Analysis of Saccharide Binding to Artocarpus integrifolia Lectin Reveals Specific Recognition of T-antigen (β-D-Gal(1→3)d-GalNAc)*

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The binding of Artocarpus integrifolia lectin to N-dansylgalactosamine (where dansyl is 5-dimethylamino-phenylalanine-1-sulfonyl) leads to a 100% increase in dansyl fluorescence with a concomitant blue shift in the emission maximum by 10 nm. This binding is carbohydrate-specific and has an association constant of $1.74 \times 10^4$ M$^{-1}$ at 20 °C. The lectin has two binding sites for N-dansylgalactosamine. The values of $-\Delta H$ and $-\Delta S$ for the binding of N-dansylgalactosamine are in the range of values reported for several lectin-mono-saccharide interactions, indicating an absence of non-polar interaction of the dansyl moiety of the sugar with the combining region of the protein. Dissociation of the lectin from methyl-α-D-galactopyranoside from its complex with the lectin and the consequent change in its fluorescence on addition of nonfluorescent sugars allowed evaluation of the association constant for competing ligands.

The thermodynamic parameters for the binding of monosaccharides suggest that the OH groups at C-2, C-3, C-4, and C-6 in the β-D-galactopyranose configuration are important loci for interaction with the lectin. The acetyl group at C-2 of 2-acetamido-2-deoxygalacto-pyranose and a methoxy group at C-1 of methyl-α-D-galactopyranoside are presumably also involved in binding through nonpolar and van der Waals' interactions. The T-antigenic disaccharide Galβ1→3GalNAc binds very strongly to the lectin when compared with methyl-β-D-galactopyranoside, the β(1→3)-linked disaccharides such as Galβ1→3GlcNAc, and the β(1→4)-linked disaccharides, N-acetylactosamine and lactose. The major stabilizing force for the avid binding of T-antigenic disaccharide appears to be a favorable enthalpic contribution. The combining site of the lectin is, therefore, extended. These data taken together suggest that the Artocarpus lectin is specific toward the Thomsen-Friedenreich (T) antigen. There are subtle differences in the overall topography of its combining site when compared with that of peanut (Arachis hypogaea) agglutinin.

The results of stopped flow spectrometry for the binding of N-dansylgalactosamine to the Artocarpus lectin are consistent with a simple single-step bimolecular association and unimolecular dissociation rate processes. The value of $k_2$ and $k_1$ at 21 °C are 8.1 × 10^4 M$^{-1}$ s$^{-1}$ and 50 s$^{-1}$, respectively. The activation parameters indicate an enthalpy-controlled association process.

The Thomsen-Friedenreich antigen is a tumor-associated antigen of non-oncotel origin and is probably one of the few chemically well-defined antigens with a proven link to malignancy; therefore, anti-T probes have enormous potentials in cancer research (1-4).

Occurrence of a lectin reactive toward Thomsen-Friedenreich antigen in the seed extract of jackfruit (Artocarpus integrifolia) has been reported based on the agar gel precipitation reaction against T-antigenic glycoproteins (5). The crude seed extract from jackfruit has also been demonstrated to be a very potent and selective mitogen of distinct T and B cell functions (6). Purification of this lectin has recently been reported (7). Among the ligands tested by these authors, MeaGal† was found to be the most effective inhibitor of the hemagglutination reaction caused by the Artocarpus lectin. This information regarding the sugar specificity is based on inhibition of hemagglutination with a limited number of monosaccharides. Moreover, these experiments, at best, provide only relative affinities.

In order to use this lectin as a sensitive probe, it is not only necessary to elucidate its carbohydrate specificity in detail but also to delineate the forces involved in its interaction with ligands (8, 9). Thermodynamic and kinetic analyses are invaluable in understanding the specificities of these interactions in addition to their relevance for a better evaluation of the binding of lectins to cells. These apart, a question of major interest is whether lectins have extended binding sites, complementary to 2 or more pyranosyl residues. With these objectives in mind, we report here the thermodynamic parameters for the binding of A. integrifolia lectin with several synthetic ligands, including Galβ1→3GalNAc, N-acetyltalactosamine, Galβ1→3GlcNAc, and N-dansylgalactose, as well as commonly available sugars, like melibiose, MeaGal, MeβGal, and GalNAc, and the kinetics of its binding to N-

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† The abbreviations used are: MeaGal, methyl-α-D-galactopyranoside; MeβGal, methyl-β-D-galactopyranoside; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DnsGalN, N-dansylgalactosamine; Gal, D-galactopyranose; Glc, D-glucopyranose; GalNAc, 2-acetamido-2-deoxygalactopyranose; N-acetylglucosamine, 2-acetamido-2-deoxy-galactopyranose. All sugars used are D-sugars unless otherwise specified.
dansylgalactosamine as studied by stopped flow spectrofluorometry.

