Binding of Multivalent Carbohydrates to Concanavalin A and Dioclea grandiflora Lectin

THERMODYNAMIC ANALYSIS OF THE “MULTIVALENCY EFFECT”

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Binding of a series of synthetic multivalent carbohydrate analogs to the Man/Glc-specific lectins concanavalin A and Dioclea grandiflora lectin was investigated by isothermal titration microcalorimetry. Dimeric analogs possessing terminal α-D-mannopyranoside residues, and di-, tri-, and tetrameric analogs possessing terminal 3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside residues, which is the core trimannoside of asparagine-linked carbohydrates, were selected in order to compare the effects of low and high affinity analogs, respectively. Experimental conditions were found that prevented precipitation of the carbohydrate-lectin cross-linked complexes during the isothermal titration microcalorimetry experiments. The results show that the value of \( n \), the number of binding sites on each monomer of the lectins, is inversely proportional to the number of binding epitopes (valency) of each carbohydrate. Hence, \( n \) values close to 1.0, 0.50, and 0.25 were observed for the binding of mono-, di-, and tetrasaccharides, respectively, to the two lectins. Importantly, differences in the functional valency of a triantennary analog for concanavalin A and \( D. \) grandiflora lectin are observed. The enthalpy of binding, \( \Delta H \), is observed to be directly proportional to the number of binding epitopes in the higher affinity analogs. For example, \( \Delta H \) of a tetravalent trimannoside analog is nearly four times greater than that of the corresponding monovalent analog. Increases in \( K_a \) values of the multivalent carbohydrates relative to monovalent analogs, known as the “multivalency effect,” are shown to be due to more positive entropy (TAS) contributions to binding of the former sugars. A general thermodynamic model for distinguishing binding of multivalent ligands to a single receptor with multiple, equal subsites versus binding to separate receptor molecules is given.

Carbohydrate-protein interactions are involved in a wide variety of biological functions including cellular growth, recognition, adhesion, cancer metastasis, bacterial and viral infections, and inflammation (1, 2). The specificity of these interactions has been an active area of research due, in part, to efforts at designing therapeutic analogs of carbohydrates (3, 4). However, attempts to design high affinity analogs for specific carbohydrate-binding proteins (lectins) have been difficult due to the intrinsic low affinity of carbohydrates in many cases (5, 6). For example, the affinity constants (\( K_a \)) for the binding of simple mono- and oligosaccharides to most lectins are between \( 10^6 \) and \( 10^9 \) M\(^{-1} \) (7, 8). This range of \( K_a \) values is too low for effective drug design. However, many naturally occurring carbohydrates and glycoconjugates including glycoproteins and glycolipids are multivalent (2) which results in their increased avidity for lectins (9). As a consequence, there has been considerable interest in designing multivalent or “clustered” carbohydrate analogs for high affinity binding to target lectin receptors (10, 11). Thus, it is important to understand the thermodynamic basis of the enhanced binding of multivalent glycoconjugates to lectins.

Binding of multivalent carbohydrates (and glycoconjugates) to lectins, which themselves are generally multivalent (2), may or may not lead to the formation of lectin-carbohydrate cross-linked complexes (12). This depends on the valencies of the two molecules, and the structural arrangement of the carbohydrate epitopes and binding sites (subunits) of the lectin. In cases where cross-linking occurs, several different types of complexes can result. When both the carbohydrate and lectin are divalent, one-dimensional soluble cross-linked complexes may form. However, when the valencies of the carbohydrate and lectin are greater than two for either molecule, two- and three-dimensional cross-linking can occur resulting in highly ordered complexes. In the latter case, precipitation of the ordered cross-linked complex generally occurs, and occasionally crystals of the complex may result. This type of cross-linked complex has been extensively described by Brewer and co-workers (12, 13). Importantly, high affinity binding occurs in this type of cross-linked complex since both the carbohydrate and lectin simultaneously bind to neighboring molecules in the lattice.

