Thermodynamic Binding Parameters of Individual Epitopes of Multivalent Carbohydrates to Concanavalin A As Determined by "Reverse" Isothermal Titration Microcalorimetry†

Tarun K. Dam,‡ René Roy,§ Daniel Pagé,§ and C. Fred Brewer*,‡

Departments of Molecular Pharmacology and of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461, and Department of Chemistry, Center for Research in Biopharmaceuticals, University of Ottawa, Ottawa, Canada

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ABSTRACT: The preceding paper [Dam, T. K., Roy, R., Pagé, D., and Brewer, C. F. (2002) *Biochemistry 41*, 1351−1358] demonstrated that Hill plots of isothermal titration microcalorimetry (ITC) data for the binding of di-, tri-, and tetravalent carbohydrate analogues possessing terminal 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside residues to the lectin concanavalin A (ConA) show increasing negative cooperativity upon binding of the analogues to the lectin. The present study demonstrates "reverse" ITC experiments in which the lectin is titrated into solutions of di- and trivalent analogues. The results provide direct determinations of the thermodynamics of binding of ConA to the individual epitopes of the two multivalent analogues. The *n* values (number of binding sites per carbohydrate molecule) derived from reverse ITC demonstrate two functional binding epitopes on both the di- and trivalent analogues, confirming previous "normal" ITC results with the two carbohydrates [Dam, T. K., Roy, R., Das, S. K., Oscarson, S., and Brewer, C. F. (2000) *J. Biol. Chem. 275*, 14223−14230]. The reverse ITC measurements show an 18-fold greater microscopic affinity constant of ConA for the first epitope of the divalent analogue versus its second epitope and a 53-fold greater microscopic affinity constant of ConA binding to the first epitope of the trivalent analogue versus its second epitope. The data also demonstrate that the microscopic enthalpies of binding of the two epitopes on the di- and trivalent analogues are essentially the same and that differences in the microscopic K_a values of the epitopes are due to their different microscopic entropies of binding values. These findings are consistent with the increasing negative Hill coefficients of these analogues binding to ConA in the previous paper.

We recently reported isothermal titration microcalorimetry (ITC)[1] data for the binding of di-, tri-, and tetravalent carbohydrate analogues possessing terminal 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside residues to the Man/Glc-specific lectins concanavalin A (ConA) and *Dioclea grandiflora* lectin (DGL) (*1*). In the previous paper in this series (*14*), ITC data for the binding of the di-, tri-, and tetravalent analogues to the two lectins were subjected to Hill plot analysis which detects cooperativity in the binding of ligands to macromolecules (cf. refs *2* and *3*). In nearly all such examples, the cooperativity has been associated with multisubunit interactions in the protein (cf. ref *4*). However, the ITC data in the previous study indicated that the observed negative cooperativity in the binding of the multivalent carbohydrate analogues to the two lectins was due to the multivalency of the sugars and not to the lectins. Furthermore, the increasing negative cooperativity observed in the Hill plots suggested that there was decreasing affinity of the multivalent analogues upon sequential binding of lectin molecules to the respective carbohydrate epitopes of the analogues.

Most ITC experiments including the above studies of the binding of multivalent carbohydrate analogues to ConA and DGL are performed by titrating a ligand (sugar) into a solution of the protein (lectin). The current study presents reverse ITC titration experiments in which the lectin (ConA) is titrated into solutions of the multivalent sugars (the di- and trivalent analogues). The results provide direct determinations of the thermodynamics of binding of the individual epitopes of the two multivalent analogues.

MATERIALS AND METHODS

Trimannoside (**1**) (Figure 1) was obtained from Sigma Chemical Co. The syntheses of the multivalent oligosaccharides **2** and **3** in Figure 1 has been previously described (*5*). The concentrations of carbohydrates were measured by the phenol−sulfuric acid method using an appropriate mixture

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* To whom correspondence should be addressed [tel, (718) 430-2227; fax, (718) 430-8922; e-mail, brewer@aecom.yu.edu].

