Thermodynamics of Lectin–Carbohydrate Interactions. Titration Microcalorimetry Measurements of the Binding of N-Linked Carbohydrates and Ovalbumin to Concanavalin A†

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ABSTRACT: The thermodynamics of binding of concanavalin A (Con A) with a series of linear and branched chain oligosaccharides including certain N-linked complex type and oligomannose type carbohydrates and a fraction of quail ovalbumin containing Man7 and Man8 oligomannose chains have been determined using titration microcalorimetry. Methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside, a branch chain trisaccharide moiety found in all N-linked carbohydrates which possesses approximately 60-fold higher affinity than methyl α-D-mannopyranoside, exhibited a change in enthalpy of binding (ΔH) of −14.4 kcal mol^{-1} as compared to −8.2 kcal mol^{-1} for the monosaccharide. This demonstrates that Con A possesses an extended binding site for the trimannoside. However, a biantennary complex type carbohydrate with terminal β(1,2)-GlcNAc residues which binds with 3-fold higher affinity than the trimannoside possesses a ΔH of only −10.6 kcal mol^{-1}. A plot of $-\Delta H$ versus $-T\Delta S$ for the carbohydrates in the present study showed positive deviations in $-T\Delta S$ for the complex type carbohydrate, as well as α(1,2)-di- and trimannosyl oligosaccharides which are part of the structures of oligomannose type carbohydrates. The relative favorable changes in binding entropies of these compounds are attributed to the presence of multiple internal and terminal residues in each molecule which can independently bind to the monosaccharide binding site of the lectin. The ΔH values for the complex type carbohydrate and the α(1,2) mannose oligosaccharides were also approximately −2.5 kcal mol^{-1} greater than that of methyl α-D-mannopyranoside, indicating some extended binding site interactions. The thermodynamics of binding of N-linked oligomannose type carbohydrates to dimeric Con A and its succinyl and acetyl derivatives were determined since these carbohydrates are bivalent and precipitate with the native tetrameric lectin but not with the dimeric protein and its two derivatives. Titration of succinyl-Con A with a Man5 oligomannose type oligosaccharide gave a ΔH of −14.5 kcal mol^{-1}, which is similar to that of the branch chain trimannoside. This indicates that the α(1,6) core arm of Man5 which contains the trimannosyl moiety is the primary binding epitope for Con A. A fraction of quail ovalbumin containing a mixture of Man7 and Man8 chains at a single glycosylation site showed univalent binding to succinyl-Con A and a ΔH of −13.6 kcal mol^{-1}. These results indicate that the trimannoside moiety on the α(1,6) arm(s) of the carbohydrate chains is the primary binding epitope and that its interactions with the lectin are relatively unaffected by the protein matrix of ovalbumin.

Interest in Con A,[1] a lectin isolated from jack bean seeds (*Canavalia ensiformis*), is due to its numerous biological properties which are related to its carbohydrate binding specificity (Goldstein & Hayes, 1978). Several reviews and monographs (cf. Bittiger & Schnebli, 1976; Lis & Sharon, 1981) have discussed its uses as a tool in different biological systems, which include probing normal and tumor cell membrane structures and dynamics (Ben-Bassat & Goldblum, 1975; Brown & Hunt, 1978), studying glycosylation mutants of transformed cells (Stanley, 1982), and yielding preparations of polysaccharides, glycopeptides, and glycoproteins from Con A affinity columns (Bittiger & Schnebli, 1976).

Goldstein and co-workers (cf. Goldstein & Poretz, 1986) showed that Con A has specificity for the α-pyranose forms of the monosaccharides Glc[2] and Man, which contain similar hydroxyl group configurations at the 3-, 4-, and 6-positions. The lectin is a tetramer above pH 7 and a dimer below pH 6, with each monomer (M_r 26 500) possessing one saccharide binding site as well as a transition metal ion site (S1) (typically Mn^{2+}) and a Ca^{2+} site (S2) (Brewer et al., 1983; Goldstein & Poretz, 1986). The three-dimensional structure of the lectin at 1.75-Å resolution has been determined by X-ray diffraction analysis (Hardman et al., 1982) and a complex with Meα-Man to 2.9-Å resolution (Derewenda et al., 1989).

Early studies by Goldstein and co-workers showed the presence of two classes of linear oligosaccharides which differed in their affinities for the protein. The first class possesses affinities similar to monosaccharide binding and includes α-(1,3), α(1,4), and α(1,6) oligosaccharides with nonreducing

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[1] Abbreviations: Con A, concanavalin A, lectin from jack bean; Meα-Man, methyl α-mannopyranoside; Meα2-dGlc, methyl α-2-deoxy-glucopyranoside; Meα-Glc, methyl α-glucopyranoside; NMR, nuclear magnetic resonance; NMRD, nuclear magnetic relaxation dispersion, the magnetic field dependence of nuclear magnetic relaxation rate (longitudinal) of solvent protons; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] All sugars are in the D-configuration.

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terminal Glc or Man residues (Goldstein et al., 1965). The second class shows higher affinities and includes the $\alpha(1,2)$ oligomannosides (So & Goldstein, 1968). The 5- and 20-fold enhanced affinities of the $\alpha(1,2)$-di- and trimannosyl oligosaccharides with respect to MeαMan, respectively, prompted speculation that Con A possessed an extended binding site that accommodated these oligosaccharides (Goldstein, 1975). The solvent proton nuclear magnetic relaxation dispersion (NMRD) technique, which measures the interactions of solvent water protons with the Mn^{2+} in Con A and is sensitive to conformational changes in the lectin induced by saccharide binding, were subsequently used to investigate the binding of a series of mono- and oligosaccharides including the $\alpha(1,2)$-oligomannosides (Koenig et al., 1973; Brewer & Brown, 1979). These studies led to the suggestion that the enhanced affinities of the $\alpha(1,2)$-oligosaccharides were primarily due to their increased probability of binding because of the presence of multiple Man residues with free 3-, 4-, and 6-hydroxyl groups in each molecule (Brewer & Brown, 1979). These findings were supported by rapid flow kinetic analysis of the binding of fluorescent labeled $\alpha(1,2)$-mannosyl oligosaccharides to the protein (Van Landschoot et al., 1980).