Multivalent binding between carbohydrates and lectins can also occur without cross-linking interactions, as in the case where the binding sites of a lectin are clustered together or face the same direction (14). An example of clustered binding sites is the hepatic asialoglycoprotein receptor (9). Binding of a monoantennary complex oligosaccharide gives an inhibition constant, \( I_{50} \) of \( 10^2 \) M\(^{-1} \), while a triantennary complex oligosaccharide possesses an \( I_{50} \) of \( 10^5 \) M\(^{-1} \) (15). The presence of subsites for each arm of the trivalent oligosaccharide on three clustered subunits of the receptor is known to be responsible for the large increase in the affinity of the triantennary carbohydrate (9). The \( 10^2 \) increase in affinity of the trivalent carbohydrate relative to the monovalent sugar is essentially the theoretical limit of the sum of the exponentials of the

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binding constants of the individual subsites (i.e. $10^3 \times 10^3 \times 10^3 = 10^9$).

More recently, clustered or "dendritic" multivalent carbohydrate analogs have been synthesized in order to obtain enhanced affinity for lectins with binding sites well separated on adjacent monomers of the protein. For example, clustered or dendritic GlcNAc-based synthetic analogs with valencies of two, four, and eight were prepared on a scaffolding of L-lysine (16). The binding potencies of the dendrimers derived from IC50 values for inhibiting wheat germ agglutinin binding to porcine stomach mucin relative to a monomeric analog were 5-fold for the divalent derivative, 25-fold for the tetrameric derivative, and 170-fold for the octameric derivative. Thus, significant enhancements in the affinities of multivalent carbohydrates for multivalent lectins have been observed. However, the thermodynamic basis for these increased affinities are not understood.

Isothermal titration microcalorimetry (ITC)1 has recently been used to study the binding interactions between carbohydrates and lectins. ITC measurements provide direct determinations of the value of $n$, the number of binding sites of the protein, $\Delta H$, the enthalpy of binding, and $K_a$, the association constant. From measurements of $K_a$, the free energy of binding, $\Delta G$, can be calculated, and hence the entropy of binding, $\Delta S$, determined. Thus, ITC measurements can determine the complete thermodynamics of binding of a carbohydrate to a lectin. As examples, the binding of mono- and oligosaccharides to the Man/Glc-specific lectin concanavalin A (ConA) and the seed lectin from Dioclea grandiflora (DGL) have been investigated using ITC measurements (8, 17–19). A large increase in the $\Delta H$ value of 3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranose, which is present in the "core" region of all asparagine-linked carbohydrates, relative to that of methyl-α-D-mannopyranoside (Me-αMan), for both lectins suggested extended binding sites in both proteins for the trimannoside (19). Importantly, these results were confirmed by x-ray crystallographic studies of ConA (20) and DGL (21). Thus, ITC measurements can provide thermodynamic as well as structural information on carbohydrate-lectin interactions. However, to date ITC measurements have not extensively been applied to the study of multivalent carbohydrate-lectin interactions.

In the present study, we report ITC measurements of the binding of synthetic multivalent carbohydrates to ConA and DGL. Synthetic dimeric analogs of α-D-mannopyranoside (Figs. 1 and 2) and di-, tri-, and tetrameric analogs of 3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (Figs. 3 and 4) were used in the study. The results show that ITC can be used to determine the functional valence of multivalent carbohydrates for ConA and DGL, and the thermodynamic basis for the enhanced affinities of the multivalent sugars.

MATERIALS AND METHODS

DGL was isolated from D. grandiflora seeds obtained from North Eastern Brazil (Albano Ferreira Martins Ltd., Rua Teodoro Souto, 718 (cambuci) 01634-000 Sao Paulo, Brazil) as described previously (22). The concentration of DGL was determined spectrophotometrically at 280 nm using $A_{280}^\text{nm} = 12.0$ at pH 5.2 and expressed in terms of monomer ($M_r = 25,000$) (22). ConA was prepared from Jack bean (Canavalia ensiformis) seeds (Sigma) according to the method of Agrawal and Goldstein (23). The concentration of ConA was determined spectrophotometrically at 280 nm using $A_{280}^\text{nm} = 12.4$ at pH 5.2 (24) and expressed in terms of monomer ($M_r = 25, 600$).

Me-αMan, p-aminophenyl-α-D-mannopyranoside, p-nitrophenyl-α-D-mannopyranoside and methyl-3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (Tri-Man), were purchased from Sigma. The synthesis of carbohydrate analogs 1 and 2 has previously been reported (25), as have analogs 4, 5, and 6 (26). The synthesis of analogs 7, 8, 9, and 10 has also been previously described (27). Synthesis of 3 will be reported elsewhere. The concentrations of the carbohydrates were determined by modification of the Dubois phenol/sulfuric acid method using appropriate monosaccharides as standards (28).

