The thermodynamics of binding of various saccharides to artocarpin, from Artocarpus integrifolia seeds, a homotetrameric lectin (M, 65,000) with one binding site per subunit, was determined by isothermal titration calorimetry measurements at 280 and 293 K. The binding enthalpies, $\Delta H_b$, are the same at both temperatures, and the values range from $-10.94$ to $-47.11$ kJ mol$^{-1}$. The affinities of artocarpin as obtained from isothermal titration calorimetry are in reasonable agreement with the results obtained by enzyme-linked lectin absorbent essay, which is based on the minimum amount of ligand required to inhibit horseradish peroxidase binding to artocarpin in enzyme-linked lectin absorbent assay (Misquith, S., Rani, P. G., and Surolia, A. (1994) J. Biol. Chem. 269, 30393–30401). The interactions are mainly enthalpically driven and exhibit enthalpy-entropy compensation. The order of binding affinity of artocarpin is as follows: mannotriose > Mano3Man > GlcNAc2Man > MeoMan > Mano6Man > Meo2Man > MeoGlc > GIC, i.e., $7.4 \times 2 > 1.4 > 1 > 0.4 > 0.3 > 0.24 > 0.11$. The $\Delta H$ for the interaction of Mano3Man, Mano6Man, and MeoMan are similar and 20 kJ mol$^{-1}$ lower than that of mannotriose. This indicates that, while Mano3Man and Mano6Man interact with the lectin exclusively through their non-reducing end monosaccharide with the subsites specific for the $\alpha$1,3 and $\alpha$1,6 arms, the mannotriose interacts with the lectin simultaneously through all three of its mannospyranosyl residues. This study thus underscores the distinction in the recognition of this common oligosaccharide motif in comparison with that displayed by other lectins with related specificity.

The thermodynamics of binding of various saccharides to artocarpin, from Artocarpus integrifolia seeds, a homotetrameric lectin (M, 65,000) with one binding site per subunit, was determined by isothermal titration calorimetry measurements at 280 and 293 K. The binding enthalpies, $\Delta H_b$, are the same at both temperatures, and the values range from $-10.94$ to $-47.11$ kJ mol$^{-1}$. The affinities of artocarpin as obtained from isothermal titration calorimetry are in reasonable agreement with the results obtained by enzyme-linked lectin absorbent essay, which is based on the minimum amount of ligand required to inhibit horseradish peroxidase binding to artocarpin in enzyme-linked lectin absorbent assay (Misquith, S., Rani, P. G., and Surolia, A. (1994) J. Biol. Chem. 269, 30393–30401). The interactions are mainly enthalpically driven and exhibit enthalpy-entropy compensation. The order of binding affinity of artocarpin is as follows: mannotriose > Mano3Man > GlcNAc2Man > MeoMan > Mano6Man > Meo2Man > MeoGlc > GIC, i.e., $7.4 \times 2 > 1.4 > 1 > 0.4 > 0.3 > 0.24 > 0.11$. The $\Delta H$ for the interaction of Mano3Man, Mano6Man, and MeoMan are similar and 20 kJ mol$^{-1}$ lower than that of mannotriose. This indicates that, while Mano3Man and Mano6Man interact with the lectin exclusively through their non-reducing end monosaccharide with the subsites specific for the $\alpha$1,3 and $\alpha$1,6 arms, the mannotriose interacts with the lectin simultaneously through all three of its mannospyranosyl residues. This study thus underscores the distinction in the recognition of this common oligosaccharide motif in comparison with that displayed by other lectins with related specificity.

Carbohydrates conjugated to proteins and lipids play key structural and functional roles in essentially all living organisms. Recognition of glycoconjugates is an important event in biological systems and is frequently in the form of carbohydrate-protein interactions. The study of how biological molecules interact with one another is fundamental to understanding the chemistry of life. Among the carbohydrate binding proteins, lectins are a group of proteins or glycoproteins which stereospecifically bind carbohydrates (1). N-Linked oligomannose-type carbohydrates constitute one class of oligosaccharide chains associated with cellular glycoproteins. The oligosaccharide chains of many of the glycoproteins appear to function as receptors for lectins in a variety of biological recognition processes, such as fertilization, embryogenesis, cell migration, organ formation, immune defense, protein folding, signal transduction, and apoptosis (2–4). Detailed insights into the specificity of carbohydrate-protein interactions, however, require not only analytical data such as inhibition assays but also thermodynamic data on the complexes. Titration microcalorimetry provides a powerful tool for investigating the binding thermodynamics of macromolecule-ligand interactions and provides important insights into the nature and magnitude of forces involved therein (5–11).