Studies of the binding of a series of oligomannose and bisected hybrid type glycopeptides and complex type oligosaccharides which possess higher affinities (~50-fold or greater) than MeαMan showed that their NMRD profiles were different from those of simple mono- and oligosaccharides (Bhattacharyya & Brewer, 1989; Bhattacharyya et al., 1987a,b; Brewer & Bhattacharyya, 1986). These results indicated different conformational changes induced in the protein upon binding the larger N-linked carbohydrates and therefore that they possessed different mechanisms of binding. These studies also observed that the trisaccharide 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannose (**9** in Figure 1) possessed nearly 100-fold higher affinity than MeαMan and gave an NMRD profile similar to those of the larger N-linked carbohydrates (Bhattacharyya & Brewer, 1989; Brewer & Bhattacharyya, 1986). These results suggested that the trimannosyl moiety in N-linked carbohydrates was responsible for their high affinity binding to Con A. Thus, the trimannosyl moieties on the outer $\alpha(1,6)$ arms of certain N-linked oligomannose type carbohydrates and the internal core region of N-linked complex type carbohydrates such as **11** were assigned as high affinity binding sites in these molecules. These conclusions were supported by structure–activity studies of Kasai and co-workers (Ohyama et al., 1985) and NMR studies of the binding of methyl trimannoside **10** by Carver et al. (1985).

Detailed insights in the specificity of carbohydrate–proteins interactions, however, require not only structural information such as that obtained from NMRD or circular dichroism measurements but also thermodynamic data on the complexes. Titration microcalorimetry provides a powerful tool for investigating the thermodynamics of binding between ligands and macromolecules (cf. Bains et al., 1992; Brummell et al., 1993). It directly measures the enthalpy change (ΔH) and association constant (K_a) and stoichiometry (n) of the binding interactions and allows calculations of the entropy change (ΔS), change in free energy (ΔG), and change in heat capacity of binding (ΔC_p) (Wiseman et al., 1989). Calorimetric studies of Con A binding to simple mono- and oligosaccharides under different pH conditions have previously been reported (Ambrosino et al., 1987; Dani et al., 1981; Munske et al., 1984). Very recently, Williams et al. (1992) have reported a titration microcalorimetric study of the binding of trimannoside **10** to Con A at pH 5.2. However, there has been considerable variation in the values of the thermodynamic parameters reported.

Using isothermal titration microcalorimetry, we herein report the thermodynamics and stoichiometry of binding of simple mono- and oligosaccharides to Con A including α-(1,2), $\alpha(1,3)$, $\alpha(1,4)$, and $\alpha(1,6)$ disaccharides and $\alpha(1,2)$-trimannoside. Thermodynamic data have also been obtained for the binding of trimannoside **9** and its methyl α-anomer **10**, complex type carbohydrate **11**, and several N-linked oligomannose type carbohydrates (**12–15**) as well as a fraction of quail ovalbumin containing Man7 and Man8 chains. The results together with previous structural studies provide detailed insight into the mechanism of binding of the carbohydrates and the glycoprotein to the lectin.

MATERIALS AND METHODS

Materials. Native Con A was prepared from jack bean seeds (Sigma) according to the method of Agrawal and Goldstein (1967). Fragment free Con A was prepared according to the procedure described by Bhattacharyya and Brewer (1990). The concentration of Con A was determined spectrophotometrically at 280 nm using $A^{1\%,1cm} = 13.7$ at pH 7.2 (Goldstein & Poretz, 1986) and 12.4 at pH 5.2 (Yariv et al., 1968) and expressed in terms of monomer ($M_r = 26\,500$) (Goldstein & Poretz, 1986). Acetyl- and succinyl-Con A were prepared as described (Mandal & Brewer, 1993). The concentrations of acetyl- and succinyl-Con A were measured spectrophotometrically at 280 nm using $A^{1\%,1cm} = 13.7$ at pH 7.2 and expressed in terms of monomer ($M_r = 28\,000$) (Gunther et al., 1973). Quail ovalbumin fraction II was purified as previously described (Mandal & Brewer, 1992) and its concentration spectrophotometrically determined using $A^{1\%,1cm} = 7.5$ at 280 nm and $M_r = 45\,000$ (Shepherd & Montgomery, 1978).

MeαGlc, MeαMan, maltose, maltotriose, and carbohydrates **2**, **5**, **7**, and **10** in Figure 1 were purchased from Sigma Chemical Co. Carbohydrates **1**, **4**, **6**, **8**, **9**, **11**, and **12** in Figure 1 were obtained from Dextra Laboratories Ltd., U.K. Meα2-dGlc and trisaccharide **3** were gifts from Dr. S. Sabesan, Du Pont Company, Wilmington, DE and Dr. Fraser Reid of Duke University, respectively. MeαGlcNAc was purchased from Toronto Research Chemicals, Ontario, Canada. Man9 glycopeptide **15** was prepared from the pronase digest of soybean agglutinin (SBA) according to a previously described procedure (Lis & Sharon, 1978). A mixture of Man7 (**13**) and Man8 (**14**) glycopeptides (~1:1 by ^{1}H NMR) was isolated from pronase digest of quail ovalbumin as described (Mandal & Brewer, 1992). The concentrations of carbohydrates were determined by modification of the phenol–sulfuric acid method (Dubois et al., 1956) using appropriate monosaccharides (glucose, mannose, or 2-deoxyglucose) as standards (Saha & Brewer, 1994). Rapid addition of sulfuric acid to the phenol sample solution within 1–2 s using a Varipet Manostat syringe (4-mL volume syringe, Teflon Tip, VWR Scientific) is essential for complete hydrolysis of glycosidic bonds to the constituent monosaccharides.

Titration Calorimetry. Isothermal titration microcalorimetry was performed using an OMEGA Microcalorimeter from Microcal, Inc. (Northampton, MA). In individual titrations, injections of 3 or 4 μL of carbohydrate were added from the computer-controlled 100-μL microsyringe at an interval of 4 min into the lectin solution (cell volume = 1.3424 mL) containing the same buffer as the saccharide, while stirring at 350 rpm. Control experiments performed by making