Isothermal Titration Microcalorimetry—ITC experiments were performed using a model MCS instrument from Microcal, Inc. (Northampton, MA). Injections of 4 μl of carbohydrate solution were added from a computer controlled 250- or 100-μl microsyringe at an interval of 4 min into the sample solution of lectin (cell volume = 1.358 ml) with stirring at 350 rpm. An example of an ITC experiment is shown in Fig. 5 for tetraivalent trimannoside 10 with ConA at 27 °C. Control experiments performed by making identical injections of saccharide into a cell containing buffer without protein showed insignificant heats of dilution. The concentration of lectins were 0.015–0.14 mM and the sugars were 0.16–4.0 mM, respectively. Titrations were done at pH 5.0–5.2 and at NaCl concentrations from 0 to 0.15 M. The experimental data were

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1 The abbreviations used are: ITC, isothermal titration microcalorimetry; ConA, lectin from Jack bean (Canavalia ensiformis); DGL, seed lectin from D. grandiflora; Me-αMan, methyl-α-D-mannopyranoside, Tri-Man, methyl-3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside; all sugars are in the b-configuration.
fitted to a theoretical titration curve using software supplied by Microcal, with $\Delta H$ (enthalpy change in kcal mol$^{-1}$), $K_n$ (association constant in M$^{-1}$) and $n$ (number of binding sites per monomer), as adjustable parameters. The quantity $c = K_n M_t(0)$, where $M_t(0)$ is the initial macromolecule concentration, is of importance in titration microcalorimetry (29). All experiments were performed with $c$ values $1 < c < 200$. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2’-monophosphate supplied by the manufacturer. Thermodynamic parameters were calculated from the equation,

$$
\Delta G = \Delta H - T\Delta S = -RT \ln K_n
$$

(Eq. 1)

where $\Delta G$, $\Delta H$, and $\Delta S$ are the changes in free energy, enthalpy, and entropy of binding. $T$ is the absolute temperature and $R = 1.98$ cal mol$^{-1}$ K$^{-1}$.

RESULTS AND DISCUSSION

Previous ITC studies have shown that values of $n$, the number of binding sites per monomer of protein, for simple mono- and oligosaccharides binding to ConA (8) and DGL (19) are close to 1.0 for both lectins. For example, the values of $n$ are 1.0 for the binding of both Me-$\alpha$Man and Tri-Man to ConA (Table I) and DGL (Table II). These values agree with the x-ray crystal data for the number of binding sites on each monomer of ConA for Me-$\alpha$Man (30) and 3,6-di-$\alpha$-mannopyranosyl-$\alpha$-mannopyranoside (20), and of DGL for Tri-Man (21).

However, $n$ values less than 1.0 have been reported for the binding of multivalent carbohydrates to ConA. An $n$ value of 0.59 was reported by Brewer and co-workers (8) for the binding of a Man9 oligomannose glycopeptide to acetyl-ConA, which is a dimer at pH 7.0 (31). An $n$ value of 0.64 was also reported for the binding of a Man5 oligomannose to acetyl-ConA (8). Both of these carbohydrates were previously shown to be divalent for ConA (32), with one binding site on the $\alpha(1\rightarrow3)$ arm of the core Man residue, and the second site on the $\alpha(1\rightarrow6)$ arm of the core Man residue. Both carbohydrates are also known to bind and precipitate tetrameric ConA at pH 7.2 (32). In addition, the Man5 oligosaccharide has also been shown to bind and precipitate with tetrameric DGL at pH 7.2 (33).

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The dimer-tetramer equilibrium of ConA has been reported to be sensitive to pH, so that the tetramer exist at pH 7 while the dimer is reported to predominate at pH 5.0 (35, 36). Acetylation or succinylation of the amino groups of ConA is reported to produce dimeric derivatives of ConA (31). We have observed that at concentrations between 15 and 140 mM ConA and DGL, both lectins at room temperature are dimers at pH 5.0 and low salt concentrations (less than 0.15 M NaCl)\(^2\). Under these con-