Artocarpin, a mannose-specific lectin isolated from jack fruit seeds is a homotetrameric protein devoid of covalently attached carbohydrates and consists of four isolectins with pI in the range of 5–6.5. Artocarpin is of considerable interest because of its potent and selective mitogenic effect on distinct T and B-cell functions, more so because of its B-cell maturation mitogenic activity (12, 13). Earlier investigations of its carbohydrate binding specificity revealed that among monosaccharides, mannose is preferred over glucose. Among mannooligosaccharides, mannotriose (Mano1–3Mano1–6Man), and mannopentaose were noted as the strongest ligands followed by Mano1–3Mano1–6Man. Substitution of both the $\alpha$1–3 and $\alpha$1–6 linked mannosyl residues of mannotriose by GlcNAc in $\beta$1–2 linkage diminishes their inhibitory potencies (9). In this investigation, isothermal titration calorimetry was employed to determine the thermodynamics of the carbohydrate-artocarpin binding reaction in terms of the binding constant ($K_b$) and change in the free energies, enthalpies, and entropies, i.e., $\Delta G^\circ_b$, $\Delta H^\circ_b$, and $\Delta S^\circ_b$.

**EXPERIMENTAL PROCEDURES**

**Materials and Sample Preparation—Glucose (Glc), Mannose (Man), methyl-α-glucose (MeoGlc), methyl-α-mannopyranoside (MeoMan), N-acetylglucosamine (GlcNAc), and N-acetylmannosamine (ManNAc) were obtained from Sigma. Methyl-β-mannopyranoside (MejMan), Mano1–2Man, Mano1–3Man, Mano1–6Man, Mano1–3Mano1–6Man, GlcNAc2Man5, and mannopentaose were the products of Dextra Laboratories, London. All other reagents and chemicals were of the highest purity available. Artocarpin, purified as previously described (9), was dialyzed overnight against 20 mM phosphate buffer at pH 7.2 containing 150 mM sodium chloride (phosphate-buffered saline) and centrifuged to remove any insoluble material. The concentrations of the protein were determined spectrophotometrically ($A_{280}^{\text{meas}} = 10.8$). Solutions of the carbohydrate were prepared by weight in the dialysate to minimize differences between the protein buffer solution and ligand buffer solution in the isothermal titration calorimetry measurements.

**Titration Calorimetry—**Isothermal calorimetric titration measurements were performed using an OMEGA titration calorimeter from Microcal Inc. as described previously (5, 10). A circulating water bath...
was used to help temperature stabilization. The instrument was allowed to equilibrate overnight. Aliquots (5–10 μl) of the ligand solution (9–36 × protein binding sites) were added from the computer-controlled 250-μl rotating syringe stirring at 395 rpm at an interval of 3 min into the lectin solution (1.34 ml) containing 0.5–8 mM binding sites. The heat of dilution was determined to be negligible in separate titrations of the ligand solution into just the buffer solution. The heat changes accompanying the ligand solutions to the lectin solution were recorded. The heat of dilution was determined to be negligible in separate titrations of the ligand solution into just the buffer solution.

The total heat, $Q_t$, was then fitted via a nonlinear least squares minimization method to the total ligand concentration ($X_t$) using Equation 1 (14),

$$Q_t = n_M \Delta H_V [1 + X/n_M + 1/n_KM_I - [(1 + X/n_M + 1/n_KM_I)]^2 - 4X/n_M]/2 \quad (\text{Eq. 1})$$

where $n$ is the number of binding sites per monomer and $V$ is the cell volume. The expression for the heat released per ith injection, $dQ(i)$, is then given by Equation 2 (15),

$$\Delta Q(i) = \Delta Q(i) + dV_i/2V[Q(i) + Q(i - 1)] - Q(i - 1) \quad (\text{Eq. 2})$$

where $dV_i$ is the volume of titrant added to the solution. The parameters $\Delta G^0$ and $\Delta S$ are calculated from the basic equations of thermodynamics according to Equations 3 and 4,