**MATERIALS AND METHODS AND RESULTS**

**DISCUSSION**

**Binding of Monosaccharides**—In this paper, we have used the fluorescence of DnsGalN to probe the interaction of saccharides with *Artocarpus* lectin. The tetrameric lectin is bivalent, and there is no interaction between the two combining sites as the Scatchard plots in the low fractional and high saturation ranges are linear. The $\Delta H$ and $\Delta S$ values of $-31.6\text{ kJ/mol}^{-1}$ and $-26.8\text{ J mol}^{-1}\text{K}^{-1}$, respectively, for the binding of DnsGalN to *Artocarpus* lectin are in the range of those obtained for several lectin-sugar interactions (20, 22, 30). A total displacement of DnsGalN complexed with lectin on addition of nonfluorescent sugar allows simultaneous determination of $K_d$ values for the competing sugar and DnsGalN. Similar values of $K_d$ obtained for DnsGalN binding to the lectin by substitution titrations and those determined according to Chipman *et al.* (19) and Scatchard (18), respectively, are consistent with the mutually exclusive binding of DnsGalN and the inhibiting ligand to a given site in the lectin.

The thermodynamic parameters for the binding of saccharides to *Artocarpus* lectin are highly informative regarding the specificity of this lectin. *Artocarpus* lectin binds MeoGal 200-fold stronger than MePGal; on the other hand, *Griffonia simplicifolia* lectin I and peanut agglutinin prefer MeoGal only by 5.5- and 1.5-fold, respectively (31–33). A methyl group in the $\alpha$ configuration appears to contribute positively for binding. The major stabilizing force is enthalpic. The change in enthalpy ($-\Delta H$) for the binding of MeoGal is $13.0\text{ kJ/mol}^{-1}$ greater than for MePGal. A relatively large value of $-\Delta S$ coupled with a smaller $-\Delta H$ value is presumably responsible for the poor binding of MeoGal. Substitutions at C-2 of Gal have marginal influence on its binding to *Artocarpus* lectin; Gal is 2 and 0.8 times more potent a ligand than 2-deoxygalactose and galactosamine, respectively. GalNAC is about 2, 5, and 2.5 times stronger binding a ligand when compared with Gal, 2-deoxygalactose, and galactosamine, respectively. The better affinity of GalNAC over Gal is presumably due to a favorable enthalpic contribution of the acetamido group for the binding, which amounts to $-17.0\text{ kJ/mol}$ and probably reflects additional van der Waals’ interactions or hydrogen bonding between the acetamido group of the sugar and the protein or potentiating of the effectiveness of the existing ones. Introduction of bulky substituents as in DnsGalN does not increase affinity very markedly over GalNAC. This is in marked contrast to soybean agglutinin where introduction of bulky N-substituents increases affinity by a factor of 30, suggesting the absence of hydrophobic interaction between the dansyl group and the corresponding binding loci in the combining region of the *Artocarpus* lectin. This is also consistent with a less dramatic enhancement in fluorescence intensity upon binding of DnsGalN to *Artocarpus* lectin as compared to soybean agglutinin (34) and *Physocarpus* tetragonolobus lectin$^3$ and poorer entropic contribution for the association of DnsGalN when compared with soybean agglutinin. A lower enthalpic contribution of DnsGalN binding to *Artocarpus* lectin suggests that the replacement of the C-2 hydroxyl group, or an acetamido group, with a dansyl group presumably abrogates hydrogen bonding at this locus.

No monosaccharide derivative with a modified substituent at C-3 of galactose was available. At C-4, inversion of the hydroxyl group as in glucose is not allowed. The C-6 hydroxyl group provides an important binding locus as shown by the observation that fucose (6-deoxygalactose) and L-arabinose do not bind to the *Artocarpus* lectin.