\(^2\)T. K. Dam, R. Roy, S. K. Das, S. Oscarson, and C. F. Brewer, unpublished results.
ditions, precipitation of both lectins by analogs 1-10 either is not observed or considerably slowed. Dimeric ConA and DGL do not precipitate with divalent sugars 1-7, since they apparently form linear cross-linked complexes with the sugars that are soluble. On the other hand, analogs 9 and 10 are potentially tri- and tetravalent, respectively, and capable of forming two- and three-dimensional cross-linked complexes even with dimeric ConA and DGL. However, using concentrations of \(-15 \mu M\) ConA and DGL at pH 5.0 and low salt concentration, complexes of 9 and 10 with both lectins are observed to be stable during the \(-1\) h required for the ITC experiments. Time-dependent precipitation of the complex occurred after the run. Thus, the ITC experiments reported in the present study were performed under conditions in which cross-linked complexes between the sugars and lectins were soluble.

**ITC Measurements of \(K_a\) and \(n\) Values of the Multivalent Carbohydrates**—Tables I and II show data for the binding of 1-10 to ConA and DGL, respectively. For comparison, data for the binding of Me-\(\alpha\)-Man, \(p\)-aminophenyl-\(\alpha\)-D-mannopyranoside, and Tri-Man, which represent the monovalent binding epitopes of the respective analogs, are also shown. As can be seen, dimeric analogs 1-6 with terminal \(\alpha\)-D-mannopyranoside residues possess much lower affinities for the two lectins than Tri-Man and dimeric analogs 7 and 8 which possess two trimannoside moieties. Tri- and tetraantennary analogs 9 and 10 have even higher affinities for both lectins. These results are
FIG. 5. ITC profile of ConA (0.020 mg) with analog 10 (0.24 mg) at 27 °C. Top, data obtained for 30 automatic injections, 4 µl each, of 10; bottom, the integrated curve showing experimental points (I) and the best fit (—). The buffer was 0.1 M sodium acetate at pH 5.2 with 0.1 M NaCl, 5 mM each of CaCl₂ and MnCl₂.

TABLE I
Thermodynamic binding parameters for concanavalin A with multivalent sugars at 27 °C

| Sugar        | $K_a \times 10^{-4}$ | $-\Delta G^\circ$ | $-\Delta H^\circ$ | $-\Delta S^\circ$ | $n^*$ |
|--------------|----------------------|-------------------|-------------------|-------------------|-------|
| Me-α-Man⁵    | 12.2                 | 5.6               | 8.4               | 2.8               | 1.0   |
| 1            | 2.2                  | 6.0               | 12.7              | 6.7               | 0.59  |
| 2            | 2.5                  | 6.0               | 11.4              | 5.4               | 0.67  |
| 3            | 5.3                  | 6.5               | 15.2              | 8.7               | 0.54  |
| p-APMan⁴     | 1.3                  | 5.6               | 7.8               | 2.2               | 1.0   |
| 4            | 4.7                  | 6.4               | 17.0              | 10.6              | 0.52  |
| 5            | 5.4                  | 6.5               | 16.6              | 10.1              | 0.52  |
| 6            | 6.8                  | 6.6               | 14.3              | 7.7               | 0.60  |
| TriMan⁵      | 39.6                 | 7.6               | 14.7              | 7.1               | 1.0   |
| 7            | 286.8                | 8.8               | 23.1              | 14.3              | 0.53  |
| 8            | 250.0                | 8.7               | 26.2              | 17.5              | 0.53  |
| 9            | 420.0                | 9.0               | 29.0              | 20.0              | 0.51  |
| 10           | 1350.0               | 9.7               | 53.0              | 43.3              | 0.26  |

† Errors in $K_a$ range from 1 to 7%.
‡ Errors in $\Delta G$ are less than 1%.
§ Errors in $\Delta H$ are 1–4%.
¶ Errors in $\Delta S$ are 1–7%.
‖ Errors in $n$ are less than 2%.
* Methyl-α-D-mannopyranoside.
* p-Aminophenyl-α-D-mannopyranoside.
* Methyl-3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside.

consistent with the reported ~40-fold lower affinity of Me-α-Man for Con A relative to that of Tri-M (8), and the nearly 250-fold lower affinity of Me-α-Man for DGL relative to that of the trimannoside (19). These differences are due to the extended binding sites of both lectins for the trimannoside as shown in their x-ray crystal structures (20, 21). Thus, analogs of the trimannoside (7–10) have intrinsically higher affinities for the two lectins relative to analogs of α-D-mannopyranoside (1–6).