‡ Albert Einstein College of Medicine.

§ University of Ottawa.

[1] Abbreviations: ConA, lectin from Jack bean (*Canavalia ensiformis*); DGL, seed lectin from *Diocleia grandiflora*; TriMan, methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; ITC, isothermal titration microcalorimetry. All sugars are in the D-configuration.

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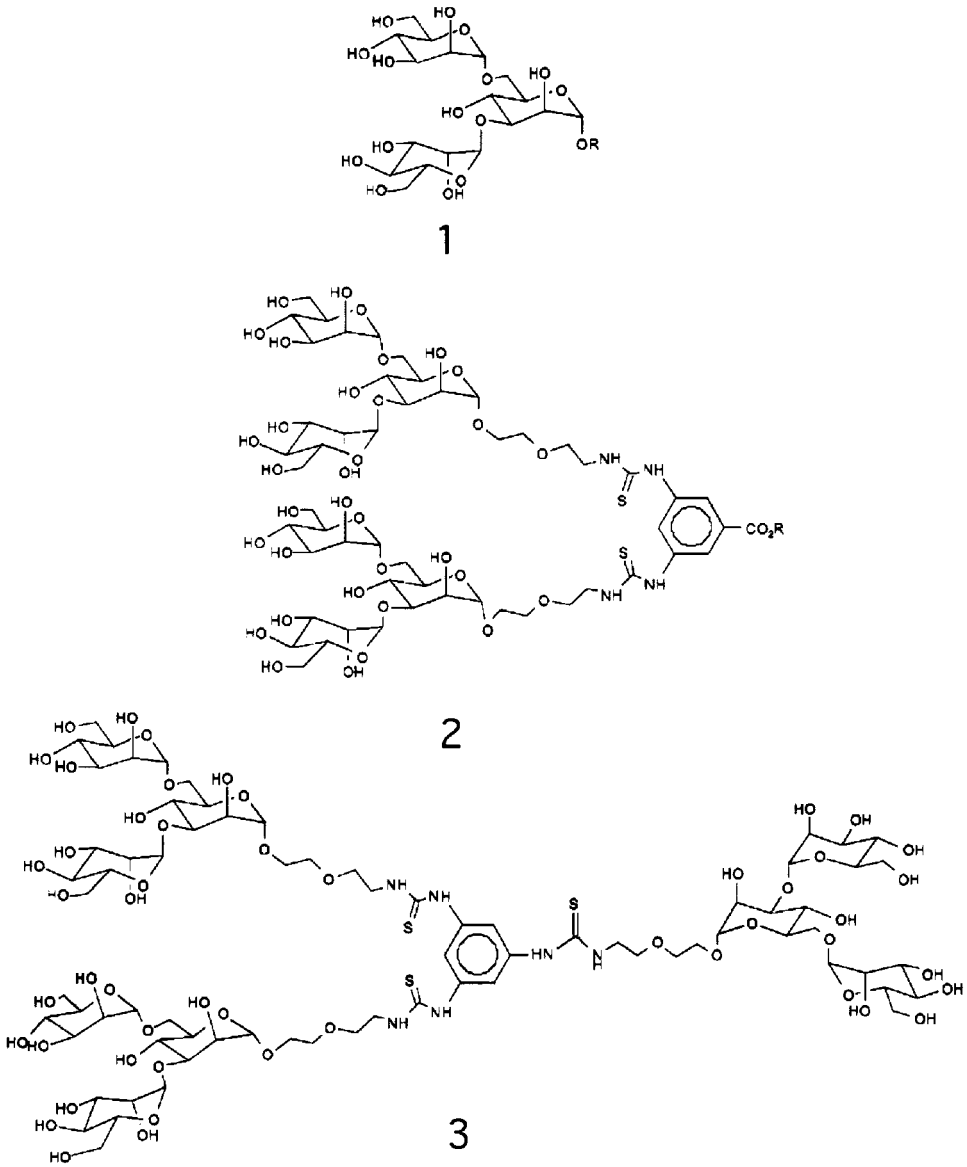

FIGURE 1: Structures of TriMan (**1**) and multivalent carbohydrate analogues **2** and **3**.

of Man, Glc, and Gal as the standard (*6*). Purity of the oligosaccharides was checked by 500 MHz ^{1}H NMR spectroscopy.

