Studies of the Binding Specificity of Concanavalin A. Nature of the Extended Binding Site for Asparagine-Linked Carbohydrates†

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ABSTRACT: In the preceding paper [Mandal, D. K., Kishore, N., & Brewer, C. F. (1994) Biochemistry (preceding paper in this issue)] the trisaccharide 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose, which is present in all asparagine-linked carbohydrates, was shown by titration microcalorimetry to bind to the lectin concanavalin A (Con A) with nearly −6 kcal mol^{-1} greater enthalpy change (ΔH) than methyl α-D-mannopyranoside (MeαMan). These results indicate that Con A possesses an extended binding site for the trisaccharide. In the present paper, we have investigated the binding of a series of synthetic analogs of the methyl α-anomer of the trisaccharide using hemagglutination inhibition, solvent proton magnetic relaxation dispersion (NMRD), near ultraviolet circular dichroism, and titration microcalorimetry measurements. Four of the analogs tested possess an α-glucosyl or α-galactosyl residue substituted at either the α(1–6) or α(1–3) position. Analysis of the data indicates that the α(1–6) residue of the parent trimannoside binds to the so-called monosaccharide site and the α(1–3) residue to a weaker secondary site. Binding at the secondary site involves unfavorable interactions of the 2-equatorial hydroxyl of the α(1–3)Glc derivative since this analog binds with 12-fold lower affinity and −3.4 kcal mol^{-1} lesser ΔH than the trimannoside, whereas the α(1–3)-2-deoxyGlc analog possesses essentially the same affinity and ΔH as the trimannoside. NMRD data show that the α(1–3) 2-, 3-, 4-, and 6-deoxy derivatives of the trimannoside induces essentially the similar conformational changes in the protein as that of the parent trimannoside. However, the calorimetry data show that only the 3-deoxy analog binds with ~10-fold lower affinity and −3.4 kcal mol^{-1} lesser enthalpy change. This indicates that the 3-hydroxyl of the α(1–3)Man makes a specific hydrogen bond with the protein at a secondary binding site. The ΔH of −11 kcal mol^{-1} for the 3-deoxy analog is still, however, greater than that of MeαMan (−8.4 kcal mol^{-1}), which indicates another site of contact between the trimannoside and Con A, most likely with the "core" Man residue. Thus, Con A has an extended binding site which includes a high-affinity site that recognizes the 3-, 4-, and 6-hydroxyl groups of the α(1–6)Man residue of the trimannoside (the monosaccharide site), a lower affinity site that binds the 3-hydroxyl of the α(1–3)Man residue, and a third site which appears to involve the "core" Man residue.

The ability of concanavalin A (Con A)[1] to bind with high affinity to certain N-linked carbohydrates has made it a widely used tool to investigate the properties of normal and transformed cells, as well as to isolate carbohydrates, glycoconjugates, and cells on Con A affinity matrixes (Bittiger & Schnebli, 1976; Lis & Sharon, 1981). The trisaccharide moiety 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose which is found in all N-linked carbohydrates is primarily responsible for the high-affinity binding of N-linked carbohydrates to Con A (cf. Brewer & Bhattacharyya, 1986). Thus, it is important to establish the nature of the molecular interactions between the trimannoside and Con A in order to understand the specificity of interactions of N-linked carbohydrates with the lectin.