**Binding of Disaccharides**—It is interesting to compare the values of association constants for monosaccharides and several of the disaccharides with those obtained for the disaccharides Galβ1→3GalNAc. This disaccharide has 3-, 36-, 100-, and 610-fold higher affinity over that observed for MeoGal, GalNAC, Gal, and MeoGal, respectively. It is observed that Galβ1→3GalNAc has a significantly higher $K_d$ than that for lactose, N-acetyllactosamine, and Galβ1→3GlCNAC. All of these disaccharides are at least 3000 times poorer ligands as compared to Galβ1→3GalNAc. It is appropriate to discuss here the carbohydrate recognition properties of *Artocarpus* agglutinin vis à vis peanut agglutinin. In contrast to the *Artocarpus* lectin, peanut agglutinin does not discriminate very markedly between fucose and galactose and between MeoGal and MeoGal. It also binds reasonably well to lactose, N-acetyllactosamine, and Galβ1→3GlCNAC and poorly to GalNAC (33, 36). For the *Artocarpus* lectin, the increase in $-\Delta H$ for the disaccharide Galβ1→3GalNAc over that for MeoGal amounts to about 63.0 kJ mol$^{-1}$, which indicates that the reducing pyranosyl residue of Galβ1→3GalNAc is bound in a subsite adjacent to the galactose-binding subsite. Other lectins specific for Gal show quite different thermodynamic parameters when compared to *Artocarpus* lectin. For example, *Ricinus communis* lectin (30, 35) and *Momordica charantia* lectin (22) show similar values of $-\Delta H$ for binding to monosaccharides and disaccharides. Peanut agglutinin, on the other hand, shows an increase in $-\Delta H$ values for binding to the disaccharides over any of the monosaccharides, although the increase in $K_d$ amounts to a factor of only 27 over that of MeoGal due to a less marked change in the $-\Delta H$ value over the $-\Delta S$ value as observed here for the *Artocarpus* lectin. This lectin, in contrast to peanut agglutinin, does not bind to the disaccharides lactose, N-acetyllactosamine, or Galβ1→3GlCNAC (36).

The situation found here for the binding of the disaccharide by *Artocarpus* lectin is reminiscent of the interaction of lysozyme to chitoooligosaccharides (37). Binding of lysozyme to chitoooligosaccharides gave $-\Delta H$ values of 26.0, 48.0, and 60.0 kJ mol$^{-1}$ for N-acetylgalcosamine, N,N'-diacetyltchitoobiose, and N,N',N"-triacetyltchitoobiose, respectively. The $-\Delta H$ values outweigh the increasingly unfavorable $-\Delta S$ values, so that there is a 1000-fold increase in the $K_d$ values for N,N'-diacetyltchitoobiose over that of N-acetylgalcosamine. Similarly, the binding of Galβ1→3GalNAc to *Artocarpus* lectin is also accompanied by such a large increase in enthalpy, which adequately compensates for the increase in the $\Delta S$ value, resulting in a 610-fold increase in $K_d$ for the binding of the disaccharide over that for MeoGal.

In order to understand the binding of disaccharides to *Artocarpus* agglutinin, we have used the thermodynamic parameters obtained by us for the binding of four disaccharides, viz. Galβ1→3GalNAc, Galβ1→3GlCNAC, lactose, and N-acetyllactosamine, and their minimum energy conformations ob-

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$^1$ Portions of this paper (including "Materials and Methods." "Results," Figs. 1–9, and Equation 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 8650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2849, cite the author, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

$^2$ M. I. Khan and A. Surolia, unpublished observations.

$^3$ G. J. Chipman and D. W. Jeffery, unpublished observations.
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tained by theoretical studies as well as the structures obtained by x-ray crystallography (36, 38–41). Fig. 10 shows ball and stick models of these four disaccharides. It is apparent that the conformation of the nonreducing residue in all the four disaccharides is identical. However, the orientation of the reducing sugar moiety with respect to that of the nonreducing residue for β(1→4)-linked disaccharides is different from that observed for β(1→3)-linked disaccharides. In β(1→4)-linked disaccharides, the C-2 substituent of the reducing sugar is on the same side as that of the hydroxymethyl group of the nonreducing sugar moiety, whereas in β(1→3)-linked sugars, it lies on the side opposite to the hydroxymethyl group of the nonreducing sugar (for example, compare the structures of Galβ1→3GalNAc and N-acetyllactosamine in Fig. 10). Despite these differences, all these disaccharides appear similar in the topography of their ring skeleton. One would, therefore, expect that these disaccharides bind to the lectin with comparable affinities. However, our observations show that the binding of Galβ1→3GalNAc is approximately 3000-fold stronger than the other disaccharides (Table I). This difference in affinity can be explained on the basis of the difference in configuration of the functional groups in the reducing sugar moiety in these disaccharides.