Purification of the Lectins. ConA was purchased Sigma Chemical Co. or prepared from Jack bean (*Canavalia ensiformis*) seeds (Sigma Chemical Co.) according to the method of Agrawal and Goldstein (*7*). The concentration of ConA was determined spectrophotometrically at 280 nm using $A^{1\%,1\ cm} = 12.4$ at pH 5.2 (*8*) and expressed in terms of monomer ($M_r = 25600$).

Isothermal Titration Microcalorimetry. ITC experiments were performed using an MCS isothermal titration calorimeter from Microcal, Inc. (Northhampton, MA). In individual titrations, injections of 4 μL of lectin solution were added from the computer-controlled 100 μL microsyringe at an interval of 4 min into the sugar solution (cell volume = 1.358 mL) dissolved in the same buffer as the lectin, while being stirred at 350 rpm. Control experiments performed by making identical injections of ConA into a cell containing buffer with no protein showed insignificant heats of dilution. The experimental data were fitted to a theoretical titration curve using Origin software version 5.0 supplied by Microcal, Inc., with ΔH (enthalpy change in kcal/mol), K_a (association constant in M^{-1}), and n (number of binding sites per molecule in the sample cell) as adjustable parameters. The quantity $c = K_a M_t(0)$, where $M_t(0)$ is the initial macromolecule concentration, is of importance in titration microcalorimetry (*9*). All experiments were performed with c values of $1 < c < 200$. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2′-monophosphate (2′-CMP) supplied by the manufacturer. Thermodynamic parameters were calculated from the equation

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

where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the absolute temperature, and $R = 1.98$ cal mol^{-1} K^{-1}.

RESULTS AND DISCUSSION

ITC measurements have previously been used to investigate binding of multivalent sugars including **2** and **3** (Figure

Table 1: Normal ITC-Derived Thermodynamic Binding Parameters for Concanavalin A with TriMan and Multivalent Sugar Analogues **1**−**3** at 27 °C (from Ref *1*)

	K_a[a] ($M^{-1} \times 10^{-4}$)	$-\Delta G$[b] (kcal/mol)	$-\Delta H$[c] (kcal/mol)	$-T\Delta S$[d] (kcal/mol)	n[e] (no. of sites/ monomer)
TriMan[f] (**1**)	39	7.6	14.7	7.1	1.0
2	250	8.7	26.2	17.5	0.53
3	420	9.0	29.0	20.0	0.51

[a] Errors in K_a range from 1% to 7%. [b] Errors in ΔG are less than 1%. [c] Errors in ΔH are 1−4%. [d] Errors in $T\Delta S$ are 1−7%. [e] Errors in n are less than 2%. [f] Methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside.

1) to ConA and DGL (*1*, *10*). The results for the binding of these two multivalent analogues, along with monovalent TriMan (**1**) (Figure 1), to ConA are shown in Table 1 (*1*). In these studies, the sugars were titrated into a solution of the lectin, as is typical in most ITC experiments. Previous ITC (*11*) and crystallographic studies (*12*, *13*) have established the existence of a single carbohydrate binding site for **1** per subunit of ConA. Therefore, ITC binding data for the multivalent sugars were fitted using a single site model with a lectin concentration based on subunit molecular weight. The resulting data determined the valency of the sugar (n values), association constant (K_a), and enthalpy of binding (ΔH) (*1*). This, in turn, allowed calculation of the binding free energy (ΔG) and entropy of binding ($T\Delta S$) of the analogues (Table 1). Importantly, the observed ΔH values scaled proportionally to the number of binding epitopes in the higher affinity multivalent carbohydrates, but not the $T\Delta S$ values (*1*). Instead, $T\Delta S$ was much more negative than if it proportionally scaled to the number of epitopes in the carbohydrates. The results suggested that the enhanced affinities of **2** and **3** were due to their more favorable entropy of binding terms (*1*).