The previous paper in this issue described titration microcalorimetry studies of the binding of methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (Man3,6) to Con A (Mandal et al., 1994). The results show that Man3,6 possesses nearly 6 kcal mol^{-1} greater change in binding enthalpy ($-\Delta H$) than the monosaccharide methyl α-mannopyranoside (Meα-Man) as well as 60-fold enhanced affinity. These results establish an extended recognition site on the lectin for the Man3,6 epitope. The present study investigates binding of a series of Man3,6 derivatives possessing either Glc[2] or Gal substituted on either arm of the trisaccharide, or deoxy analogs of the α(1–3)Man residue of the trimannoside, to Con A using

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[1] Abbreviations: NMR, nuclear magnetic resonance; NMRD, nuclear magnetic relaxation dispersion; CD, circular dichroism; Con A, concanavalin A with unspecified metal ion content; CMPL, Con A with Mn^{2+} and Ca^{2+} at the S1 and S2 sites, respectively; MeαMan, methyl α-D-mannopyranoside; Man(3,6), methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; Glc(6), methyl 3-*O*-(α-D-mannopyranosyl)-6-*O*-(α-D-glucopyranosyl)-α-D-mannopyranoside; Glc(3), methyl 3-*O*-(α-D-glucopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; Gal(6), methyl 3-*O*-(α-D-mannopyranosyl)-6-*O*-(α-D-galactopyranosyl)-α-D-mannopyranoside; Gal(3), methyl 3-*O*-(α-D-galactopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; 2-dMan(3,6), methyl 3-*O*-(α-2-deoxy-D-mannopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; 3-dMan(3,6), methyl 3-*O*-(α-3-deoxy-D-mannopyransoyl)-6-*O*-(α-D-mannopyransoyl)-α-D-mannopyranoside; 4-dMan(3,6), methyl 3-*O*-(α-4-deoxy-D-mannopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; 6-dMan(3,6), methyl 3-*O*-(α-6-deoxy-D-mannopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] All sugars are in the D configuration.

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hemagglutination inhibition, nuclear magnetic relaxation dispersion (NMRD), circular dichroism (CD), and titration microcalorimetry measurements. The results provide insight into the extended recognition site of Con A for the Man3,6 epitope.

MATERIALS AND METHODS

Con A was prepared from jack bean seeds (Sigma) as described (Agrawal & Goldstein, 1967). The lectin containing Mn^{2+} and Ca^{2+} (CMPL) was prepared and characterized as reported (Brown et al., 1977). Protein concentrations were 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). Man(3,6) and MeαMan were obtained from Sigma Chemical Co. Glc(6), Glc(3), Gal(6), and Gal(3) were synthesized as described (Garegg et al., 1990). The synthesis of 2-dMan(3,6), 3-dMan-(3,6), 4-dMan(3,6) and 6-dMan(3,6) will be described elsewhere. The concentrations of carbohydrates were determined by the phenol–sulfuric acid method (Dubois et al., 1956) using appropriate mixtures of Man, Glc, and Gal as standards except for the deoxy derivatives whose concentrations were measured on dry weight basis. The purity of the samples was checked by high-resolution ^1H NMR at 500 MHz.

Hemagglutination–Inhibition Assays. Hemagglutination–inhibition was assayed at 22 °C by 2-fold serial dilution in 10 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, using 3% suspensions of rabbit erythrocytes (Osawa & Matsumoto, 1972).

NMRD Measurements. Measurements at 5 and 25 °C of the magnetic field dependence of the longitudinal (spin–lattice) relaxation rates ($1/T_1$), i.e., NMRD profile, of the solvent water protons were made using a field cycling relaxometer (Brown et al., 1977; Koenig & Brown, 1987). Reproducibility of the data was better than ±1%. Sample solutions (200 μL) contained known concentrations of CMPL together with sufficient amounts of carbohydrate to saturate the protein binding sites in pH 5.6 buffer.

Circular Dichroism. Near-UV CD spectra were obtained at 23 °C with a Jasco J-500C or Jasco J-700 spectropolarimeter using 1-cm path-length cells (2.5 mL for the Jasco J-500C and 0.9 mL for the J-700). The instrument was standardized with a 1 mg/mL aqueous solution of d_{10}-camphor sulfonic acid, as specified by the manufacturer. Data are presented as molar ellipticities. Sample solutions contained Con A, together with a large excess of carbohydrate to saturate the protein binding sites, in 0.1 M potassium acetate buffer, pH 5.6, containing 0.9 M KCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$.