Analogs 1–3 have 2–4-fold higher $K_a$ values for Con A relative to Me-α-Man (Table I), and 4–20-fold higher affinities for DGL (Table II). Importantly, the $n$ values for 1–3 binding to Con A are considerably lower than 1.0, with values of 0.59, 0.67, and 0.54, respectively. The $n$ values for 1–3 binding to DGL are 0.61, 0.70, and 0.56, respectively. The lowest $n$ values are for 3 which possesses the highest $K_a$ values of the three analogs for each lectin. Since the theoretical value of $n$ for divalent binding of a carbohydrate to Con A is $n = 1/2 = 0.5$, the $n$ values for 3 of 0.54 and 0.56 for Con A and DGL indicate that it predominately exists in divalent cross-linked complexes with each lectin. The somewhat higher values of $n$ for 1 and 2 indicate a lower percentage of cross-linked molecules in solution relative to that for 3. Thus, the flexible hexyl chain in 3 appears to favor slightly higher affinity binding and the formation of a higher percentage of cross-linked complexes of the analog with the two lectins, relative to the more rigid acetylenic spacer groups in 1 and 2.

Analogs 4–6 show 4–5-fold higher $K_a$ values for Con A (Table I) relative to Me-α-Man, and 2–5-fold higher $K_a$ values for DGL (Table II) relative to Me-α-Man. The $n$ values for 4–6 binding to Con A are also lower than 1.0, with values of 0.52, 0.52, and 0.60, respectively. The $n$ values for 4–6 binding to DGL are 0.60, 0.57, and 0.70, respectively. The somewhat higher $n$ values for DGL relative to Con A appear to correlate with the somewhat higher $K_a$ values of the analogs for Con A relative to DGL. For both lectins the highest $K_a$ values are for 6 which possesses the longest spacer group in this series. However, the lowest $n$ values for Con A are for 4 (0.52) and 5 (0.52), while the lowest $n$ value for DGL is for 5 (0.57). These results indicate that 4 and 5 exists predominately as divalent cross-linked complexes with Con A, and that 5 exists as the highest percentage of cross-linked complex with DGL among the three analogs. Hence, the shorter chain analogs, 4 and 5, yielded the highest percentage of cross-linked complexes with Con A. Analog 4 and 5, with nearly the same $n$ values for DGL, also gave the highest percentages of cross-linked complexes.

| Sugar        | $K_a \times 10^{-4}$ | $-\Delta G^\circ$ | $-\Delta H^\circ$ | $-\Delta S^\circ$ | $n^*$ |
|--------------|----------------------|-------------------|-------------------|-------------------|-------|
| Me-α-Man⁵    | 0.46                 | 4.9               | 8.2               | 3.3               | 1.0   |
| 1            | 2.0                  | 5.9               | 11.2              | 5.3               | 0.61  |
| 2            | 1.6                  | 5.7               | 11.0              | 5.3               | 0.70  |
| 3            | 10.6                 | 6.8               | 14.8              | 8.0               | 0.56  |
| p-APMan⁴     | 0.7                  | 5.2               | 7.3               | 2.1               | 1.0   |
| 4            | 1.6                  | 5.7               | 14.3              | 8.6               | 0.60  |
| 5            | 2.5                  | 6.0               | 14.8              | 8.8               | 0.57  |
| 6            | 3.7                  | 6.2               | 12.0              | 5.8               | 0.70  |
| TriMan⁵      | 122.0                | 8.3               | 16.2              | 7.9               | 1.0   |
| 7            | 600.0                | 9.3               | 24.7              | 15.4              | 0.50  |
| 8            | 590.0                | 9.2               | 27.5              | 18.3              | 0.51  |
| 9            | 1000.0               | 9.6               | 32.2              | 22.6              | 0.40  |
| 10           | 6500.0               | 10.6              | 58.7              | 48.1              | 0.25  |

