average of seven measurements) for the dissociation of SBA-Dns-GalN complex by *N*-acetylgalactosamine. A mixture of Dns-GalN (15 μ M) and SBA (17 μ M) in phosphate-buffered saline was allowed to mix with a 10 mM solution of *N*-acetylgalactosamine in the same buffer in the stopped-flow instrument at 20 °C. The straight line in the inset for the plot of $\ln(\Delta F_\infty - \Delta F_t)$ versus time is consistent with a single-step mechanism for the dissociation reaction. The slope of the line yielded the dissociation rate as 0.2 s⁻¹.

Table 3. Association (k_{+1}) and dissociation (k_{-1}) rate constants for the interaction of Dns-GalN and SBA

The equilibrium constants, K_a , were obtained from k_{+1} and k_{-1} .

Temp. (°C)	$k_{+1} \times 10^5$ ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	$10^{-5} \times K_a$ (M^{-1})
10	1.62	0.06	27.0
20	2.40	0.2	12.0
30	4.38	0.41	10.7
40	8.40	1.66	5.1

Fig. 8. Arrhenius plots for the association (○) and dissociation (●) rate constants**Table 4. Activation parameters for the binding of Dns-GalN to SBA**

The units are: E_A , $\text{kJ} \cdot \text{mol}^{-1}$; ΔG , $\text{kJ} \cdot \text{mol}^{-1}$; ΔH , $\text{kJ} \cdot \text{mol}^{-1}$; ΔS , $\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$.

Parameter	From kinetic studies	From equilibrium studies
$\Delta H_{\text{total}}^0$	-38.3	-37.9
E_{Aa}	41.6	
E_{Ad}	79.9	
ΔH_a	39.2	
ΔH_d	77.4	
$\Delta S_{\text{total}}^0$	-13.3	-10.9
ΔS_a	-7.4	
ΔS_d	5.9	
$\Delta G_{\text{total}}^0$	-34.4	-34.7
ΔG_a	41.3	
ΔG_d	75.7	

those obtained by equilibrium titrations. Values of ΔG , ΔH and ΔS were also determined from the kinetic data and are reported in Table 2.

The activation energies for the association and dissociation reactions were obtained from Arrhenium plots

(Fig. 8). The thermodynamic parameters $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for these reactions were obtained from the following expressions (Laidler, 1979):

$$\Delta H^\ddagger = E_A - RT \quad (1)$$

$$\ln\left(\frac{k}{T}\right) = \frac{\Delta H^\ddagger}{R} \cdot \frac{1}{T} + \frac{\Delta S^\ddagger}{R} + \ln\left(\frac{k'}{h}\right) \quad (2)$$

$$\Delta G^\ddagger = \Delta H^\ddagger - T \cdot \Delta S^\ddagger \quad (3)$$

where k is the rate constant, k' is the Boltzmann constant and h is the Planck constant.

Plots of $\ln(k/T)$ versus $1/T$ gave straight lines, from the slopes of which the $\Delta H^\ddagger$ values for the association and dissociation reactions were calculated. Substitution of these values in eqn. (2) yielded the values for $\Delta S^\ddagger$, and in eqn. (3) gave the $\Delta G^\ddagger$ values from the corresponding $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values. These values are presented in Table 4.

DISCUSSION

Binding of Dns-GalN to SBA

We have used the fluorescence properties of Dns-GalN to probe the interaction of various saccharides with SBA. The reversal of fluorescence enhancement when inhibiting saccharides are added to SBA-Dns-GalN complex proves that the binding of this fluorescent sugar to SBA is saccharide-specific. The large blue-shift in emission maximum observed when Dns-GalN binds to SBA indicates that on binding to the protein the dansyl moiety is going into a less-polar environment.