To provide further insight into the mechanisms of the enhanced affinities of multivalent analogues including **2** and **3**, their raw ITC binding data were subjected to Scatchard and Hill plot analyses (*14*). While **1** showed no evidence of cooperative binding to ConA or DGL, both Scatchard and Hill plots revealed negative cooperativity for the binding of **2** and **3**. In fact, the curvilinear Hill plots for **2** and **3** showed increasing negative cooperativity, suggesting that the affinities of both multivalent analogues decreased with increasing number of bound lectin molecules.

In the present study, "reverse" ITC experiments were performed with ConA titrated into solutions of **2** and **3**. The data were fitted using Origin version 5.0 software from Microcal. The number of binding epitopes on the carbohydrates was selected from the "normal" ITC data previously obtained in Table 1. Importantly, selection of other values for the number of epitopes failed to provide a fit of the data.

As an important control, ConA was titrated into a solution of monovalent TriMan (**1**). The ITC profile is shown in Figure 2, and the data are given in Table 2. The n value for **1** in the reverse experiment (Table 2) is 0.99, which agrees well with the n value of 1.0 for the normal titration (Table 1). The K_a value for **1** in the reverse titration (Table 2) is 6.3×10^5 M^{-1} as compared to 3.9×10^5 M^{-1} for the normal titration (Table 1). The ΔH value for **1** in the reverse experiment is −13.1 kcal/mol (Table 2) as compared to

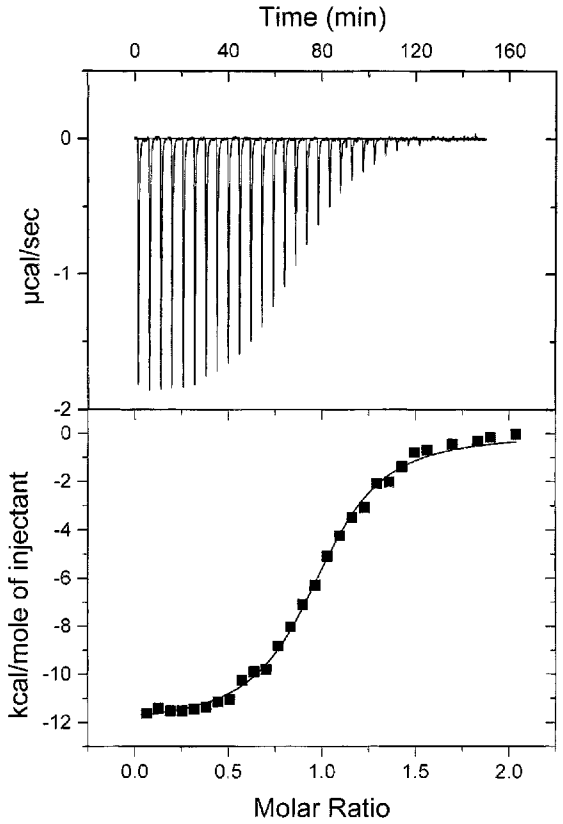

FIGURE 2: Reverse ITC profile of ConA (1 mM) titrated into a solution of TriMan (**1**) (50 μM) at 27 °C. The buffer was 0.1 M sodium acetate and 150 mM sodium chloride, pH 5.2.

−14.7 kcal/mol for the normal titration (Table 1). These two values are close to the error limits of the measurements. Therefore, the results of the reverse ITC of ConA with **1** agree with previously reported normal ITC results.

The reverse ITC profile of ConA with **2** is shown in Figure 3. The data were fitted using a two-site model since data in Table 1 from the normal ITC experiment demonstrated two binding sites for **2**. The data from the reverse ITC experiment are given in Table 2 and show individual thermodynamic data for the two binding epitopes of **2** with ConA. The n value for the first binding epitope ($n1$) of **2** is 0.97 and for the second epitope ($n2$) is 0.94. This indicates that both trimannosyl epitopes of **2** are essentially fully bound to ConA. This supports the conclusion derived from the data in Table 1 for **2** (*1*).