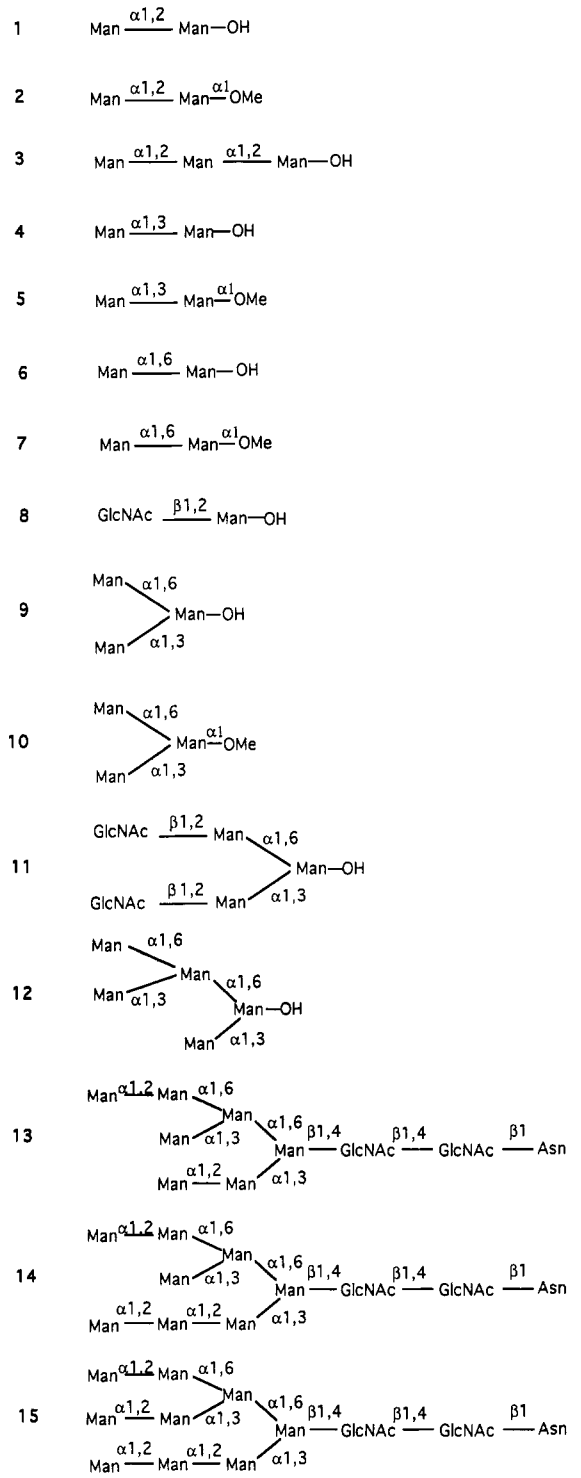

FIGURE 1: Structures of oligosaccharides **1–11** and oligomannose type carbohydrates **12–15**. Man, GlcNAc, and Asn represent mannose, *N*-acetylglucosamine, and asparagine residues, respectively.

identical injections of saccharide into a cell containing buffer with no protein showed insignificant heat of dilution. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (the enthalpy change in kcal mol^{-1}), K_a (the association constant in M^{-1}), and n (the number of binding sites per monomer) 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 (Wiseman et al., 1989). All experiments were performed with c values $1 < c < 200$ in the present study. In the case of maltotriose ($c = 1.2$), the data were fitted with n fixed and K_a and ΔH as floating parameters. The instrument was calibrated by using the calibration kit containing ribonuclease A (Rnase A) and cytidine 2′-monophosphate (2′-CMP) supplied by the manufacturer. In order to confirm the reliability of the ΔH measurements in the present study, calorimetric titration experiments with the same stock solutions of **9** and native Con A at pH 7.2 were carried out in the laboratories of Dr. J. Brandts (Microcal, Inc.) and Dr. P. R. Connelly (Vertex Pharmaceuticals Inc.) under identical conditions. The ΔH values in these laboratories were found to be −14.4 and −14.2 kcal mol^{-1}, respectively, which compare favorably to our value of −14.1 kcal mol^{-1}.

Scatchard Analysis. Scatchard plots were constructed from the calorimetric titration data as follows. The heat evolved on the ith injection $Q(i)$ (μcal) and the total concentration of ligand $X_t(i)$ (mM) and macromolecule (protein) $M_t(i)$ (mM) after the ith injection were readily obtained from the Omega worksheet used in the curve fitting software, which takes into account the correction for displaced volume effects which occur with each injection. The concentration of bound ligand $X_b(i)$ after the ith injection is obtained by

$$X_b(i) = \Delta X Q(i)/Q_{max} + X_b(i-1) \quad (1)$$

where ΔX is the increase in concentration (mM) of ligand in the cell produced by the ith injection and Q_{max} (μcal) is the heat which would be evolved for complete binding of ligand. Q_{max} is calculated from

$$Q_{max} = X_t(1)\Delta H V_0 \quad (2)$$

where $X_t(1)$ is the concentration of ligand (mM) after the 1st injection, ΔH the enthalpy change (cal mol^{-1}), and V_0 the active cell volume (mL). (Alternatively, Q_{max} may be obtained by extrapolation.) The total concentration of free ligand $X_f(i)$ is then given by

$$X_f(i) = X_t(i) - X_b(i) \quad (3)$$

Finally, a plot of $r(i)/X_f(i)$ vs $r(i)$ where $r(i) = X_b(i)/M_t(i)$, the fraction of ligand bound per mole of protein after the ith injection, is the Scatchard plot of the data.

RESULTS AND DISCUSSION

Binding of Monosaccharides and Disaccharides. Figure 2 shows the data for a typical isothermal microcalorimetry experiment for the titration of native Con A with disaccharide **1** (Figure 1) in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. As shown in Figure 2A, exothermic heat is produced after each injection of the saccharide. The magnitude of the released heat decreases progressively with each new injection until complete saturation is achieved. Figure 2B shows the experimental data and the calculated best-fit binding curve which passes very closely through the experimental points. The best fit values of the parameters are $n = 0.98$, $K_a = 4.17 \times 10^4$ M^{-1}, and $\Delta H = -9.9$ kcal mol^{-1} (Table 1). The calculated value of $T\Delta S$ is also given in Table 1.

Data were obtained for the binding of simple mono- and oligosaccharides to Con A since in many cases literature values differ substantially (cf. Ambrosino et al., 1987; Dani et al., 1981; Munske et al., 1984). The thermodynamic parameters derived for MeαGlc, MeαGlcNAc, Meα2-dGlc, the α(1,4)-linked glucose oligosaccharides maltose and maltotriose, MeαMan, disaccharides **1–2**, **4–8**, and α(1,2)-trimannoside **3** at 25 °C and pH 7.2 are summarized in Table 1, along with the concentrations of lectin and saccharide used. The average deviations for multiple determinations at the same or different lectin concentrations were typically 1–4% for all three