Titration Calorimetry. Isothermal titration calorimetry was performed using the OMEGA microcalorimeter from Microcal, Inc. (Northampton, MA) as described in the preceding paper. Briefly, in each titration, 3 μL of saccharide solution was injected successively from the computer-controlled 100-μL microsyringe at an interval of 4 min into Con A solution (cell volume = 1.3424 mL) dissolved in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, while stirring at 350 rpm. Control experiments performed in absence of protein showed negligible ligand heat of dilution. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (the enthalpy change in cal mol^{-1}), K_a (the association constant in M^{-1}), and n (the number of binding sites per monomer) as adjustable 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.

Table 1: Inhibitory Potencies of the Saccharides in Figure 1 for Con A Mediated Hemagglutination of Rabbit Erythrocytes

saccharide	minimum concn required for complete inhibition[a]	relative inhibitory potency[b]
MeαMan	3.1 mM	1
Man(3,6)	25 μM	124
Glc(6)	0.15 mM	21
Glc(3)	0.88 mM	3.5
Gal(6)	0.68 mM	4.6
Gal(3)	1.0 mM	3.1
2-dMan(3,6)	25 μM	124
3-dMan(3,6)	0.29 mM	11
4-dMan(3,6)	26 μM	119
6-dMan(3,6)	32 μM	97

[a] In each case the lectin concentration was adjusted to a hemagglutination titer of 8. [b] All data normalized to that of MeαMan. Higher values indicate greater inhibitory potency.

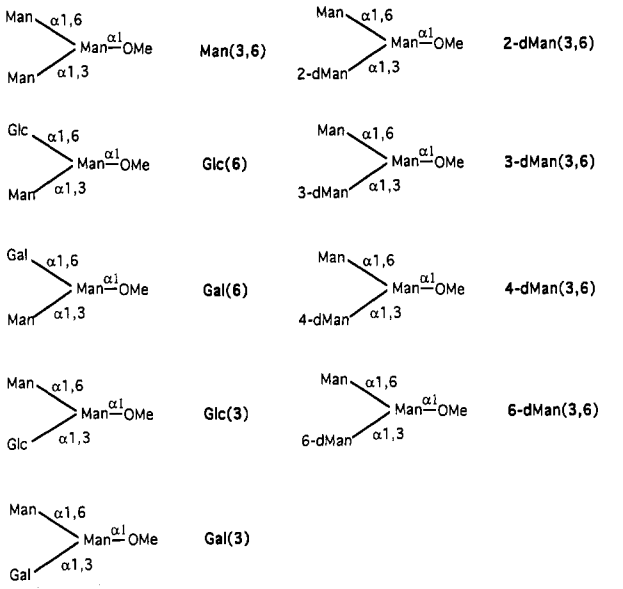

FIGURE 1: Structures of the trimannoside and its derivatives. Man, Glc, Gal, 2-dMan, 3-dMan, 4-dMan, and 6-dMan represent mannose, glucose, galactose, 2-deoxymannose, 3-deoxymannose, 4-deoxymannose, and 6-deoxymannose, respectively.

RESULTS

Hemagglutination Inhibition. Table 1 shows the relative binding affinities of the Glc and Gal analogs as well as deoxy analogs of Man(3,6) (Figure 1) for Con A, with respect to MeαMan, as determined by hemagglutination inhibition measurements. Man(3,6) binds with approximately 100-fold greater affinity than MeαMan, as previously reported (Brewer & Bhattacharyya, 1986). Substitution of Glc on the α(1–6) arm of the parent trimannoside to give Glc(6) results in a 6-fold reduction in binding affinity. However, substitution of Glc on the α(1–3) arm to give Glc(3) results in an affinity only slightly greater than MeαMan. Substitution of Gal on either arm of the trimannoside to give Gal(6) and Gal(3) also results in loss of high-affinity binding. Table 1 also shows that the 2-dMan(3,6), 4-dMan(3,6) and 6-dMan(3,6) derivatives of Man(3,6) possess the same inhibitory potencies as that of the parent trimannoside, but 3-dMan(3,6) shows a 11-fold loss in relative affinity.