In the four disaccharides mentioned above, the C-4 hydroxyl group of the reducing sugar moiety in the β(1→3)-linked disaccharides occupies a position similar to that of the C-3 hydroxyl group of the reducing pyranosyl residue of the β(1→4)-linked disaccharides (Fig. 10). However, the configuration of these hydroxyl groups in these disaccharides is not the same. The C-3 hydroxyl groups of the reducing residue in β(1→4)-linked disaccharides and the C-4 hydroxyl group in Galβ1→3GlcNAc are in the equatorial configuration, whereas the C-4 hydroxyl group of the reducing residue in Galβ1→3GalNAc is in the axial configuration. This difference alone seems to be responsible for the very weak binding of Galβ1→3GlcNAc, N-acetyllactosamine, and lactose when compared with Galβ1→3GalNAc. The structure and conformation of Galβ1→3GalNAc and Galβ1→3GlcNAc are identical except for the configuration of the C-4 hydroxyl group of the reducing residue. It, therefore, appears probable that the change in

![Fig. 10. Ball and stick models of disaccharides: Galβ1→4Glc (lactose) (a), Galβ1→4GlcNAc (N-acetyllactosamine) (b), Galβ1→3GlcNAc (c), and Galβ1→3GalNAc (d). Note that the conformation of the nonreducing sugar moiety is the same in all these disaccharides. In the (1→4)-linked sugars (a and b), the reducing residue is rotated by about 180° when compared to that in the (1→3)-linked sugars (c and d) (as a result of this, the numbering scheme for the ring skeleton in the reducing sugar residue is in the counterclockwise direction for a and b, whereas it is in the clockwise direction for c and d). Due to this, in a and b, the hydroxymethyl group on the reducing sugar occupies a position topographically similar to that occupied by the acetamido group in c and d. The C-4 hydroxyl group in d (marked with an open arrow) is in the axial orientation, whereas the hydroxyl groups in the topographically similar position in a−c (marked with closed arrows) are in the equatorial orientation. This difference alone is responsible for the very poor binding of a−c when compared with d. The acetamido group and the C-4 hydroxyl group of the reducing sugar in d constitute strong binding loci in its interaction with the Artocarpus lectin.](image-url)
The lectin is qualitatively consistent with a single-step binding controlled reactions, and the kinetics of the sugar binding to the phobic region in the ligand-binding site of the lectin. The nature of Galpl+3-arabinose.

Fluorogenic ligands to concanavalin A (23, 25-27), a system that involves a two-step process, can be utilized to study the association and dissociation rates. The possibility of formation of reaction intermediate within the dead time of the instrument is, therefore, ruled out. However, the presence of association constants determined from kinetic measurements between 12 and 27 °C are in agreement with values determined from the fluorescence titrations.

The value of $-\Delta H$ determined from kinetic measurements also agrees well with the value from equilibrium measurements. This indicates that the enthalpy change is related to the total binding event, and there does not exist any faster process which we are not observing, but which contributes significantly to the reaction enthalpy for saccharide binding. Linearity of $\ln(k_i/T)$ versus $(1/T)$ plot indicates that dramatic conformational transitions in the lectin molecule are absent in the temperature range studied.

The large activation energy is also consistent with the slow reaction rates observed here. The apparent activation entropy is quite large when compared with facile bimolecular reactions. This large activation entropy for the binding reaction suggests that a specific configuration of reactants is required for sugar binding to the lectin. However, the principal barrier for the sugar binding appears to be energetic.

Generally, when the second order rate constants for the ligand binding to a protein differ by several orders of magnitude from that expected for a diffusion-controlled reaction, the binding is presumed to involve a putative intermediate complex as depicted in the equation below (D represents W-dansylgalactosamine and P represents lectin).

$$P + D \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} P\cdot D \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} P\cdot D$$

$K_i = k_i/k_{-i}$, $K_D = k_2/k_{-2}$

where $P$, $D$, and $P\cdot D$ are the intermediate and final complexes, respectively. Formation of $P\cdot D$ proceeds much faster than $P\cdot D$. Our failure to observe the $P\cdot D$ complex could be due to an unobservable signal change in the faster step. If the first step is always kinetically uncoupled from the second step, $P\cdot D \rightleftharpoons P\cdot D$ is rate-controlling, i.e. $k_1 \gg k_2$, then $k_{app}$ would not be a linear function of $[P]$. In such a case, $k_{app}$ should progress from a first order dependence to a zero order dependence as the concentration of the excess component (which is $P$ in our case) increases from a value much lower