$$\Delta G^0 = -RT \ln K$$

$$T\Delta S = \Delta H - \Delta G$$

RESULTS

The structures of ligands used in titration calorimetry experiments are depicted in Fig. 1. The results of a typical titration calorimetry measurement, which consisted of addition of 5-μl aliquots of Manα3Man (5 mM) to artocarpin (0.5 mM) in phosphate-buffered saline, pH 7.2, at 281 K are shown in Fig. 2. The results exhibit a monotonic decrease in the exothermic heat of binding till saturation is achieved. A least squares fit of the total heat released as a function of ligand concentration to the identical site model described by Equation 1 is also shown in Fig. 2. The close fit of the data to the identical site model shows that a molecule of ligand binds to each of the four sites of artocarpin independently and with the same binding constant and stoichiometry. The thermodynamic binding parameters of mannopyranosides and mannooligosaccharides to artocarpin are listed in Table I. All experiments were performed in the C value ($C = K_n \times M_I$, where $M_I$ is the macromolecular concentration) range of 1–<C>20, except for Manα4Man where a C value of approximately 0.75 could only be achieved (binding site ~ 8 mM). In most cases the standard deviations in the values of $K_n$ and $\Delta H$ were within 5%, except for ligands such as glucose, mannose, MeGlc, Manα2Man, etc. which have low affinity for artocarpin.

The thermodynamic parameters for the binding of mannose and mannooligosaccharide show that the binding reactions are essentially enthalpically driven with little dependence of the enthalpy on temperatures from 280.1 to 293.5 K. The values for $\Delta H$ range from 47.11 kJ for mannotriose to 9.84 kJ for Manα2Man. The binding constants for different sugars range from 21,200 M$^{-1}$ for mannopentaose to 150 M$^{-1}$ for glucose. The binding reactions for artocarpin to monosaccharides and mannooligosaccharides exhibit enthalpy-entropy compensation as shown in Fig. 3.

Whereas glucose and mannose bind to the lectin, albeit weakly, GlcNAc and ManNAc do not bind at all even at high concentrations (8 mM protein binding sites and 160 mM sugars). Binding of the lectin with Manα4Man is barely detected at the above concentrations, so much so that the thermodynamic parameters for its binding could not be ascertained. MeaMan, Manα3Man, and Manα6Man display similar changes in enthalpies (~28–28 kJ mol$^{-1}$), whereas mannotriose and mannopentaose exhibit 45–47 kJ mol$^{-1}$ of $\Delta H$ values, viz. 20 kJ more favorable change in enthalpy over them.
Trimannoside Binding Specificity of Artocarpin

### Table I

| Sugar          | Temperature | $K_n$  | $-\Delta H_n$ | $-\Delta G_n$ | $-\Delta S_n$ |
|---------------|-------------|--------|---------------|---------------|---------------|
| Glucose       | 283.00      | 150 ± 20 | 16.20 ± 2.00  | 11.94 ± 0.66  | 4.26          |
| Mannose       | 280.40      | 1640 ± 36| 24.59 ± 1.37  | 17.26 ± 0.05  | 7.33          |
| GlcNAc        | 281.00      | NB     |               |               |               |
| MannNAc       | 280.50      | 1378 ± 61| 25.10 ± 1.63  | 17.61 ± 0.11  | 7.49          |
| Methyl-αMan   | 280.50      | 2500 ± 91| 28.12 ± 0.84  | 18.24 ± 0.08  | 9.88          |
| Methyl-βMan   | 281.00      | NB     |               |               |               |
| Methyl-αGlc   | 281.00      | NB     |               |               |               |
| Man1,2Man     | 281.00      | 1864 ± 112| 28.77 ± 0.97  | 18.37 ± 0.15  | 10.40         |
| Man1,3Man     | 281.00      | NB     |               |               |               |
| Mannotriose   | 281.00      | ND     |               |               |               |
| Man1,4Man     | 281.00      | 1864 ± 112| 28.77 ± 0.97  | 18.37 ± 0.15  | 10.40         |
| Mannopentaose | 281.00      | 1864 ± 112| 28.77 ± 0.97  | 18.37 ± 0.15  | 10.40         |