NMRD Measurements. Figure 2 shows the NMRD profiles of Con A in the absence and presence of the Glc and Gal derivatives of Man(3,6) in Figure 1. Figure 2A shows data in the absence of saccharide, in the presence of Glc(6), and

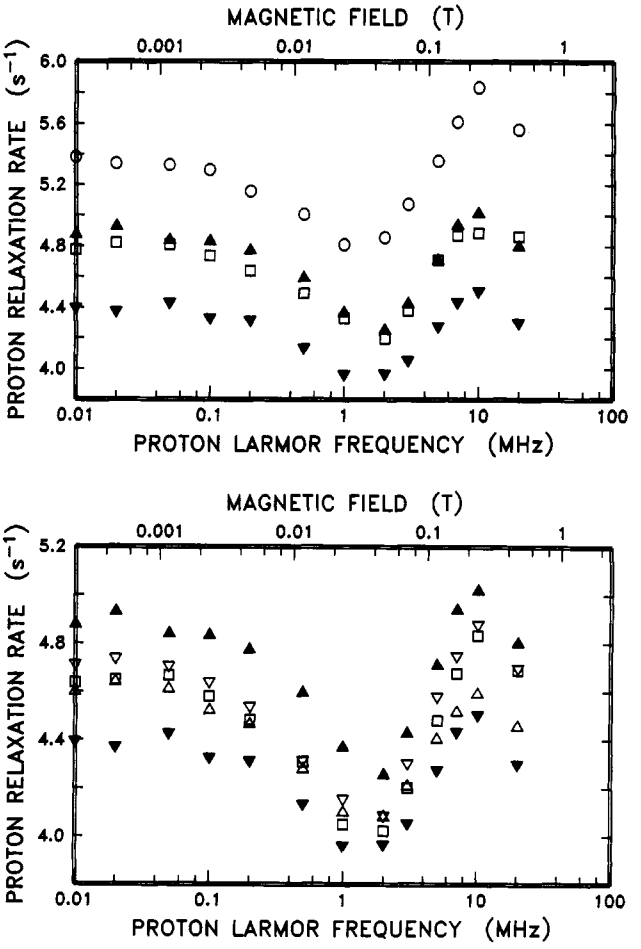

FIGURE 2: Solvent proton relaxation rates from 0.01 to 50 MHz of CMPL (0.40 mM) (A, top) at 25 °C in the absence (○) and presence of MeαMan (10 mM) (▼), Man(3,6) (10 mM) (▲), and Glc(6) (10 mM) (□); and (B, bottom) in the presence of MeαMan (10 mM) (▼), Man(3,6) (10 mM) (▲), Glc(3) (10 mM) (▽), Gal(3) (10 mM) (△), and Gal(6) (10 mM) (□).