† Errors in $K_a$ are 7–10%.
‡ Errors in $\Delta G$ are 1%.
§ Errors in $\Delta H$ are 1–7%.
¶ Errors in $\Delta S$ are 5–13% for analogs 1–6 and 1–2% for analogs 7–10.
‖ Errors in $n$ are 2–7% for analogs 1–6 and less than 1% for analogs 7–10.
* Methyl-α-D-mannopyranoside.
* p-Aminophenyl-α-D-mannopyranoside.
* Methyl-3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside.
The ITC data in Table I for analogs 7 and 8 show 7- and 6-fold higher $K_a$ values for ConA, respectively, relative to monovalent Tri-Man. Both analogs show 5-fold greater $K_a$ values for DGL (Table II) relative to Tri-Man. Hence, the absence or presence of the methyl ester on the benzoic acid moieties of the central phenyl ring has little effect on the affinity of these two analogs. The $n$ values for 7 and 8 binding to ConA are 0.53 for both analogs, and for binding to DGL are 0.50 and 0.51, respectively. These values are close to the theoretical value of 0.50 for binding of a divalent ligand to the two lectins. This indicates that 7 and 8 are primarily involved in divalent cross-linking interactions with the lectins.

Analog 9 and 10 show 11- and 35-fold higher $K_a$ values for ConA, respectively, and 8- and 53-fold higher $K_a$ values for DGL relative to the trimannoside. Thus, ConA and DGL exhibit substantially higher affinities for tetraantennary analog 10 relative to the corresponding monovalent ligand, Tri-Man. Importantly, $n$ values for 10 binding to ConA and DGL are 0.26 and 0.25, respectively. These values are consistent with the theoretical value of a tetravalent carbohydrate binding to either lectin which is $n = 1.0 / 0.25$. This indicates that all four trimannoside moieties of 10 bind to ConA and DGL. The fact that both lectins precipitate with 10 after the ITC experiments suggests that each molecule of 10 cross-links four lectin molecules, respectively, and forms a two- or three-dimensional cross-linked complex which is generally required for precipitation of the complex (12).

The Functional Valency of 9 Differs from Its Structural Valency for ConA and DGL—The above results show that the values of $n$ for binding of 7, 8, and 10 to ConA and DGL are inversely proportional to the number of binding epitopes in the analogs. However, the value of $n$ for the binding of 9 to ConA is 0.51 instead of the predicted value of 0.33 based on the structural valency of the triantennary analog which possesses three trimannoside residues. Thus, 9 is functionally bivalent in binding to ConA as indicated by its ITC derived value of $n$. On the other hand, the $n$ value for 9 binding to DGL is 0.40 which is less than 0.50 for divalent binding but higher than 0.33 for trivalent binding. The $n$ value of 0.40 suggest that greater than half of the molecules of 9 are involved in trivalent binding to DGL and less than half are involved in bivalent binding to the lectin. Thus, the ITC results show that analog 9 is divalent in binding to ConA, and divalent and trivalent in binding to DGL. The reason for this difference in functional valency of 9 for the two lectins is unknown, but there has been a report of differences in the binding of divalent C-glycosides to ConA and DGL (34). This difference in functional valency of 9 for ConA and DGL is also interesting in light of the similarity in overall structures of the two lectins (21). The results for 9 also indicate the importance of ITC measurements in determining the functional valency of a multivalent carbohydrate for specific lectins, which may differ from the structural valence of the carbohydrate.

$\Delta H$ Increases in Direct Proportion to the Valency of High Affinity Carbohydrate Analogs—The present study indicates that multivalent analogs with relatively high affinities possess $n$ values for ConA and DGL that reflect the functional and, most often, nominal structural valency of the ligands. Present results also show that for higher affinity multivalent analogs, the observed value of $\Delta H$ per mole of the analog is approximately the sum of the $\Delta H$ values of the individual epitopes. Similar observations have been made for the binding of a trivalent system of receptor and ligand derived from vancomycin and D-Ala-D-Ala (37). Examples in the present study include divalent analogs 4 and 5 binding to ConA ($n = 0.52$ for both) (Table I), which have $\Delta H$ values of $-17.0$ kcal mol$^{-1}$ and $-16.6$ kcal mol$^{-1}$, respectively. Assuming that $p$-aminophenyl-$\alpha$-$D$-mannopyranoside approximates the binding thermodynamics of the monovalent epitopes in 4 and 5, the $\Delta H$ value of $p$-aminophenyl-$\alpha$-$D$-mannopyranoside is $-7.8$ kcal/mol which is nearly half that of 4 and 5 (Table I).