### Table I

| Sugar                | $10^{-3} \times K_a$ at: | $\Delta H^\circ$ | $\Delta G^\circ$ | $\Delta S^\circ$ |
|---------------------|--------------------------|------------------|-----------------|-----------------|
|                     | 15 °C | 20 °C | 25 °C | 30 °C | hJ·mol$^{-1}$ | hJ·mol$^{-1}$ | J·mol$^{-1}$·K$^{-1}$ |
| DnsGalN             | 24    | 17.4  | 14.1  | 11.5  | -31.6 (±2.3) | -23.8 (±1.2) | -26.6 (±2.0) |
| Galactose           | 1.34  | 1.22  | 0.93  | 0.61  | -38.0 (±1.5) | -17.3 (±1.0) | -70.6 (±2.2) |
| MesGal              | 63    | 40    | 24    | 21.3  | -55.0 (±1.8) | -25.8 (±1.1) | -99.6 (±2.2) |
| 2-Deoxy-Gal         | 0.285 | 0.20  | 0.16  | 0.10  | -42.0 (±1.2) | -12.9 (±0.8) | -99.3 (±5.5) |
| GalN                | 1.44  |       |       |       | -17.7 (±2.1) |          |         |
| GalNAc              | 4.5   | 3.3   | 2.3   | 1.9   | -55.0 (±2.7) | -19.7 (±2.0) | -120.4 (±5.6) |
| Fucose              |        |       |       |       | -8.28 (±1.5) |          |         |
| L-Arabinose         |        |       |       |       | -8.7 (±1.7)  |          |         |
| Lactose             |        |       |       |       | -9.0 (±2.0)  |          |         |
| N-Acetyllactosamine |        |       |       |       | -21.3 (±1.8) |          |         |
| Gal|3GlcNAc           | 0.041  |     |       |       | -28.5 (±1.5) | -261.0 (±8.4) |         |
| Melibiose           | 8.25   | 8.44  | 8.44  | 8.44  | -261.0 (±8.4) |          |         |

* Values are calculated for 20 °C.

### Table II

| Temperature | $10^{-3} \times k_1$ | $k_2$ | $10^{-4} \times K_a$ |
|-------------|----------------------|-------|----------------------|
| °C          | $M^{-1} s^{-1}$      | s$^{-1}$ | $M^{-1}$          |
| 12          | 4.0 (±0.4)           | 17.7 (±2.0) | 2.26                 |
| 15          | 5.3 (±0.3)           | 25.0 (±2.3) | 2.12                 |
| 18          | 6.8 (±0.2)           | 34.2 (±1.8) | 1.98                 |
| 21          | 9.3 (±0.7)           | 50.0 (±2.7) | 1.86                 |
| 24          | 11.7 (±1.5)          | 83.5 (±5.4) | 1.4                  |
| 27          | 13.9 (±2.2)          | 105.0 (±4.6) | 1.27                 |

$\Delta H_a = 56.4 (±3.2) \text{ kJ} \cdot \text{mol}^{-1}$

$\Delta S_a = 59.94 (±4.4) \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$

$\Delta G_a = 38.78 (±2.1) \text{ kJ} \cdot \text{mol}^{-1}$

$E_a = 62.36 (±3.7) \text{ kJ} \cdot \text{mol}^{-1}$

$\Delta S = \Delta S_a - \Delta S_b = -23.76 (±2.9) \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$

$E_a = E_b = 27.0 (±3.1) \text{ kJ} \cdot \text{mol}^{-1}$

$K_a$ is the association constants obtained from the association ($k_1$) and dissociation ($k_2$) rate constants.

| Sugar     | $10^{-3} \times K_a$ at: | $\Delta H^\circ$ | $\Delta G^\circ$ | $\Delta S^\circ$ |
|-----------|--------------------------|------------------|-----------------|-----------------|
| 11729     |                         |                  |                 |                 |

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**Kinetic Studies**—The forward rate constants listed in Table II are several orders of magnitude slower than the diffusion-controlled reactions, and the kinetics of the sugar binding to the lectin is qualitatively consistent with a single-step binding mechanism (Equation 1). Bimolecular association rate constants which are slower than the diffusion-controlled process have also been reported for the binding of chromogenic/fluorogenic ligands to concanavalin A (23, 25-27), R. communis agglutinin (24), and soybean agglutinin (34). The fluorescence change appearing in stopped flow traces on completion of the reaction is equivalent to that observed in equilibrium titrations for association and dissociation rates. The possibility of formation of reaction intermediate within the dead time of the instrument is, therefore, ruled out. The values of overall association constants determined from kinetic measurements between 12 and 27 °C are in agreement with values determined from the fluorescence titrations.