| Sugar          | Temperature | $K_n$  | $-\Delta H_n$ | $-\Delta G_n$ | $-\Delta S_n$ |
|---------------|-------------|--------|---------------|---------------|---------------|
| GlcNAc-Man1   | 283.00      | 150 ± 20 | 16.20 ± 2.00  | 11.94 ± 0.66  | 4.26          |
| MannNAc-Man1  | 280.50      | 1378 ± 61| 25.10 ± 1.63  | 17.61 ± 0.11  | 7.49          |
| Methyl-αMan   | 280.50      | 2500 ± 91| 28.12 ± 0.84  | 18.24 ± 0.08  | 9.88          |
| Methyl-βMan   | 281.00      | NB     |               |               |               |
| Methyl-αGlc   | 281.00      | NB     |               |               |               |
| Man1,2Man     | 281.00      | 1864 ± 112| 28.77 ± 0.97  | 18.37 ± 0.15  | 10.40         |
| Man1,3Man     | 281.00      | NB     |               |               |               |
| Mannotriose   | 281.00      | ND     |               |               |               |
| Man1,4Man     | 281.00      | ND     |               |               |               |
| Mannopentaose | 281.00      | ND     |               |               |               |

![Enthalpy-entropy compensation plot](image)

**Fig. 3.** Enthalpy-entropy compensation plot for the binding of artocarpin to monosaccharides and mannoooligosaccharides at 293 K. The plot shows a linear relationship with a slope of 1.2 with a correlation coefficient to 0.93.

### Discussion

In an earlier study, artocarpin was demonstrated to differ considerably from all the other mannose/glucose binding lectins studied so far. Using an enzyme based assay, it was shown that among mannoooligosaccharides artocarpin binds best to mannotriose and mannopentaose, highlighting the possibility of an extended combining region for these saccharides in the lectin (9). To examine in greater detail the nature of these interactions, the binding of several monosaccharides and mannoooligosaccharides to artocarpin was studied by isothermal titration calorimetry.

In Table I, the values of the binding constants and resultant relative affinities determined by titration calorimetry are listed together with the relative potencies determined by inhibition assays using enzyme-linked lectin absorbent essay (ELLA) (9). The relative affinities for binding of these saccharides are in good agreement with the inhibition studies, except for GlcNAc-Man3 which gives higher value of $K_b$ from that expected from inhibition data (9).

Thermodynamics of the interaction of artocarpin with mannose and mannoooligosaccharides are essentially enthalpically driven and exhibit compensatory changes in $\Delta H_n$ and $\Delta S_n$ as shown in Fig. 3. This compensatory behavior has been attributed to the accompanying changes in solvent reorganization. Reduction in soft vibrational modes restricting mobility of water molecules involved in the binding reaction at the interface between the lectin and the sugar molecules could account for enthalpy-entropy compensation. Displacement of water molecules from the interacting complementary surfaces account for the favorable entropic contribution. However, this gain in entropy from previously imposed motional restriction is offset by loss of a certain amount of enthalpic interactions, which gives rise to a weakening of hydrogen bonds and van der Waals interactions in the combining site. If a group of similar ligands interact by the same mechanism, then a linear relationship between enthalpy and entropy can be expected, with a slope of unity reflecting a complete compensation. Our $\Delta H$ versus $\Delta S$ plots show linearity with slope of 1.2 (correlation coefficient $r = 0.93$) indicating an underlying common mechanism in artocarpin-sugar reaction, unlike ConA where profound deviations have been noted, for example, between the other sugars used in these studies and GlcNAc-Man3 (16, 17). A value of $>1$ for the slope $(s = 1.2)$ of enthalpy-entropy compensation plot indicates the primacy of enthalpic forces in determining the overall free energies of artocarpin-ligand interactions. This indicates that the major driving force for artocarpin-sugar interactions is hydrogen bonding and Van der Waals interactions. Additionally, temperature independence of enthalpies highlights that these reactions for the most part occur with little changes in the conformation of either the ligand or the protein.

A comparison of the thermodynamic parameters for the binding of artocarpin with carbohydrates provide interesting insights on the topography of the combining site of artocarpin. As

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1 The abbreviations used are: ConA, concanavalin A; GNA, Galanthus nicaeensis agglutinin.
the artocarpin-sugar interactions are enthalpically driven and enthalpy-entropy compensation is observed, the differences in binding enthalpies can be explained by considering that the initial interaction of the carbohydrate ligands with the solvent is similar for all the ligands.