The reverse ITC measurements show two K_a values for **2** in Table 2. K_a1 is 1.6×10^7 M^{-1} and K_a2 is 8.8×10^5 M^{-1}. Hence, the microscopic affinity constant of the first epitope of **2** is 18 times greater than that of the second epitope. The latter value, in turn, is close to the affinity constant of TriMan (**1**) (Tables 1 and 2). These findings indicate that binding of the first molecule of ConA to **2** occurs with much higher affinity than binding of the second molecule of ConA. These results are consistent with the increasing negative cooperativity of **2** binding to ConA and DGL observed in the previous paper (*14*).

Importantly, Table 1 shows that the observed ΔG value for **2** binding to ConA in the normal ITC experiment is −8.7 kcal/mol. Table 2 shows that $\Delta G1$ is −9.8 kcal/mol and $\Delta G2$ is −8.1 kcal/mol for the two epitopes of **2**. The average value of $\Delta G1$ and $\Delta G2$ is −9.0 kcal/mol, which is very similar to

Table 2: Reverse ITC-Derived Thermodynamic Binding Parameters for TriMan (**1**) and Multivalent Sugar Analogues **2** and **3** at 27 °C

	K_a1^a ($M^{-1} \times 10^{-5}$)	ΔK_a2^a ($M^{-1} \times 10^{-5}$)	$-\Delta G1^b$ (kcal/mol)	$-\Delta G2^b$ (kcal/mol)	$n1^c$ (no. of sites)	$n2^c$ (no. of sites)	$\Delta H1^d$ (kcal/mol)	$-\Delta H2^d$ (kcal/mol)	$-T\Delta S1^e$ (kcal/mol)	$-T\Delta S2^e$ (kcal/mol)
TriMan[f] (**1**)	6.2		7.9		0.99		13.1		5.2	
2	161	8.8	9.8	8.1	0.97	0.94	12.5	12.3	2.7	4.2
3	460	8.6	10.4	8.1	1.05	1.09	13.3	12.2	2.9	4.1

[a] Errors in K_a are less than 7%. [b] Errors in ΔG are less than 5%. [c] Errors in n are less than 4%. [d] Errors in ΔH are less than 4%. [d] Errors in $T\Delta S$ are less than 7%. [f] Methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside.

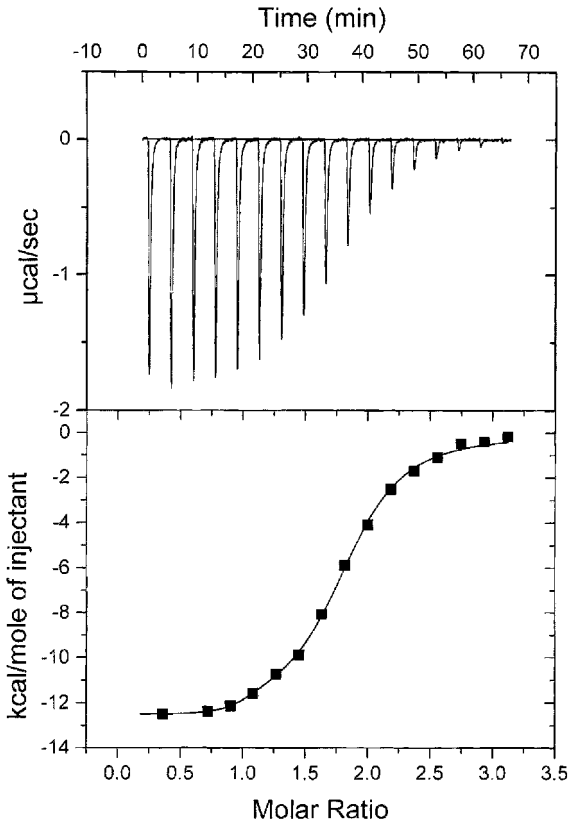

FIGURE 3: Reverse ITC profile of ConA (970 μM) titrated into a solution of carbohydrate analogue **2** (16 μM) at 27 °C. The buffer was 0.1 M sodium acetate and 150 mM sodium chloride, pH 5.2.