The association constant of $1.5 \times 10^6 \text{ M}^{-1}$ at 20 °C obtained for the binding of Dns-GalN to SBA indicates a much stronger association between them than that observed for any other SBA-monosaccharide complex. This large difference in the affinity is probably due to a favourable contribution from the dansyl moiety. It is interesting to compare the thermodynamic parameters obtained for this complex with many other lectin-mono-saccharide complexes. The ΔH value of $-37.9 \text{ kJ} \cdot \text{mol}^{-1}$ falls in the range of ΔH values obtained for several other lectin-monosaccharide complexes (Van Landschoot *et al.*, 1977; Neurohr *et al.*, 1980, 1981; Khan *et al.*, 1981). ΔH values obtained for the interaction of methyl α -galactoside, methyl α -N-acetylgalactosaminide and N-acetylgalactosamine with SBA are in the range -30 to $-40 \text{ kJ} \cdot \text{mol}^{-1}$ (Table 2). The ΔS value for the SBA-Dns-GalN complex is $-10.9 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, which is much smaller than the values obtained for other monosaccharides, namely -32.8 , -49.1 and $-38.6 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ respectively for methyl α -galactoside, methyl α -N-acetylgalactosaminide and N-acetylgalactosamine. ΔS values obtained for several other lectin-monosaccharide complexes are also much larger than the values obtained for the SBA-Dns-GalN complex (Van Landschoot *et al.*, 1977; Neurohr *et al.*, 1981; Khan *et al.*, 1981). Thus the binding of Dns-GalN to SBA is characterized by a very low ΔS value, resulting in the high affinity for this complex, which is larger by about two orders of magnitude than that for any of the lectin-monosaccharide complexes mentioned above.

Binding of non-fluorescent saccharides to SBA

Association constants are the largest for N-acetylgalactosamine and its 1-O- α -methyl derivative among

the simple saccharides studied (Table 1). Binding of *N*-acetylgalactosamine is 40-fold stronger than that of galactose, indicating that the 2-acetamido moiety contributes strongly towards binding, presumably owing to hydrophobic interactions with the binding locus in the lectin. This is consistent with the current thinking that in the binding of carbohydrates to antibodies and lectins hydrophobic interactions play a substantial role (Kabat *et al.*, 1981; Lemieux, 1982). In the light of this concept it appears that Dns-GalN has the most complementary surface to the binding site of SBA. A substituent in the equatorial position on the C-2 of galactose seems to favour the binding considerably, as illustrated by the poor binding of 2-deoxygalactose. Thus the affinities of 2-deoxygalactose, galactose, *N*-acetylgalactosamine and Dns-GalN increase in that order, indicating the probability of a hydrophobic pocket corresponding to the C-2 position. The poor binding of fucose and *L*-arabinose as compared with galactose suggests that the 6-hydroxy group also favours the binding process. The presence of a hydrophobic group at the anomeric position also favours the binding when compared with the free sugar. Thus the association of methyl α -*N*-acetylgalactosaminide is 1.5-fold stronger than that of *N*-acetylgalactosamine. A bulkier group increases the affinity even further, since *p*-nitrophenyl α -*N*-acetylgalactosaminide binds with a K_a value that is 10-fold larger than that of *N*-acetylgalactosamine. It may also be noted that methyl α -galactoside as well as its β -anomer bind with more avidity than their parent sugar galactose, though the binding of the α -anomer is more favoured.

Kinetic studies

The binding of Dns-GalN follows first-order kinetics, as evidenced by linear plots obtained when $\log(\Delta F_t - \Delta F_i)$ was plotted as a function of time (Fig. 7). The rate constant for the association process was temperature-dependent; in this respect it differs from that for peanut agglutinin, to which the binding of monosaccharides has been observed, from n.m.r. studies, to be temperature-independent (Neurohr *et al.*, 1981). The thermodynamic parameters obtained from the temperature-dependence of the association rate constant show that the association of SBA and Dns-GalN is enthalpically driven with a $\Delta H^\ddagger$ value of $39.2 \text{ kJ} \cdot \text{mol}^{-1}$ and that the entropic contribution is very small. The dissociation reaction with a rate constant of 0.2 s^{-1} at 20°C is the slowest reported so far for any lectin-sugar complex. The dissociation process is also strongly temperature-dependent, with a $\Delta H^\ddagger$ value of $77.4 \text{ kJ} \cdot \text{mol}^{-1}$. The values of $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ were used to calculate the equilibrium thermodynamic parameters $\Delta G_{\text{total}}^0$, $\Delta H_{\text{total}}^0$ and $\Delta S_{\text{total}}^0$. When compared with the values obtained by the equilibrium studies these values are in good agreement. These results are diagrammatically represented in Fig. 9.