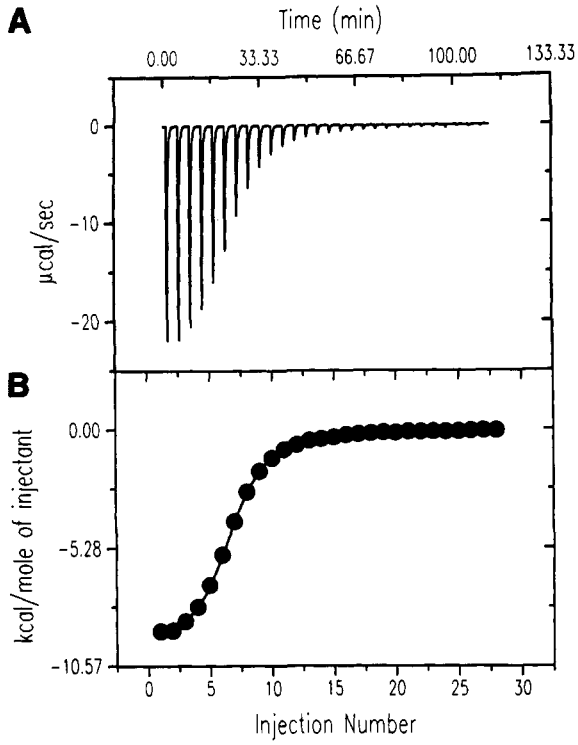

FIGURE 2: Calorimetric titration of native Con A (0.39 mM) with disaccharide **1** (26.7 mM) at 25 °C: (A) raw data obtained for 30 automatic injections, each of 3 μL, of **1**; and (B) the integrated curve showing experimental points (●) and the best fit (—). The buffer was 0.1 M HEPES containing 0.9 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ at pH 7.2. For details, see Materials and Methods.

parameters. We wish to emphasize that it is essential that the concentrations of the individual carbohydrates be determined by the phenol–sulfuric acid method (Dubois et al., 1956), where possible, using a calibration curve of the appropriate monosaccharides (Sanat & Brewer, 1994). Failure to accurately determine the concentration of the carbohydrate will lead to large errors in some of the thermodynamic parameters such as ΔH.

Table 1 shows that Meα-Glc, maltose, and maltotriose have similar K_a and ΔH values. The ΔH values are somewhat less than −7.3 and −7.2 kcal mol^{-1} reported for Meα-Glc and maltose, respectively, by Magnuson and co-workers (Munske et al., 1984). The data, however, are consistent with binding of the nonreducing terminal glucose residues of α(1,4)-glucosyl oligosaccharides to Con A which agree with studies by Goldstein et al. (1965). Meα-GlcNAc also has a ΔH similar to that of Meα-Glc. Table 1 shows that Meα-Man has a greater ΔH (−8.2 kcal mol^{-1}) than Meα-Glc as well as 4-fold higher affinity, which agrees with previous precipitation inhibition studies (Goldstein et al., 1965). Similar results were obtained for Meα-Man binding to dimeric succinyl-Con A at pH 7.2 (Table 2). Meα2-dGlc has a ΔH of −7.3 kcal mol^{-1}, which is between that of Meα-Glc and Meα-Man, as is its affinity constant (Table 1) (Goldstein & Poretz, 1986).

The affinity and thermodynamic parameters for α(1,6)-dimannoside **7** are very similar to those for Meα-Man (Table 1). Disaccharide **6** which lacks the α-anomeric methyl group of **7** has a somewhat greater $-\Delta H$ (−1 kcal mol^{-1}) and ~2-fold higher affinity than **7**. α(1,3)-Mannobiose **4** and its α-methyl derivative (**5**) possess ΔH values about −2 kcal mol^{-1} greater and affinities 4-fold higher than that of Meα-Man, which indicate extended site interactions with these two disaccharides.

Table 1: Thermodynamic Parameters Derived from the Titration of Con A with Various Saccharides at 25 °C

carbohydrate	carbohydrate conc. (mM)	lectin conc. (mM)	K_a[a] (M^{-1})
	Native Con A at pH 7.2[b]		
Meα-Glc	70.0	0.895	1.96 (±0.05) × 10^3
Meα-GlcNAc	72.0	0.918	1.08 (±0.04) × 10^3
maltose	68.8	0.918	1.31 (±0.07) × 10^3
maltotriose	80.6	0.904	1.34 (±0.03) × 10^3
Meα-Man	46.0	0.483	0.82 (±0.02) × 10^4
Meα2-dGlc	61.7	0.730	2.75 (±0.07) × 10^3
1	26.7	0.390	4.17 (±0.08) × 10^4
2	26.5	0.380	1.41 (±0.04) × 10^5
3	5.15	0.125	3.79 (±0.26) × 10^5
4	20.8	0.256	1.41 (±0.02) × 10^4
5	14.8	0.247	3.35 (±0.12) × 10^4
6	42.9	0.483	1.34 (±0.04) × 10^4
7	14.8	0.247	0.81 (±0.03) × 10^4
8	35.9	0.420	0.67 (±0.01) × 10^4
9	5.93	0.145	3.37 (±0.14) × 10^5
10	7.04	0.132	4.90 (±0.15) × 10^5 [d]
11	8.00	0.145	1.40 (±0.10) × 10^6
	Native Con A at pH 5.2[c]		
10	11.0	0.195	5.10 (±0.21) × 10^5 [d]
15	1.10	0.025	1.10 (±0.10) × 10^6 [d]
	Fragment free Con A at pH 7.2[b]		
9	5.90	0.167	3.44 (±0.17) × 10^5

carbohydrate	$-\Delta H$[a] (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})	n[a] (no. of sites/monomer)
	Native Con A at pH 7.2[b]		
Meα-Glc	6.6 (±0.1)	2.1	0.98 (±0.02)
Meα-GlcNAc	6.2 (±0.2)	2.1	1.03 (±0.03)
maltose	6.2 (±0.3)	1.9	0.99 (±0.04)
maltotriose	6.4 (±0.4)	2.1	1.00 (held fixed)
Meα-Man	8.2 (±0.1)	2.9	1.04 (±0.01)
Meα2-dGlc	7.3 (±0.1)	2.6	1.05 (±0.01)
1	9.9 (±0.1)	3.6	0.98 (±0.01)
2	10.5 (±0.1)	3.5	1.04 (±0.01)
3	10.7 (±0.1)	3.1	1.05 (±0.01)
4	10.2 (±0.1)	4.5	1.01 (±0.01)
5	10.7 (±0.1)	4.5	1.01 (±0.01)
6	9.4 (±0.1)	3.8	1.00 (±0.01)
7	8.4 (±0.2)	3.1	0.99 (±0.02)
8	5.3 (±0.1)	0.1	0.97 (±0.01)
9	14.1 (±0.1)	6.6	1.03 (±0.01)
10	14.4 (±0.1)	6.6	1.02 (±0.01)
11	10.6 (±0.1)	2.2	0.99 (±0.01)
	Native Con A at pH 5.2[c]		
10	14.3 (±0.1)	6.5	0.96 (±0.01)
15	18.0 (±0.3)	9.8	0.91 (±0.01)
	Fragment free Con A at pH 7.2[b]		
9	14.3 (±0.1)	6.7	1.03 (±0.01)

[a] Values in parentheses indicate the standard deviation of fit between the experimental binding curve and the calculated curve obtained with the fitted thermodynamic parameters. [b] The buffer was 0.1 M HEPES containing 0.9 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ at pH 7.2. [c] The buffer was 0.05 M dimethyl glutarate containing 0.25 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ at pH 5.2. [d] Data taken from Mandal and Brewer (1993) and included here for comparison.