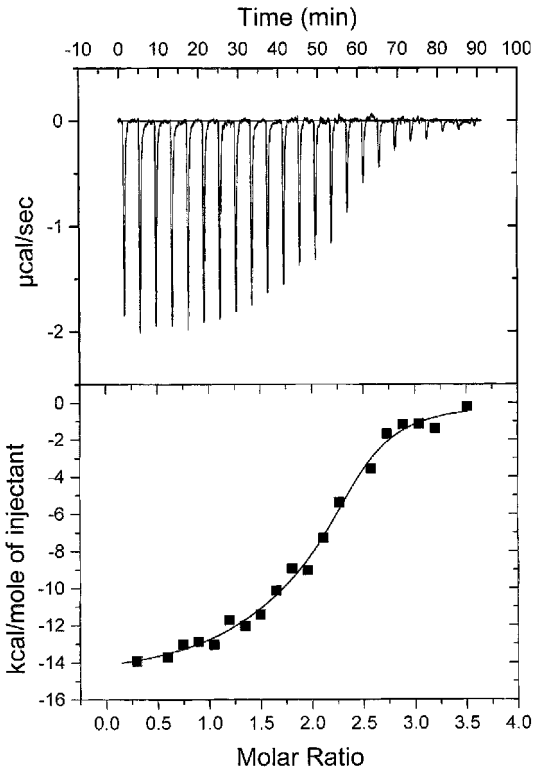

FIGURE 4: Reverse ITC profile of ConA (1 mM) titrated into a solution of carbohydrate analogue **3** (20 μM) at 27 °C. The buffer was 0.1 M sodium acetate and 150 mM sodium chloride, pH 5.2.

−8.7 kcal/mol observed in Table 1. Thus, the observed macroscopic K_a for **2** in Table 1 agrees with the average of the two microscopic K_a values for **2** reported in Table 2. This is an important result confirming the validity of the two microscopic K_a values determined in the reverse ITC measurement.

The microscopic enthalpies of binding (ΔH) of the two sites of **2** are also given in Table 2. $\Delta H1$ is −12.5 kcal/mol and $\Delta H2$ is −12.3 kcal/mol. This indicates that the enthalpy of binding of the two epitopes of **2** is essentially the same. These values are very similar to that for TriMan in Table 2 (−13.1 kcal/mol), indicating constant ΔH values for the trimannosyl moieties of **1** and **2** in binding to ConA. These findings agree with our previous results using normal ITC measurements (Table 1) (*1*).

Knowing the microscopic $\Delta G1$ and $\Delta G2$ values for the two epitopes of **2** along with the microscopic $\Delta H1$ and $\Delta H2$ values (Table 2) allows calculation of the corresponding microscopic entropy of binding ($T\Delta S$) values of the two epitopes. The calculated $T\Delta S1$ value is −2.7 kcal/mol and $T\Delta S2$ is −4.2 kcal/mol. Hence, there is a 1.5 kcal/mol more favorable entropy of binding for the first epitope of **2** relative to the second epitope. These results provide a direct demonstration of the enhanced entropy effects for binding of the first epitope of a divalent carbohydrate to a lectin that leads to overall enhanced affinity of the sugar. These findings also agree with previous conclusions using normal ITC measurements (Table 1) (*1*).