The entropy of activation for the association process is very small, indicating that the association process does not involve a highly ordered transition state. This could mean that the sugar can approach the binding pocket in several ways. This is in contrast with the situation with peanut agglutinin (Neurohr *et al.*, 1981), where a highly ordered transition state has been implicated in an entropically driven binding reaction ($\Delta S_a^\ddagger = -160 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). Binding of concanavalin A to saccharides is also accompanied by considerably large $\Delta S_a^\ddagger$ values (Farina & Wilkins, 1980). It appears that SBA

Fig. 9. Energy diagram for the binding of Dns-GalN to SBA

differs considerably in this respect from concanavalin A and peanut agglutinin.

The value of activation energy (E_A) for the association reaction is $41.6 \text{ kJ} \cdot \text{mol}^{-1}$, indicating that sufficiently large amounts of energy have to be expended for the binding process to take place. This can be utilized in overcoming some steric constraints, such as some conformational changes of the protein and/or in breaking hydrogen bonds between the solvent and the sugar as well as the protein. A conformational change in the protein seems to be very probable in view of the relatively lower rates of the association reaction as compared with the normally expected diffusion-controlled process and the considerable ligand-induced changes in the c.d. pattern of the protein (Thomas *et al.*, 1979). However, such conformational changes are likely to take place only at the tertiary structural level and not at the secondary structural level, as no change in the c.d. spectra was observed in the secondary structure region (Thomas *et al.*, 1979).

The association constants, K_a , determined from the kinetic data are in agreement with those obtained from steady-state titrations (Table 1), suggesting that no significant reaction has occurred during the dead time of the instrument.

When this manuscript was in preparation we came across a paper by De Boeck *et al.* (1984a) reporting the kinetics of saccharide binding to SBA assessed from the absorbance changes of 4-methylumbelliferyl β -*N*-acetylgalactosaminide on binding. They did not, however, study the activation parameters for the binding process. The association rate constant of $1.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ reported by them is about 20-fold slower than the rate constant obtained by us for the binding of Dns-GalN to SBA. Such a difference could arise from the use in these studies of different reporter groups attached at different places to the parent sugar. However, the observations made by De Boeck *et al.* (1984a), contrary to our findings, suggest that Dns-GalN binds to SBA at a very low rate. These differences, in addition to the differences observed in the thermodynamic properties associated with the binding of monosaccharides (see Table 2), suggest that the lectin used in our study differs appreciably from that used by De Boeck *et al.* (1984a). The reasons for these differences are not very clear. However, it is known that certain strains of *Glycine max* do not express the lectin gene (Pull *et al.*, 1978). In view of this observation, it

appears probable that the different strains of this plant that express the lectin gene may contain lectins differing from each other in their primary structure by a few residues, leading to a difference in their saccharide-binding properties. In the light of these observations, it is imperative to characterize the lectins with respect to their saccharide-binding properties before their use in studies on cell surfaces etc., or, in all such studies, lectin isolated from the same strain should be used.

The rate constant of $2.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ obtained for the association process at 20°C is about three to four orders of magnitude slower than that expected for a diffusion-controlled process. In this respect it is similar to several other lectin-sugar interactions (Lewis *et al.*, 1976; Podder *et al.*, 1978; Neurohr *et al.*, 1981; Loontjens, 1983). It has been suggested (Neurohr *et al.*, 1981) that such slow kinetics could be due to the steric constraints that the system has to overcome in order for binding to take place. The lectin may have to undergo unfavourable conformational changes in order to accommodate the saccharide moiety in the binding site. Another possibility is that the lectin may bind to its most complementary ligand at a higher rate than a simple saccharide, and it is possible that the receptors of the lectins in biological systems are not the simple saccharides studied in the laboratory.