α(1,2)-Dimannoside **2** possesses a ΔH similar to that of **5** (Table 1); however, the affinity of **2** is 4-fold greater. The increase in affinity arises, in part, from entropic effects since $T\Delta S$ is 1 kcal mol^{-1} more positive for **2**. Similar results were obtained for α(1,2)-mannobiose **1** relative to **4**. The enhanced entropic effects of the α(1,2)-dimannosides arise from the presence of two Man residues with free 3-, 4-, and 6-hydroxyl groups which can independently bind to the lectin and thus increase the number of binding modes. These observations are consistent with NMRD studies of the binding of the α-(1,2)-mannose oligosaccharides to Con A (Brewer & Brown, 1979). It should be noted that the binding affinity of **1** is

Table 2: Thermodynamic Parameters Derived from the Titration of Acetyl- and Succinyl-Con A with Various Oligosaccharides/Glycopeptides/Glycoprotein at 25 °C

carbohydrate	carbohydrate conc. (mM)	lectin conc. (mM)	K_a[a] (M^{-1})
	Succinyl-Con A at pH 7.2[b]		
MeαMan	37.0	0.620	0.99 (±0.03) × 10^4 [e]
10	7.66	0.155	5.13 (±0.22) × 10^5 [e]
12	1.60	0.031	6.63 (±0.40) × 10^5 [e]
13/14 mixture[d]	1.40	0.030	3.03 (±0.41) × 10^5 [e]
15	0.93	0.031	8.96 (±0.14) × 10^5 [e]
ovalbumin fraction II	0.98	0.055	7.79 (±0.12) × 10^5
	Acetyl-Con A at pH 7.2[c]		
9	1.40	0.028	3.10 (±0.43) × 10^5 [e]
12	1.10	0.031	3.51 (±0.27) × 10^5 [e]
15	1.10	0.032	5.45 (±0.43) × 10^5 [e]

carbohydrate	$-\Delta H$[a] (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})	n[a] (no. of sites/monomer)
	Succinyl-Con A at pH 7.2[b]		
MeαMan	8.3 (±0.1)	2.9	1.04 (±0.01)
10	14.3 (±0.1)	6.5	1.01 (±0.01)
12	14.5 (±0.1)	6.6	1.01 (±0.01)
13/14 mixture[d]	18.3 (±0.6)	10.8	0.92 (±0.02)
15	17.1 (±0.4)	9.0	0.92 (±0.01)
ovalbumin fraction II	13.6 (±0.2)	5.6	0.99 (±0.01)
	Acetyl-Con A at pH 7.2[c]		
9	13.8 (±0.5)	6.3	0.93 (±0.02)
12	18.4 (±0.4)	10.8	0.64 (±0.01)
15	18.4 (±0.3)	10.6	0.59 (±0.01)

[a] Values in parentheses indicate the standard deviation of fit between the experimental binding curve and the calculated curve obtained with the fitted thermodynamic parameters. [b] The buffer was 0.1 M HEPES containing 0.9 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ at pH 7.2. [c] The buffer was 0.02 M HEPES containing 0.15 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ at pH 7.2. [d] Man7 and Man8 glycopeptides isolated from pronase digest of quail ovalbumin (Mandal & Brewer, 1992). [e] Data taken from Mandal and Brewer (1993) and included here for comparison.

about 5-fold higher than that of MeαMan, while that of **2** is about 17-fold higher, since α-anomeric pyranosides bind to the monosaccharide binding site of the lectin better than the corresponding free sugars. The greater $-\Delta H$ values for **1** and **2** relative to MeαMan also contribute to their enhanced affinities which agree with previous observations by Goldstein and co-workers (Williams et al., 1981). Interestingly, α(1,2)-trimannoside **3** binds with a ΔH (−10.7 kcal mol^{-1}) similar to that of **2**, but with nearly 3-fold higher affinity (Table 1). The enhanced affinity of **3** relative to **2** is due primarily to the positive entropy contribution (0.4 kcal mol^{-1}) of the third α(1,2)Man residue with free 3-, 4-, and 6-hydroxyl groups.

*Binding of Trimannosides **9** and **10**.* Methyl 3,6-di-*O*-(α-mannopyranosyl)-α-mannopyranoside (**10**) binds to tetrameric Con A at pH 7.2, 25 °C, with a ΔH of −14.4 kcal mol^{-1} and $K_a = 4.9 \times 10^5$ M^{-1} (Table 1). These parameters are similar to those previously obtained by equilibrium dialysis measurements at pH 5.4, 25 °C (ΔH = −14.8 kcal mol^{-1}; $K_a = 6.7 \times 10^5$ M^{-1}) (Mackenzie, 1986). Trisaccharide **9** which lacks an α-anomeric methyl group possesses a ΔH of −14.1 kcal mol^{-1}, similar to that of **10**. The ΔH and K_a values for **9** and **10** are greater than that of MeαMan, as well as those of disaccharides **4**–**7** which comprise the two arms of the 3,6-trimannoside. This provides direct evidence that the two nonreducing Man residues of the trimannoside make extending site binding interactions with Con A, which agrees with similar conclusions derived from NMRD studies (Bhattacharyya & Brewer, 1989; Brewer & Bhattacharyya, 1986).

Williams et al. (1992) recently reported a ΔH of −9.8 kcal mol^{-1} and a ΔG of −7.2 kcal mol^{-1} for **10** binding to Con A

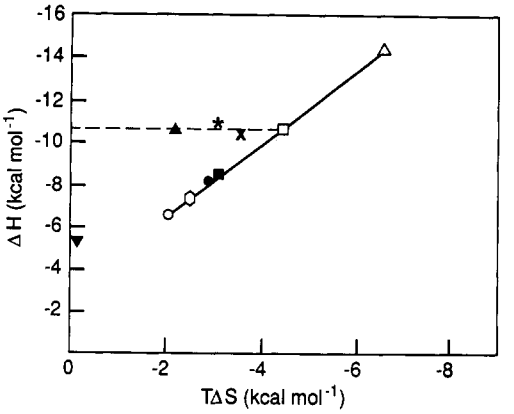

FIGURE 3: Plot of ΔH versus $T\Delta S$ for the binding of MeαGlc (○), Meα2-dGlc (○), MeαMan (●), **5** (□), **7** (■), and **10** (△) to native Con A at 25 °C (298 K). Data points for **2** (×), **3** (★), **8** (▼), and **11** (▲) are also shown.