The fact that glucose is about 9-fold poorer a ligand, as compared with mannose, and displays $\Delta H_b$, which is 9 kJ mol$^{-1}$ less positive than the latter, emphasizes the importance of an axial orientation for the hydroxyl group at C2 of a hexapyranose for its interaction with the corresponding locus on the combining site of artocarpin. The preference of the lectin for mannopyranoside is also reflected when one considers the binding of MeaMan and MeaGlc, where the former is 6-fold better a ligand and exhibits 7.7 kJ mol$^{-1}$ more favorable binding enthalpy as compared with the latter. The preference for mannopyranoside over glucopyranoside is apparently the highest for artocarpin as compared with the other mannose/glucose-specific lectins such as those from pea, lentil, ConA, or Dioeclea family of lectins (18–20). Me8Man was inactive, highlighting the indispensability of mannopyranoside in a configuration for its recognition by artocarpin. Nonbinding of N-acetylmannosamine suggests that the bulky acetalmo group at C2 in manno configuration leads to a steric hindrance.

It is interesting to compare the binding potencies of various mannooligosaccharides with ConA, Dioeclea lectins, and snowdrop lectin with that of artocarpin. Dioeclea lectins and especially ConA recognize mannobioses in the order Man1–2Man $\gg$ Man1–6Man $>$ Man1–3Man in a site that essentially accommodates a monosaccharide, a fact also borne out by their nearly similar enthalpies of binding (21, 22). The greater affinity of ConA for Man1–2Man has been related to statisti-

![Image](image350x601to512x729)

FIG. 4. Schematic representation of artocarpin extended binding site for mannnotriose. The α1–3-linked mannose occupies the primary binding site, and the α1–6 Man occupies the secondary subsite with both sites being specific to the α1–3 and α1–6 arms of the two oligosaccharides. They together with core mannose constitute the extended combining site of artocarpin.

both sites being specific to the α1,3 and α1,6 arms of the two oligosaccharides. They, together with 3,6-disubstituted mannose residue, constitute the extended combining site of artocarpin. The equivalence of binding constants ($\approx$9000–10,800 M$^{-1}$) and $\Delta H$ ($\approx$46–47 kJ mol$^{-1}$) values for mannnotriose and mannopentaose suggests that artocarpin binds to the trimannosyl moiety located at the α1,6 arm of mannopentaose. Poor binding of GlcNAc2Man3 ($K_b = 2902$ M$^{-1}$), a complex type glycan, and especially its low values of $\Delta H$ (10.94 kJ mol$^{-1}$) underscores the unfavorable consequences of substituting the α1,3- and α1,6-linked mannoseyl residues at their C2-positions for interactions with artocarpin. In this respect, artocarpin differs strikingly from ConA where this structure binds better than mannnotriose (17). More so, its binding mechanism also differs significantly from those of mannooligosaccharides, whereas for artocarpin its location on the enthalpy-entropy compensation plot attests to its mechanism of interaction akin to the other ligands used (16).

At a superficial level, the combining site of artocarpin would appear similar to that of GNA. Nevertheless a detailed examination of thermodynamic parameters reveals that for GNA the binding of Man1–3Man and mannnotriose are accompanied by values of $\Delta H$ that differ very little from each other, i.e. 12.97 and 16.32 kJ mol$^{-1}$, respectively (27). For artocarpin the situation is different. The order of binding affinity is as follows: mannnotriose $>$ Man3Man $>$ GlcNAc2Man3 $>$ MeaMan $>$ ManosMan $>$ Man6Man $>$ Manol2Man $>$ MeaGlc $>$ Glc, i.e. 7.4–26–1.4–1.7–0.4–0.3–0.24–0.11. Moreover, the $\Delta H_b$ values for the binding of MeaMan, ManosMan, and Manol6Man are close to each other (27–29 kJ mol$^{-1}$), which are about 20 kJ mol$^{-1}$ lower than that of mannotriose. This indicates that, while the two disaccharides may be binding to artocarpin mostly through their nonreducing end monosaccharides to the respective subsites specific for α1–3 and α1–6 arms of the combining site, the addition of core mannose makes approximately ~20 kJ mol$^{-1}$ contribution to the binding enthalpy. In other words, in contrast to GNA where the core mannose makes little contribution, 3.2 kJ mol$^{-1}$, to the overall binding process, in artocarpin it seems to make significant contribution to the interaction. Taken together, these data suggest that the extended binding site of artocarpin has features that diverge from both ConA family of lectins and GNA (16, 17, 20, 27).

In summary, the extended combining site of artocarpin exhibits interesting differences from the mannose/glucose lectins studied so far and provides an additional paradigm for investigation of the recognition of the trimannoside motif found commonly in the N-linked glycoproteins.
Trimannoside Binding Specificity of Artocarpin

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