The value of $-\Delta H$ determined from kinetic measurements also agrees well with the value from equilibrium measurements. This indicates that the enthalpy change is related to the total binding event, and there does not exist any faster process which we are not observing, but which contributes significantly to the reaction enthalpy for saccharide binding. Linearity of $\ln(k_i/T)$ versus $(1/T)$ plot indicates that dramatic conformational transitions in the lectin molecule are absent in the temperature range studied.

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Generally, when the second order rate constants for the ligand binding to a protein differ by several orders of magnitude from that expected for a diffusion-controlled reaction, the binding is presumed to involve a putative intermediate complex as depicted in the equation below (D represents W-dansylgalactosamine and P represents lectin).

$$P + D \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} P\cdot D \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} P\cdot D$$

$K_i = k_i/k_{-i}$, $K_D = k_2/k_{-2}$

where $P$, $D$, and $P\cdot D$ are the intermediate and final complexes, respectively. Formation of $P\cdot D$ proceeds much faster than $P\cdot D$. Our failure to observe the $P\cdot D$ complex could be due to an unobservable signal change in the faster step. If the first step is always kinetically uncoupled from the second step, $P\cdot D \rightleftharpoons P\cdot D$ is rate-controlling, i.e. $k_1 \gg k_2$, then $k_{app}$ would not be a linear function of $[P]$. In such a case, $k_{app}$ should progress from a first order dependence to a zero order dependence as the concentration of the excess component (which is $P$ in our case) increases from a value much lower.
than 1/K, to P ≫ 1/K. Since our plots are linear up to 500 μM P, the association constant of the first step (K) has to be lower than 2000 M⁻¹. Alternatively, steric factors may be involved in lowering the bimolecular association rate constant. Such steric effects have been demonstrated by Cramer et al. (42) in the binding of dyes to α-cyclodextrins.

Conclusions—Considering the thermodynamic parameters in the light of suggestions by Ross and Subrahmanian (43), the major forces for the binding of sugars to Artocarpus lectin are hydrogen bonding and van der Waals' interactions coupled with some contributions from nonpolar interactions (44). Thus, several points of hydrogen bonding and nonpolar interactions between the Artocarpus lectin and saccharides may be inferred from these studies. The C-2, C-4, and C-6 hydroxyl groups of Gal are probably involved in hydrogen bonding. Nonpolar contacts play an important role in the association of M6Gal through its methyl group (since M6Gal binds about 30 times stronger than galactose). The T-antigenic disaccharide binds to the lectin very strongly due to a favor-able enthalpic contribution. In contrast to the peanut agglu-tinin, the only other anti-T lectin reported, Artocarpus agglu-tinin binds extremely poorly to other disaccharides such as lactose, N-acetyllactosamine, and Galβ1→3GalNAc, which are topographically similar to Galβ1→3GalNAc. The differences in the binding property of Artocarpus lectin when compared with peanut agglutinin reflect subtle differences in the topographies of their combining region; and this lectin should, therefore, prove to be a valuable probe for studying the expression of the Thomsen-Friedenreich antigen on cell surfaces.

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Materials: The reagents for polyacrylamide gel electrophoresis, sodium dodecylsulfate, p-mercaptoethanol, polyacrylamide, dithiothreitol, glucose, and sodium dodecyl sulfate were obtained from Sigma Chemical Co., St. Louis, U.S.A. All the other reagents were used as analytical grade.

Methods

Preparation of A. cochinchinensis lectin: Concanavalin A (Con A) was derived from the root of A. cochinchinensis by a modification of the method of Levan and Streicher (12). The purified lectin was dialyzed extensively against PBS containing 0.9% sodium chloride and 0.02% sodium azide, and then lyophilized. The lectin solution was adjusted to a final concentration of 1 mg/ml in PBS. Emission was excited at 380 nm under a nitrogen atmosphere. The protein concentration was determined according to Lowry et al. (13) using bovine serum albumin as standard.

Preparation of A. cochinchinensis lectin: DnsGalN was prepared by the method of Dervan and coworkers (14). Dns-p-Galactoamnino obtained by neutralizing its hydrochloride with an equal amount of dry metal was allowed to react with a 0.8% w/v aqueous solution of freshly prepared d-aminonirnethyl chloride for 12 hr under nitrogen atmosphere. The precipitate was washed with methanol and recrystallized from 20% methanol, 0.05 M sodium chloride. The purified DnsGalN was eluted with methanol in dichloromethane. The extinction coefficient was measured by means of a double beam spectrophotometer (UV-110, Shimadzu, Japan) on the report of flavins and nicotinamide (16).