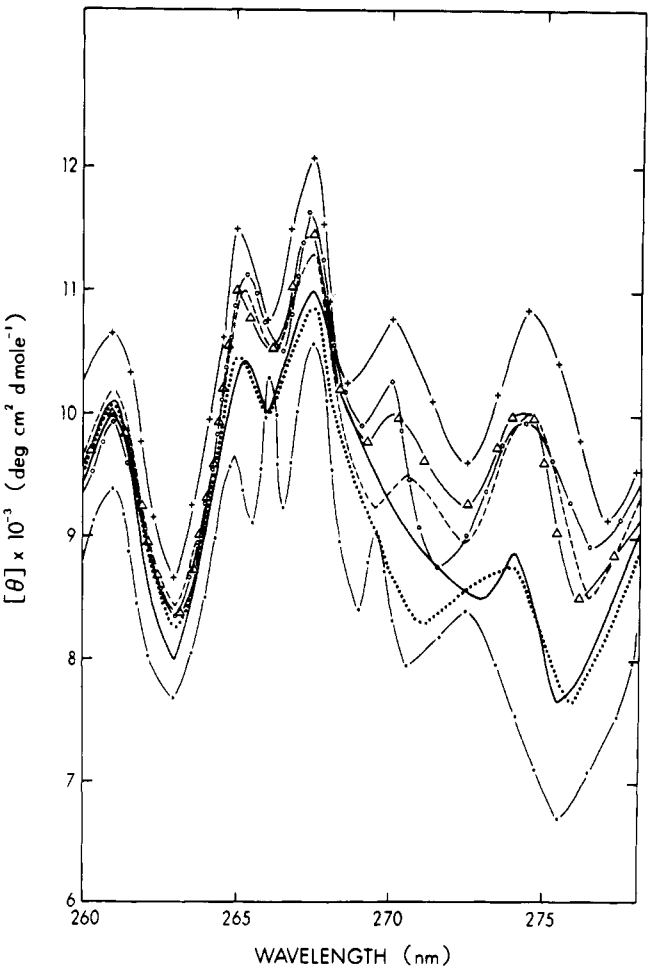

FIGURE 3: Near-UV circular dichroism molar ellipticity values of Con A (55 μM) in the absence (– · –) and presence of MeαMan (8.0 mM) (– + –), Glc(3) (1.3 mM) (- - -), Glc(6) (0.90 mM) (···), Gal-(3) (1.5 mM) (– ○ –), Gal(6) (1.4 mM) (– △ –), and Man(3,6) (0.60 mM) (—) at 23 °C.

in the presence of Man(3,6) and MeαMan for comparison. As previously found (Koenig et al., 1973; Brewer & Brown, 1979), binding of MeαMan results in a nearly 20% reduction in the NMRD profile of Con A at 25 °C, while Man(3,6) produces a nearly 10% reduction (Brewer & Bhattacharyya, 1986). Glc(6) shows a reduction in the profile similar to that for Man(3,6). The same results were obtained at 5 °C (not shown). Figure 2B shows the NMRD profiles of Con A in the presence of Glc(3), Gal(6), and Gal(3) at 25 °C. The profiles for all three are between those of Man(3,6) and MeαMan. Similar results were obtained at 5 °C (not shown). The NMRD profiles of Con A at 25 °C in the presence of 2-dMan(3,6), 3-dMan(3,6), 4-dMan(3,6), and 6-dMan(3,6) (Figure 1) are all similar to that of Man(3,6) (not shown).

Near-UV CD Measurements. The CD spectra of Con A in the near-UV region in the absence and presence of the Glc and Gal derivatives of Man(3,6) at 23 °C are shown in Figure 3. As previously shown, binding of MeαMan increases the molar ellipticity values of the protein by about 17% (Bhattacharyya et al., 1991; Pflumm et al., 1971; Young et al., 1982). In addition, the band at 267 nm disappears, and there are small shifts in the bands at 270 and 273 nm. Binding of Man(3,6) induces approximately half of the increase in molar ellipticity values as that of MeαMan, as we have recently reported (Bhattacharyya et al., 1991). In addition, the band at 270 nm of the lectin is absent in the presence of Man(3,6).

Binding of Glc(6) induces a change in the CD spectrum of Con A similar to that of Man(3,6) (Figure 3). However, binding of Glc(3), Gal(6), or Gal(3) produce changes in the ellipticity values of Con A in the region 265 to 280 nm that are between those induced by Man(3,6) and MeαMan. In addition, in the presence of Glc(3), Gal(6), and Gal(3) the band at 271 nm observed in the MeαMan complex is also observed, although less intensely. The CD spectrum of Con A in the presence of 2-dMan(3,6) is essentially superimposable on that of Man(3,6) (not shown).