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REFERENCES

- Barondes, S. H. (1981) *Annu. Rev. Biochem.* **50**, 207–231
 Bohool, B. B. & Schmidt, E. L. (1974) *Science* **185**, 269–271
 Chipman, D. M., Grisaro, V. & Sharon, N. (1967) *J. Biol. Chem.* **242**, 4388–4394
 De Boeck, H., Lis, H., Sharon, N. & Loontjens, F. (1983) *Proc. FEBS Meet.* 15th p. 213, Abstr. 5-05-TH-233
 De Boeck, H., Lis, H., van Tilbeurgh, H., Sharon, N. & Loontjens, F. G. (1984a) *J. Biol. Chem.* **259**, 7064–7074
 De Boeck, H., Loontjens, F. G., Lis, H. & Sharon, N. (1984b) *Arch. Biochem. Biophys.* **254**, 297–304
 Farina, R. D. & Wilkins, R. G. (1980) *Biochim. Biophys. Acta* **631**, 428–438
 Goldstein, I. J. & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.* **35**, 127–340
 Gordon, J. A., Blumberg, S., Lis, H. & Sharon, N. (1972) *Methods Enzymol.* **28 B**, 365–368
 Hemperly, J. J. & Cunningham, B. A. (1983) *Trends Biochem. Sci.* **8**, 100–102
 Kabat, E. A., Liao, J., Burzynska, M. H., Wong, T. C., Thogersen, H. & Lemieux, R. U. (1981) *Mol. Immunol.* **18**, 873–881
 Khan, M. I., Mazumder, T., Pain, D., Gaur, N. & Surolia, A. (1981) *Eur. J. Biochem.* **113**, 471–476
 Laidler, K. J. (1979) *Theories of Chemical Reaction Rates*, pp. 41–55, R. E. Krieger Publishing Co., Huntington
 Lartey, P. A. & Derechin, M. (1979) *Prep. Biochem.* **9**, 85–95
 Lemieux, R. U. (1982) *Frontiers in Chemistry*, pp. 3–24, Pergamon Press, Oxford
 Lewis, S. D., Shafer, J. A. & Goldstein, I. J. (1976) *Arch. Biochem. Biophys.* **172**, 689–695
 Loontjens, F. G. (1983) *FEBS Lett.* **162**, 193–196
 Lotan, R., Siegelman, H. W., Lis, H. & Sharon, N. (1974) *J. Biol. Chem.* **249**, 1219–1224
 Neurohr, K. J., Young, N. M. & Mantsch, H. H. (1980) *J. Biol. Chem.* **255**, 9205–9209
 Neurohr, K. J., Young, N. M., Smith, I. C. P. & Mantsch, H. H. (1981) *Biochemistry* **20**, 3499–3504
 Novogrodsky, A. & Katchalski, E. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2515–2518
 Podder, S. K., Surolia, A. & Bachhawat, B. K. (1978) *FEBS Lett.* **85**, 313–316
 Pull, S. P., Pueppke, S. G., Hymowitz, T. & Orf, J. H. (1978) *Science* **200**, 1277–1279
 Rapin, A. M. C. & Burger, M. M. (1974) *Adv. Cancer Res.* **20**, 1–78
 Reinhold, A., Baues, R. F. & Gray, G. R. (1977) *J. Biol. Chem.* **252**, 57–60
 Reisner, Y. & Sharon, N. (1980) *Trends Biochem. Sci.* **5**, 29–32
 Roberts, D. D. (1983) Ph.D. Thesis, University of Michigan
 Sarkar, M. & Kabat, E. A. (1979) *Carbohydr. Res.* **69**, 143–149
 Sela, B. A., Lis, H., Sharon, N. & Sachs, L. (1970) *J. Membr. Biol.* **3**, 267–279
 Sela, B. A., Lis, H., Sharon, N. & Sachs, L. (1971) *Biochim. Biophys. Acta* **249**, 564–568
 Shaanan, B., Shoham, M., Yonath, A., Lis, H. & Sharon, N. (1984) *J. Mol. Biol.* **174**, 723–725
 Thomas, M. W., Rudzki, J. E., Walborg, E. F., Jr. & Jirgensons, B. (1979) *ACS Symp. Ser.* **88**, 67–75
 Van Landschoot, A., Loontjens, F. G., Clegg, R. M., Sharon, N. & De Bruyne, C. K. (1977) *Eur. J. Biochem.* **79**, 275–283
 Vodkin, L. O., Rhodes, P. R. & Goldberg, R. B. (1983) *Cell (Cambridge, Mass.)* **34**, 1023–1031

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