Fluorescence measurements: The fluorescence spectra of DnsGalN (100 μM) in the absence and presence of A. cochinchinensis lectin were recorded in a Perkin-Elmer model 400 spectrophotometer with a 400-nm xenon arc lamp in the excitation range of 300-500 nm. The samples were excited at 370 nm and the emission spectra recorded about 400-500 nm with 5 nm slit widths on both monochromers. Fluorescence titrations were performed on a Omnicon Gilson FS 750. A 1-cm path-length quartz cuvette was excited at 370 nm with a 5 nm slit and emission was monitored by means of a quartz interference band-pass filter (370-470 nm) at 500 nm along with a 100 nm cutoff filter. The temperature of the samples was maintained within 0.5°C. The fluorimeter is controlled by a microcomputer which allows averaging several readings. Typically, three sets of 10 measurements were taken and the standard deviation of these averaged values of absorbance at 370 nm was 0.005. Determination of the number of binding sites and the association constants, K, for the binding of DnsGalN to the lectin was done by the method of Taft et al. (17) with the use of a Job's plot analysis. The total reaction volume was 1.2 ml. The temperature was maintained at 25°C. The solution was stirred by a magnetic stirrer and the reaction solutions were mixed with 1.2 ml of 0.01 M sodium phosphate buffer (pH 7.4). A plot of the fluorescence intensity of DnsGalN upon binding to A. cochinchinensis lectin was constructed by means of a double beam spectrophotometer (UV-110, Shimadzu, Japan) on the report of flavins and nicotinamide (16). The extinction coefficient was measured by means of a double beam spectrophotometer (UV-110, Shimadzu, Japan) on the report of flavins and nicotinamide (16).

Results

The fluorescence intensity of DnsGalN in the absence of A. cochinchinensis lectin increased progressively on addition of A. cochinchinensis lectin (Fig. 1). The fluorescence intensity of DnsGalN at pH 7.4 in the absence of A. cochinchinensis lectin was determined to be 0.50 X 10^-4 M. The observed fluorescence change with constant protein and increasing sugar concentration was independent of particular protein concentration. The dissociation rate constant of A. cochinchinensis lectin-DnsGalN complex was determined by mixing the complex with methyl violet (17 aM).

Discussion

Fast reaction kinetic studies were performed on a Union Faraicell LF-320 spectrophotometer working in the fluorescence mode. Samples were excited at 370 nm and emission was collected above 450 nm using a cutoff filter at 450 nm. The total reaction volume was 1.2 ml. The temperature was maintained at 25°C. The solution was stirred by a magnetic stirrer and the reaction solutions were mixed with 1.2 ml of 0.01 M sodium phosphate buffer (pH 7.4). A plot of the fluorescence intensity of DnsGalN upon binding to A. cochinchinensis lectin was constructed by means of a double beam spectrophotometer (UV-110, Shimadzu, Japan) on the report of flavins and nicotinamide (16). The extinction coefficient was measured by means of a double beam spectrophotometer (UV-110, Shimadzu, Japan) on the report of flavins and nicotinamide (16).

The association of covalent nonfluorescent ligands was assessed by monitoring the change in fluorescence upon addition of sugar. The extinction coefficient of DnsGalN at 450 nm was defined earlier (17 aM). In this reaction, the fluorescence change was evaluated by assuming the following reaction scheme (20, 22).

Fig. 1. Fluorescence emission spectra of DnsGalN in the presence and absence of A. cochinchinensis lectin. The solutions were excited at 370 nm with 5 nm slit on both monochromers. (a) Fluorescence emission spectrum of DnsGalN (1 μM) upon addition of A. cochinchinensis lectin (30 μM) (final concentration). (b) Fluorescence emission spectrum of DnsGalN obtained by addition of both A. cochinchinensis lectin (30 μM) and DnsGalN (1 μM) resulting in TcJ reaction. (d) Controls 1. Buffer, 2. Protein and 3. Meas.