Titration Calorimetry. The concentrations of lectin and saccharide in the individual titrations and the thermodynamic parameters derived for the carbohydrates are shown in Table 2. For multiple determinations at either the same or different protein concentrations, the average deviations are typically 1–4% for all three parameters. The results show that Glc(6) binds with a ΔH of –12.5 kcal mol^{-1}, which is about 2 kcal mol^{-1} lower than that of Man(3,6), and that Glc(6) possesses a K_a about 3.5-fold lower than that of the trimannoside and 18-fold higher than MeαMan (Table 2). Gal(6), Glc(3), and Gal(3) bind to Con A with similar ΔH values (~11 kcal mol^{-1}) and K_a values that are similar and only 5-fold higher than MeαMan (Table 2). The relative K_a values agree with those determined by hemagglutination inhibition measurements (Table 1).

The results for binding of a series of α(1–3)deoxy analogs of Man(3,6) (Figure 1) show that 2-dMan(3,6), 4-dMan-(3,6), and 6-dMan(3,6) have essentially same values for ΔH and K_a as the trimannoside but that 3-dMan(3,6) binds with

Table 2: Thermodynamic Parameters Derived for the Binding of Con A with Man(3,6) and Its Derivatives Obtained by Titration Microcalorimetry at 25 °C[a]

carbohydrate	carbohydrate concn (mM)	lectin concn (mM)	K_a[b] (M^{-1})
MeαMan[c]	46.0	0.483	0.82 (±0.02) × 10^4
Man(3,6)[c]	7.04	0.132	4.90 (±0.15) × 10^5
Glc(6)	8.42	0.247	1.48 (±0.05) × 10^5
Gal(6)	12.8	0.247	4.35 (±0.13) × 10^4
Glc(3)	14.8	0.245	4.25 (±0.14) × 10^4
Gal(3)	13.7	0.249	4.81 (±0.14) × 10^4
2-dMan(3,6)	6.20	0.136	5.68 (±0.29) × 10^5
3-dMan(3,6)	9.0	0.140	5.39 (±0.26) × 10^4
4-dMan(3,6)	7.20	0.165	5.02 (±0.15) × 10^5
6-dMan(3,6)	6.40	0.154	3.96 (±0.12) × 10^5

carbohydrate	$-\Delta H$[b] (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
MeαMan[c]	8.2 (±0.1)	2.9
Man(3,6)[c]	14.4 (±0.1)	6.6
Glc(6)	12.5 (±0.1)	5.4
Gal(6)	10.5 (±0.1)	4.2
Glc(3)	11.0 (±0.1)	4.7
Gal(3)	11.0 (±0.1)	4.6
2-dMan(3,6)	14.5 (±0.1)	6.7
3-dMan(3,6)	11.0 (±0.2)	4.6
4-dMan(3,6)	14.3 (±0.1)	6.5
6-dMan(3,6)	14.0 (±0.1)	6.4

[a] Values of n were between 0.95 and 1.05 in all cases. [b] Values in parentheses indicate the standard deviation of fit between the experimental binding curve and the calculated curve obtained with the fitted thermodynamic parameters. 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] Data taken from the preceding paper.

a lesser enthalpy change (ΔH = −11 kcal mol^{-1}) and lower affinity (K_a = 5.4 × 10^4 M^{-1}) relative to Man(3,6) (Table 2).

DISCUSSION

In the previous paper in this issue (Mandal et al., 1994), Man(3,6) was shown to bind to Con A with ~6 kcal mol^{-1} greater enthalpy change relative to MeαMan, indicating extended site binding interactions with the trimannoside. In order to probe the nature of these extended site interactions, we have investigated the binding of the Glc and Gal analogs of Man(3,6) in Figure 1 to the lectin using a combination of hemagglutination inhibition, titration microcalorimetry, CD, and NMRD measurements. The results with the Glc and Gal analogs, in turn, led to the synthesis of α(1–3) 2-, 3-, 4-, and 6-deoxy derivatives of Man(3,6) (Figure 1) and studies of their interactions with the lectin. The findings of this study have provided new insights into the extended binding site of Con A for N-linked carbohydrates.