Divalent analog 3 possesses an $n$ value of 0.54 for binding to ConA and a $\Delta H$ of $-15.2$ kcal/mol. Me-o-Man ($n = 1.0$) as the corresponding monovalent epitope binding to ConA has a $\Delta H$ of $-8.4$ kcal/mol, which is nearly half that of 3. Analogs 1 and 2 with higher $n$ values than that of 3 show $\Delta H$ values between that of Me-o-Man and 3, indicative of partial divalent binding of the former two analogs to ConA. Divalent analogs 7 ($n = 0.53$ to ConA, and 0.50 for DGL) and 8 ($n = 0.53$ to ConA, and 0.51 for DGL) are also relatively high affinity divalent carbohydrates. 7 and 8 have $\Delta H$ values of $-23.1$ and $-26.2$ kcal/mol, respectively, for binding to ConA which are less than twice the $\Delta H$ value of $-14.7$ kcal/mol for Tri-Man (Table I). The same is true for 7 and 8 binding to DGL (Table II). The $\Delta H$ value for tetravalent analog 10 binding to ConA is $-53.0$ kcal/mol, which is approximately four times as great as the $\Delta H$ value of the free trimannoside ($-14.7$ kcal/mol). The $\Delta H$ value for 10 binding to DGL is $-58.7$ kcal/mol, which is also nearly four times as great as the $\Delta H$ value of the free trimannoside ($-16.2$ kcal/mol). The $\Delta H$ values of analog 9 are more complicated since the analog appears to be close to bivalent for ConA ($n = 0.51$), and partially trivalent for DGL ($n = 0.40$). The above results, therefore, provide evidence that the $\Delta H$ values of relatively high affinity multivalent carbohydrates binding to ConA and DGL are approximately the sum of the $\Delta H$ values of the individual binding epitopes in the analogs.

$T\Delta S$ Does Not Directly Increase in Proportion to the Valency of High Affinity Carbohydrate Analogs—The $K_a$ values for analogs 1-10 binding to ConA and DGL in Tables I and II, respectively, are greater than those of the corresponding monovalent carbohydrates. The largest increases in $K_a$ values are for tetravalent analog 10 binding to ConA (35-fold) and DGL (53-fold). However, these enhancements are small compared with the very large increases in binding that occur when a multivalent ligand binds to a single molecule possessing multivalent binding sites. Examples of the latter are binding of a triantennary complex carbohydrate to the hepatic asialoglycoprotein receptor which has a $10^{-10}$ M inhibition constant relative to the $10^{-13}$ M inhibition constant of the corresponding monovalent oligosaccharide (15). Even more dramatic is the increase in affinity to $10^{17}$ M$^{-1}$ of a trivalent derivative of vancomycin binding to a trivalent derivative of D-Ala-D-Ala in which the affinity of the corresponding monovalent analogues is $10^6$ M$^{-1}$ (37). In the latter study, thermodynamic measurements showed that both $\Delta H$ and $T\Delta S$ scaled proportionally to the number of binding epitopes in the molecules. These thermodynamic findings are characteristics of the binding of a multivalent ligand to a single multivalent receptor molecule.

However, in the present study, $\Delta H$ scales proportionally to the number of binding epitopes in the higher affinity multivalent carbohydrates in Tables I and II (compare 7, 8, and 10), but $T\Delta S$ does not. Instead, $T\Delta S$ is much more negative than if it proportionally scaled to the number epitopes in the carbohydrates. For example, 10 contains four trimannosyl binding epitopes, and to a first approximation, the $\Delta H$ value for ConA of $-53$ kcal/mol is four times the $\Delta H$ of $-14.7$ kcal/mol for Tri-Man (Table I). However, the observed $T\Delta S$ value for 10 is $-43.3$ kcal/mol, not $-28.4$ kcal/mol if it scaled with the $T\Delta S$ value of $-7.1$ kcal/mol for Tri-Man (Table I). The resulting $\Delta G$ value of 10 would also be much greater if $T\Delta S$ scaled with valency since the difference between $\Delta H$ and $T\Delta S$ would be greater. However, the observed $\Delta G$ value(s) for 10 are much smaller. The
same is true for the other multivalent carbohydrates in Tables I and II. Hence, the finding in the present study that ΔH values scale but not ΔS values is characteristic of the binding of a multivalent ligand to separate, unconnected receptor molecules. In the present case, binding of a single molecule of 10 to four separate ConA or DGL molecules.