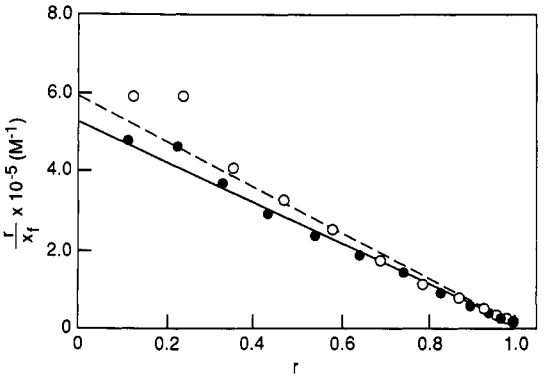

FIGURE 4: Scatchard plots for the binding of **10** with tetrameric Con A (0.132 mM) (○) and dimeric succinyl-Con A (0.155 mM) (●) at pH 7.2 and 25 °C. The concentrations of **10** in the titrations of tetrameric Con A and dimeric succinyl-Con A are 7.04 and 7.66 mM, respectively. The values for Q_{max} were calculated according to eq 2. For details, see Materials and Methods.

at pH 5.2. However, we obtain the same results at pH 5.2 (dimeric Con A) and pH 7.2 (tetrameric Con A) (Table 1). They also proposed positive cooperativity for binding of the trisaccharide based on several points of a Scatchard plot based on microcalorimetry data which exhibited downward deviations at low fractional occupancy of the protein. However, Scatchard plots of microcalorimetry data are sensitivity to small errors in the initial data points, and hence the presence of curvature in this region does not provide strong evidence for cooperativity effects. Scatchard plots derived from microcalorimetric data for binding of **10** to tetrameric Con A and dimeric succinyl-Con A in the present study are shown in Figure 4. The plots are essentially linear except for the first data points. The amount of heat evolved for binding of a high affinity ligand such as **10** is essentially constant for the first few injections where the added ligand is almost completely bound (~99%). Deviations in this region of the Scatchard plot can occur because the first injection of ligand is susceptible to volume errors. An error in heat measurement by as little as 0.4% generates a data point which is well below the second point along the ordinate in the plot. Because of this, the first data point in Figure 4 is calculated by assuming the heat evolved for first and second injection to be the same. Furthermore, the intercept for the plots at $r = 0$ shows values of K_a to be 5.8×10^5 M^{-1} and 5.2×10^5 M^{-1} for native Con A and succinyl-Con A, respectively, which are in good agreement with those obtained by the Omega curve fitting software. The data in Figure 4 also agree with a linear

Scatchard plot of equilibrium dialysis data for binding of **10** to Con A at pH 5.4, 25 °C (Mackenzie, 1986).

*Binding of Complex Type Oligosaccharide **11** and Disaccharide **8**.* Biantennary complex type oligosaccharide **11** possesses a terminal β(1,2)GlcNAc residue on each arm of the core trimannoside moiety and is a naturally occurring homolog of **9**. Table 1 shows that, although **11** possesses nearly 4-fold higher affinity than **9**, its ΔH is only −10.6 kcal mol^{-1} as compared to −14.1 kcal mol^{-1} for **9**. On the other hand, $T\Delta S$ for **11** is −2.2 kcal mol^{-1} whereas that for **9** is −6.6 kcal mol^{-1}. These results indicate that the increased affinity of **11** relative to **9** is due to its relatively favorable change in entropy of binding which offsets its reduced change in enthalpy of binding. Thus, even though NMRD studies have suggested similar mechanisms of binding of **9** and **11** to Con A (Bhattacharyya et al., 1987b), the calorimetry data indicate different mechanisms of binding.

Disaccharide **8**, which constitutes the individual chains of **11**, also shows a relatively small ΔH but a favorable $T\Delta S$ contribution (Table 1). Although the affinity of **8** is only slightly less than that of Meα-Man, ΔH is −5.3 kcal mol^{-1} which is about 3 kcal mol^{-1} less than that of Meα-Man and half of that observed for **11**. The change in entropy of binding of the disaccharide is relatively positive ($T\Delta S$ = −0.1 kcal mol^{-1}) which provides a substantial contribution to the free energy of binding. These results suggest common binding mechanisms for **8** and **11**.

The relatively positive entropy contributions for **8** and **11** are apparent in a plot of ΔH versus $T\Delta S$ (Figure 3) for many of the carbohydrates in Table 1. The plot for Meα-Glc, Meα-2-dGlc, Meα-Man, **5**, **7**, and **10** shows a linear relationship with a correlation coefficient better than 0.99. Such enthalpy–entropy compensations for the carbohydrate–protein interactions have long been recognized and attributed to the unique properties of water (cf. Lemieux et al., 1991). The thermodynamics binding parameters of these carbohydrates are similar to those reported for many other lectin–carbohydrate interactions in that they have favorable enthalpic values and relatively unfavorable entropic contributions (Lemieux, 1989; Munske et al., 1984). However, **8** and **11** deviate from the linear plot of the above carbohydrates due to more favorable (less negative) entropy contributions. The α(1,2)-di- and trimannosides (**2** and **3**) also show similar deviations from the plot and lie on a line with **11** which intersects the plot for the other carbohydrates at the data point for **5**. The (dashed) line formed by **2**, **3**, **5**, and **11** in Figure 3 intersects the ordinate at $\Delta H \sim -10.5$ kcal mol^{-1}, indicating a common heat of binding and hence apparent binding loci to the protein. However, it is important to note that **2**, **3**, **8**, and **11**, which all show positive entropy deviations, possess multiple internal and terminal residues with free 3-, 4-, and 6-hydroxyl groups which have been shown by Goldstein (1975) to be capable of independently binding to Con A. This has led to the conclusion that molecules like **2** and **3** show enhanced affinity for Con A, in part, due to "sliding" or "jumping" between such residues in a molecule before full dissociation which decreases their off-rates (Brewer & Brown, 1979). The presence of such residues, the terminal β-GlcNAc residue and internal α-Man residue(s), in **8** and **11** suggests that similar mechanisms exist for these two oligosaccharides.