Fig. 2. Scatchard plot for the binding of DnsGalN to A. cochinchinensis lectin at 25°C and a fixed concentration of DnsGalN (0.01 M) (Inset in lowercase). The fluorescence of the ligand protein mixture was compared with that of equal concentration of ligand in the absence of protein.
The binding of D and L to the same site is mutually exclusive and thus PDL = D and Kp = Kp = 0. Lectin-sugar complexes [PO] and [PL] are then characterized by the following relationship

\[ [P]_0 \gamma = \frac{[P]_0 [P]_0}{[P]_0 + [P]_0} \]

where \([P]_0\) is the total concentration of lectin, \([P]_0\) is the concentration of protein-fluorescence sugar complex, [O] and [L] are free D and L concentrations respectively, when L is mixed with P. If \(Kp\) is the observed change in fluorescence intensity on addition of an aliquot of the competing ligand and \(F_0\) is the change in fluorescence intensity for totally bound D, then according to the above scheme

\[ \frac{[P]_0}{[P]_0} = \left( \frac{[P]_0}{[P]_0} + [P]_0 \right) \]

The distribution of competing ligand L in various complexes is given by

\[ [L]_0 = \frac{[L]_0}{[L]_0} + \frac{[L]_0}{[L]_0} \]

\[ \frac{[L]_0}{[L]_0} = \frac{[L]_0}{[L]_0} \cdot \frac{[L]_0}{[L]_0} \]

The value of [L]_0 at each point is given by [L]_0 \(1 - \frac{[L]_0}{[L]_0}\).

A plot of \(\frac{[P]_0}{[P]_0} - 1\) [L]_0 gives a straight line for the binding of GalG->3GalNAc shown as a representative case in Fig. 5. From the slope and intercept of this plot the \(Kp\) values for DnsGall and GalG->3GalNAc were determined to be 2.2 x 10^-6 and 1.2 x 10^-6 respectively. The \(Kp\) value for the fluorescent saccharide thus determined is also consistent with that obtained by Scottard analysis and by the relationship of Lussier et al. (12), supporting our conclusions that D and L bind to the same site on the lectin. \(Kp\) values for the binding of several of the saccharides thus determined at various temperatures are listed in Table 1.

A glance at Table 1 reveals that Artocarpus agglutinin binds to GalG->3GalNAc, ManGal, and GalNAc and binds very poorly to Lactose, N-acetylgalactosamine, GalG->4GalNAc, fucose, and galactose. From the temperature-dependent association constants, thermodynamic parameters were calculated for several saccharides by using half plots as shown in Fig. 4 and are listed in Table 1. It can be seen from the Table that the binding of 12-0.5mM (Kp) leads to more pronounced enthalpy changes than the binding of the same 12 values. A significantly larger value for the change in activity for the binding of GalG->3GalNAc suggests that Artocarpus lectin has an extended binding site.

The rate constant \(k_p\) was evaluated from the slopes of linear plots of \(\ln F_0 - F_0\) against time where \(F_0\) and \(F_0\) are the fluorescence at time t and at the end of reaction respectively. Association (\(k_p\)) and dissociation (\(k_1\)) rates were obtained from the slopes and intercepts, respectively, of the linear plots of \(k_p\) vs [P]. The value of \(k_1\) could also be determined directly and therefore with slightly greater precision, by displacing the sugar from its complex with protein by adding excess neutral and following the rate of disappearance of fluorescence of the displaced DnsGalN. For these reactions the following schemes apply:

\[ P_0 + L \rightarrow P_0 \]

\[ P_0 + L \rightarrow P_0 \]

The value of \(k_1\) as determined from the slope of this plot is equal to 8.1 x 10^6 M^-1 s^-1. Values of \(k_1\) as determined from y-intercept of this plot are consistent with those obtained from the first order rate constant for the displacement of D by a large excess of Neutral. The value of \(k_1\) thus determined is 5.0 x 10^6 s^-1.

The bimolecular association rate constants which are considerably slower than the diffusion controlled reactions have also been reported for other lectins (24-28). However these rate constant are slower than those reported for the binding of saccharides to wheat germ agglutinin (20).

Stopped flow experiments were carried out at six temperatures in order to obtain activation parameters. To compare the resulting reaction enthalpy changes with those determined from the equilibrium experiments. The activation energy (\(E_a\)) parameters were evaluated from the plots shown in Fig. 9.
Combining Site of Artocarpus Lectin

The activation parameters $\Delta H^*$, $\Delta S^*$ and $\Delta F^*$ were then calculated from the following equations [20].

$$
\Delta H^* = E_a - RT
$$

$$
\ln(k/T) = \omega/k_b - 1/T + \Delta S^*/R + \ln(k'/h)
$$

$$
\Delta F^* = \Delta H^* - T\Delta S^*
$$

where $k$ is the appropriate rate constant, $k'$ is the Boltzman constant and $h$ is Planck's constant. The values are listed in Table 2.

Fig. 5. $\ln(k_1/T)$ and $\ln(k_2/T)$ values obtained were plotted against inverse of temperature (1/T).