Table 2 shows the data from the reverse ITC experiment of ConA with the triantennary analogue **3**. Our previous ITC study (*1*) showed that the functional valency of **3** with ConA was 2 rather than 3 (Table 1). Hence, **3** is functionally bivalent with ConA as is **2**. Importantly, the reverse ITC data of ConA with **3** could only be fit with a two binding site model ($n = 2$ per molecule of **3**) but not a three site model, consistent with our previous findings (*1*). The values of n in Table 1 are $n1$ is 1.05 and $n2$ is 1.09, which is consistent with two sites of binding on **3** to ConA. The association constants of the two sites are $K_a1 = 4.6 \times 10^{-7}$ M^{-1} and $K_a2 = 8.6 \times 10^{-5}$ M^{-1}. Thus, there is a 53-fold higher affinity of the first binding site of **3** versus its second binding site for ConA. Interestingly, the affinity of the second site on **3** is similar to that of **2**, while the affinity of the first site on **3** is 2.5-fold greater than the first site of **2**. This may relate to the greater structural valency of **3** relative to **2**,

although their functional valencies are the same.

Table 1 shows that the observed ΔG value for **3** binding to ConA in the normal ITC experiment is −9.0 kcal/mol. Table 2 shows that $\Delta G1$ is −10.4 kcal/mol and $\Delta G2$ is −8.1 kcal/mol for the two epitopes of **3** that bind two molecules of ConA. The average of $\Delta G1$ and $\Delta G2$ is −9.3 kcal/mol, which is very similar to the observed ΔG value of −9.0 kcal/mol for **3** in Table 1. Thus, the observed macroscopic K_a for **3** in Table 1 agrees with the average of the two microscopic K_a values for **3** reported in Table 2. This is an important means of confirming the validity of the two microscopic K_a values determined in the reverse ITC measurement.

The enthalpies of binding of the two sites of **3** are also given in Table 2. $\Delta H1$ is −13.3 kcal/mol and $\Delta H2$ is −12.2 kcal/mol. This indicates that the enthalpy of binding of the two epitopes of **3** is nearly the same as found for **2**. These values are also very similar to that for TriMan in Table 2 (−13.1 kcal/mol), indicating constant ΔH values for ConA binding to the trimannoside moieties of TriMan, **1**, and **2**. These findings agree with the conclusion reached in our previous study using normal ITC measurements (*1*) that the two functional binding epitopes of **3** possess essentially equal ΔH values and are additive in the observed ΔH value (Table 1).

Knowing the microscopic $\Delta G1$ and $\Delta G2$ values for the two epitopes of **3** along with the microscopic $\Delta H1$ and $\Delta H2$ values allows calculation of the corresponding microscopic entropy of binding ($T\Delta S$) values of the two sites. The calculated $T\Delta S1$ value is −2.9 kcal/mol and $T\Delta S2$ is −4.1 kcal/mol. Hence, there is a 1.2 kcal/mol more favorable entropy of binding value for the first epitope of **3** relative to the second epitope of **3**. These results also provide a direct demonstration of the enhanced entropy effect for binding of the first epitope of a functionally divalent carbohydrate to a lectin.

Summary. The reverse ITC experiments in the present study provide direct determinations of the microscopic thermodynamic parameters for ConA binding to multivalent analogues **2** and **3**. Table 2 shows that the functional valencies of **2** and **3** are proportional to their respective n values. Thus, both analogues are functionally bivalent with two values of n, $n1$ and $n2$. The results in Table 2 show that the first trimannosyl binding epitope of **2** possesses a microscopic K_a1 value that is 18-fold greater than that of the second trimannosyl epitope. The difference between K_a1 and K_a2 for **3** is 53-fold, which is even greater than that for **2**. Importantly, the microscopic ΔH values, $\Delta H1$ and $\Delta H2$ for **2** and **3**, respectively, are essentially the same as that of TriMan. On the other hand, the entropy of binding ($T\Delta S$) of the first epitope of **2** and **3** to ConA ($T\Delta S1$) is more favorable than binding of the second epitope ($T\Delta S2$) (Table 2), which supports our previous conclusion from normal ITC experiments (*1*). The overall enhancements in affinities of ConA for **2** and **3** relative to **1** are due to favorable entropy effects associated with binding of the first epitopes of these multivalent sugars. The physical mechanism for this enhancement is suggested to be due to kinetic effects in the off-rates of the first bound complex, as discussed in the previous paper in this series (*14*).

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