Binding Interactions of Acetyl- and Succinyl-Con A with Oligomannose Type Carbohydrates. The thermodynamic parameters for binding of trisaccharide **10** to tetrameric native Con A and dimeric succinyl-Con A in the presence of high salt buffer at pH 7.2 as well as dimeric form of native Con A in presence of low salt buffer at pH 5.2 are virtually identical (Tables 1 and 2). Moreover, the binding of **9** to dimeric acetyl-Con A in low salt buffer at pH 7.2 is similar to that observed for tetrameric Con A in high salt buffer at pH 7.2 (Tables 1 and 2). These results show that the thermodynamic parameters of binding of the trimannoside to tetrameric and dimeric Con A as well as its acetyl and succinyl derivatives are essentially identical, as recently reported (Mandal & Brewer, 1993), and that pH (5.2–7.2) and salt concentration (0.15–0.9 M NaCl) have essentially no effect.

Oligomannose type glycopeptides have previously been shown to bind and precipitate with tetrameric Con A as divalent ligands (Bhattacharyya & Brewer, 1989; Bhattacharyya et al., 1987a). Structure–activity studies have shown that the two lectin binding sites are located on the α(1,6) and α(1,3) arms of the core β-mannosyl residue of the glycopeptides. The α(1,6) arm was identified as the high affinity or primary site and the α(1,3) arm as the low affinity or secondary site (Bhattacharyya et al., 1987a). Since divalent carbohydrates do not precipitate with dimeric lectins (cf. Bhattacharyya et al., 1988) (although they may form soluble cross-linked complexes), titration microcalorimetry experiments with oligomannose type carbohydrates were carried out with dimeric Con A which exists at relatively low pH (below pH 6.0) as well as its acetyl and succinyl derivatives which are pH independent (Gunther et al., 1973). Maintaining relatively low concentrations of the protein and glycoproteins, in certain cases, permitted determination of the thermodynamics of binding of only the high affinity α(1,6) arm of certain glycopeptides.

The thermodynamic parameters for binding of the Man5 oligosaccharide (**12**) (Figure 1) with dimeric succinyl-Con A are given in Table 2. The value of n is 1.01 indicating univalent interactions of **12** with the lectin, and ΔH is −14.5 kcal mol^{-1} and K_a is 6.6×10^5 M^{-1}, which are values similar to those for **10**. These results provide direct evidence that **12** binds to succinyl-Con A via the trimannosyl moiety located on the α(1,6) arm of the molecule. In contrast, the thermodynamic parameters for binding of **12** with acetyl-Con A (Table 2) include a lower n value (0.64) and a greater ΔH (−18.4 kcal mol^{-1}) as compared to succinyl-Con A. [An n value of 1.0 indicates that 1 mol of saccharide is bound per mole of lectin monomer, while an n value of 0.5 implies bivalency of the carbohydrate, that is, 1 mol of bound saccharide per 2 mol of lectin monomers. The latter may imply binding of 1 mol of saccharide to 1 mol of lectin dimer where one of the subunits is unavailable or inactive for binding. This is ruled out by the fact that both acetyl- and succinyl-Con A have similar agglutination activities with cells (Gunther et al., 1973; Mandal & Brewer, 1993) and that acetyl-Con A has been shown to be divalent in cross-linking studies with glycoproteins (Mandal & Brewer, 1992)]. The results suggest that binding of **12** leads to cross-linking of some acetyl-Con A molecules producing an equilibrium mixture of soluble cross-linked and non-cross-linked molecules. It is apparent that binding of both the α(1,6) arm possessing the trimannosyl moiety and α(1,3) arm leads to a greater enthalpy change per mole of bound saccharide compared to that obtained for binding of the trimannosyl group only, which explains the greater ΔH for acetyl-Con A (−18.4 kcal mol^{-1}) relative to succinyl-Con A (−14.5 kcal mol^{-1}). At much higher concentrations of succinyl-Con A, the value of n also begins to decrease below 1.0 (data not shown), due to binding of the lower affinity α(1,3)Man residue of **12** and formation of soluble cross-linked complexes. These results are consistent with previous studies

on the interactions of acetyl- and succinyl-Con A with glycoproteins which show that the precipitating activities of succinyl-Con A are much weaker than those of acetyl-Con A, possibly due to unfavorable electrostatic interactions (Khan et al., 1991; Mandal & Brewer, 1992).

Ambrosino et al. (1987) using titration microcalorimetry reported an n value of 0.5 for binding of the Man5 oligomannose type glycopeptide to Con A at pH 4.5. Their value of $-\Delta H$ (−18.8 kcal mol^{-1}) agrees with the value for **12** in Table 2. However, the authors failed to recognize the bivalency of the carbohydrate and instead postulated anticooperativity effects upon its binding based on its fractional n value. These effects were also reported for a Man6 oligomannose type and bisected hybrid type glycopeptide in the same study; however, both glycopeptides are also bivalent for Con A (Bhattacharyya & Brewer, 1989; Bhattacharyya et al., 1987a).

The thermodynamic parameters for binding of the Man9 glycopeptide (**15**) to succinyl-Con A are shown in Table 2. The value of n is 0.92, which suggests some bivalent binding of the glycopeptide via the high affinity pentamannosyl moiety of the α(1,6) arm and the lower affinity α(1,2)-trimannosyl moiety on the α(1,3) arm (Bhattacharyya & Brewer, 1989). The ΔH of −17.1 kcal mol^{-1} for **15** is higher than that for trisaccharide **10**, which may also reflect the formation of a fraction of molecules in solution in cross-linked complexes. The value of K_a is found to be 9×10^5 M^{-1}, which is only about 2-fold higher than that for trisaccharide **10** (Table 2). Calorimetric data for binding of **15** with tetrameric Con A at pH 7.2 could not be obtained since the protein readily precipitates with the glycoprotein under these conditions (Bhattacharyya & Brewer, 1989). Con A also precipitates with **15** in high salt buffer at pH 5.2, suggesting the presence of some tetrameric lectin under these conditions. However, binding of **15** to low concentrations of Con A (25 μM) in low salt buffer (0.05 M dimethyl glutarate containing 0.25 M NaCl) at pH 5.2 results in no precipitation, indicating that the lectin is essentially all dimer under these conditions. The thermodynamic binding parameters for **15** under these conditions are similar to those observed with succinyl-Con A (Tables 1 and 2). These results indicate similar modes of binding of **15** to dimeric Con A at pH 5.2 and to dimeric succinyl-Con A at pH 7.2. Titration of acetyl-Con A (32 μM) with **15** in low salt buffer at pH 7.2 shows a binding stoichiometry of 0.59 (Table 2) demonstrating predominant bivalent binding interactions of **15**. These results are consistent with those for **12** in which acetyl-Con A shows a greater tendency to cross-link with the oligosaccharide than succinyl-Con A. However, under any of the above conditions, the calorimetrically determined thermodynamic parameters of binding of **15** are hampered by the formation of a fraction of soluble cross-linked molecules in solution, and therefore its values cannot be directly compared with those for **10** or **12**.