Relative Contributions of ΔH and ΔS to the Increased Affinities of Multivalent Carbohydrates 1–10—The enhancements in affinities of 1–10 to ConA and DGL, relative to their monovalent analogs, are not due to their binding to a single tetrameric lectin molecule in each case (above results). For example, the enhancements in affinity of 10 for ConA (35-fold) and DGL (53-fold) relative to Tri-Man are not due to 10 binding to single molecules of ConA or DGL that possess four binding sites. Rather, the increases in affinity of 10 are due to binding of four separate lectin molecules to 10. This means that the observed $K_v$ value for 10 is actually the average of the four microscopic $K_v$ values at each of its four epitopes, since each epitope is involved in binding to a separate lectin molecule. It therefore follows that if $\Delta H$ is constant at each epitope, as determined above, and is approximately the same as that of Tri-Man, then increases in the overall microscopic $K_v$ values at the four epitopes require more favorable $\Delta S$ contributions than that of Tri-Man. A detailed analysis of these microscopic equilibria parameters will be presented elsewhere. However, an estimate of the favorable macroscopic $\Delta S$ contribution to the enhanced affinity of 10 relative to Tri-Man can be obtained by considering that the differences in $\Delta G$ values between 10 and Tri-Man for the two lectins is the enhancement in $\Delta S$ values. The difference in $\Delta G$ values for ConA and DGL binding 10 are 2.1 and 2.3 kcal/mol, respectively, which can be considered as the favorable contribution to binding of $\Delta S$ for 10 relative to Tri-Man to the two lectins.

In terms of a physical model for the enhanced affinities of the multivalent carbohydrates in Tables I and II, increased favorable $\Delta S$ contributions for binding of the analogs can be analyzed in kinetic terms. Since $K_v = k_f/k_{-1}$, the ratio of the forward and reverse rate constants for binding, the enhanced affinity constants of the multivalent analogs can be due to either an increase in $k_f$ or a decrease in $k_{-1}$, or both. Since each analog has microscopic $K_v$ values at each epitope, it follows that an increase in the corresponding microscopic rate constant $k_f$ or decrease in the microscopic off-rate, $k_{-1}$, will enhance binding at each epitope and hence the overall microscopic $K_v$ value. These changes in microscopic rate constants associated with the more favorable $\Delta S$ contributions at each epitope are associated with physically “clustering” binding epitopes in a molecule. More details of this phenomena will be presented elsewhere.

Entropy-Enthalpy Compensation Plots—Enthalpy-entropy plots ($\Delta H$ versus $\Delta S$) of the data in Tables I and II for analogs 1–10 are shown in Fig. 6A. Remarkably, straight line fits of the data for both lectins are observed with slopes of 1.11 for ConA and 1.13 for DGL (correlation coefficients of 0.99 for both). For comparison, Fig. 6B shows enthalpy-entropy plots for binding of a series of monovalent carbohydrates to ConA and DGL which are listed in the figure legend. All of the monovalent sugars used in the two plots in Fig. 6B bind to ConA and DGL with n values close to 1.0 (8, 38). The slopes of their $\Delta H$ versus $\Delta S$ plots are 1.52 for ConA and 1.55 for DGL, with correlation coefficients of 0.98 and 0.99, respectively. The plots for both lectins are similar as previously noted (8, 38). The slopes for 1–10 binding to ConA (1.11) and DGL (1.13) are less than those for the monovalent carbohydrates because the magnitude of the $\Delta H$ and $\Delta S$ values for 1–10 are larger relative to their $\Delta G$ values as compared with the monovalent sugars which possess smaller $\Delta H$ and $\Delta S$ values relative to their $\Delta G$ values.

Summary—The present ITC study indicates that the value of n, the number of binding sites on each monomer of ConA or DGL, is inversely proportional to the number of binding epitopes in relatively high affinity multivalent carbohydrates. The value of n for these carbohydrates can therefore determine the functional valency of a sugar as opposed to its structural valency, as observed for binding of 9 to ConA and DGL. The observed $\Delta H$ values of relatively high affinity multivalent carbohydrates are approximately the sum of the $\Delta H$ values of the individual epitopes in the molecules. Evidence indicates that the greater $K_v$ values of multivalent carbohydrate 1–10 to ConA and DGL, relative to monovalent analogs, are due to more positive contributions in their $\Delta S$ terms. A thermodynamic model for distinguishing binding of a multivalent ligand to a single receptor with multiple, equal subsites (37) versus binding of a multivalent ligand to separate receptor molecules is presented.

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