Binding of Quail Ovalbumin to Succinyl-Con A. Ovalbumin isolated from different species is a monomeric glycoprotein of M_r 45 000 and exhibits microheterogeneity with respect to the N-linked carbohydrate chain which is present at a single glycosylation site (Huang & Montgomery, 1972). In the case of quail ovalbumin, only high mannose type chains are associated with the protein, and glycoprotein molecules containing different high mannose chains can be fractionated by Con A-Sepharose affinity chromatography (Iwase et al., 1983). The most tightly bound fraction has been designated as bound fraction II which possesses Man7 and Man8 oligomannose type chains in ~1:1 ratio, as determined by high-resolution ^{1}H NMR spectroscopy (Mandal & Brewer, 1992).

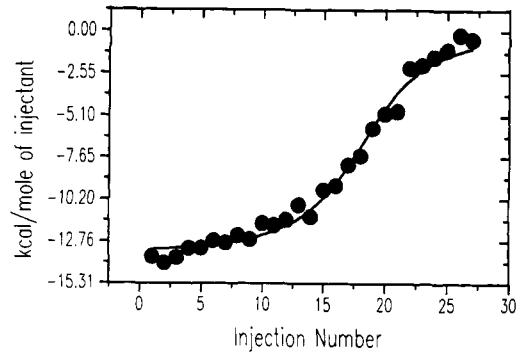

FIGURE 5: Integrated titration data at 25 °C, showing experimental points (●) and the best fit (—) obtained for 27 automatic injections, each of 4 μL, of quail ovalbumin bound fraction II (0.98 M) into a solution of succinyl-Con A (55 μM) in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$.

Titration of succinyl-Con A with a nearly 1:1 mixture of Man7 (**13**) and Man8 (**14**) glycopeptides isolated from quail ovalbumin fraction II by pronase digest (Mandal & Brewer, 1992) yielded thermodynamic parameters and a stoichiometry of binding that are similar to that of **15** (Table 2). Both glycopeptides have been shown to be bivalent for tetrameric Con A (Bhattacharyya & Brewer, 1989), which is consistent with the low value of n (0.92) as observed for **15**. The thermodynamic binding parameters for the mixture of **13** and **14** thus reflect formation of a fraction of soluble cross-linked complexes in solution with succinyl-Con A and, like **15**, cannot be directly compared with the values for **10** and **12**.

On the other hand, titration of succinyl-Con A with quail ovalbumin fraction II gives an n value of 0.99 (Table 2) which indicates predominantly univalent interactions of the two glycoprotein species with the lectin. The titration calorimetry curve shown in Figure 5 yields a ΔH of −13.6 kcal mol^{-1} and a K_a of 7.8×10^5 M^{-1}. These values are similar to those for **10** and **12**, which indicates that the trimannosyl moiety located on the α(1,6) arm of the core β-mannosyl residue of the oligosaccharide chain(s) of the glycoprotein(s) is the primary recognition epitope in their respective complexes with the lectin. Thus, the protein matrix of quail ovalbumin does not significantly affect the mode of binding of the Man7 and Man8 oligomannose type carbohydrate chains to succinyl-Con A. It should be mentioned, however, that under the appropriate conditions, the oligomannose chain(s) present on ovalbumin fraction II exhibits bivalency by precipitating tetrameric Con A (Mandal & Brewer, 1992). It is also apparent that the presence of the protein matrix of quail ovalbumin reduces the bivalent activities of the Man7 and Man8 chains somewhat since the free glycopeptides show a greater tendency to form cross-linked complexes with succinyl-Con A. It should also be pointed out that while the affinity of quail ovalbumin fraction II is essentially the same as that of **10** for succinyl-Con A, the Man7 and Man8 glycopeptides have been reported to have nearly 7-fold higher affinities for tetrameric Con A relative to **10** (Bhattacharyya & Brewer, 1989; Mandal & Brewer, 1993). This is most likely related to the higher affinities of Man7, Man8, and Man9 oligomannose type glycopeptides for tetrameric Con A relative to dimeric Con A and succinyl- and acetyl-Con A (Mandal & Brewer, 1993).

*Heat Capacity of Binding of MeαMan, Disaccharide **5**, and Trisaccharide **10**.* The results of calorimetry determinations at two different temperatures (25 and 37 °C) gave an

estimate of ΔC_p, the heat capacity of binding, for MeαMan, disaccharide **5**, and trisaccharide **10**, which are −90, −80, and −110 cal K^{-1} mol^{-1}, respectively. Though the data were not collected over a large temperature range, these values of ΔC_p are negative and relatively small in comparison with a wide variety of other binding phenomena involving proteins (Sturtevant, 1977). In many cases, however, binding of saccharides is coupled to changes in solvent accessibilities that result in negative, albeit small, ΔC_p values (Bains et al., 1992).

Summary. The present study provides direct thermodynamic evidence that the 3,6-trimannoside moiety (**9**) found in all N-linked carbohydrates binds to Con A via extended site interactions and that it is the primary recognition epitope for the lectin in the Man5 oligomannose type carbohydrate (**12**) as well as in a fraction of quail ovalbumin containing Man7 and Man8 oligomannose type chains. The presence of the protein matrix of quail ovalbumin did not significantly affect the thermodynamics of binding of the Man7 and Man8 chains to succinyl-Con A, thus demonstrating preservation of the primary binding epitope in these covalently linked carbohydrates for Con A. The present data also demonstrate that addition of terminal GlcNAc residues to **9** to give complex type carbohydrate **11** results in a substantial change in the thermodynamics of binding of the two carbohydrates with the latter possessing a more favorable entropy change upon binding. A plot of ΔH vs $T\Delta S$ demonstrated that **11**, disaccharide **8**, and the $\alpha(1,2)$-mannosyl di- and trisaccharides all show positive entropy deviations from the linear plot of six other mono- and oligosaccharides. The positive entropy deviations can be related to the presence of multiple internal and terminal residues in these molecules which can independently bind to the lectin and facilitate internal recapture mechanisms and hence increase their number of binding modes.

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