Binding of Glc(6), Glc(3), Gal(6), and Gal(3) to Con A. Using a precipitation inhibition assay, Goldstein and co-workers (Kaku et al., 1991) recently demonstrated that analogs of Man(3,6) containing either Glc or Gal substituted on the α(1–6) or α(1–3) arms (Figure 1) possessed substantially lower affinities for Con A. The results of hemagglutination inhibition studies (Table 1) confirm the pattern of binding of the derivatives as observed by these workers. Substitution of Glc on the α(1–6) arm results in about a 5–6-fold loss of binding, relative to Man(3,6), while substitution of Glc on the α(1–3) arm results in a larger loss in affinity. Substitution of Gal on either arm also results in a substantial loss in affinity for the lectin. In order to gain greater insight into the molecular basis of their binding interactions with the lectin, NMRD, CD, and titration calorimetry studies were performed.

NMRD and CD Measurements. Nuclear magnetic relaxation dispersion (NMRD) measurements, which record the magnetic field dependence of solvent proton relaxation (generally spin–lattice), have previously been used to investigate the saccharide binding properties of Con A (Brewer & Brown, 1979; Brewer & Bhattacharyya, 1986, 1988; Bhattacharyya et al., 1991). The NMRD profile of CMPL shows a strong paramagnetic relaxation contribution due to exchange of the water ligands at both metal ion sites (Koenig et al., 1973). The NMRD profile of the lectin has also been shown to be sensitive to conformational changes induced in the protein by saccharide binding (Koenig et al., 1973; Brewer & Brown, 1979). Binding of monosaccharides such MeαMan and simple linear oligosaccharides with nonreducing α-Glc or α-Man residues uniformly reduces the NMRD profile of the protein at 25 °C by approximately 20%, as shown in Figure 2A for MeαMan. However, a series of high-affinity N-linked oligosaccharides and glycopeptides induce a smaller reduction (approximately 10%) in the paramagnetic relaxtivity values of Con A (Brewer & Bhattacharyya, 1986, 1988; Bhattacharyya et al., 1991). The trimannoside 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose [and Man(3,6)] was also observed to induce the same change in the NMRD profile as the larger N-linked glycopeptides [see Figure 2A for Man(3,6)]. The near-UV CD spectrum of the protein in the presence of the trimannoside or oligomannose type glycopeptides also shows similar smaller changes in the molar ellipticity values as compared to MeαMan (Bhattacharyya et al. 1991). Thus, the NMRD and CD profiles of the lectin indicate unique interactions between Man(3,6) and Con A, as observed with larger N-linked carbohydrates. These unique interactions correlate with the greater ΔH of Man(3,6) (−14.4 kcal mol^{-1}) and certain N-linked carbohydrates to Con A (Mandal et al., 1994), relative to MeαMan (−8.2 kcal mol^{-1}) and appear to be a marker of extended binding site interactions between the protein and these carbohydrates.

The NMRD profile of Con A in the presence of Glc(6) (Figure 2A) is very similar to that for Man(3,6). This indicates that the conformational changes induced in the protein upon saccharide binding are similar for the two carbohydrates, which, in turn, suggest similar binding modes. The CD spectra of the protein in the presence of the two carbohydrates are also similar (Figure 3), consistent with the NMRD data. The NMRD data for Glc(3), Gal(6), and Gal(3) in Figure 2B show profiles between those of Man(3,6) and MeαMan, suggesting somewhat different binding modes than either of the latter two carbohydrates. The CD spectra of the lectin in the presence of Glc(3), Gal(6), and Gal(3) also show similar intermediate ellipticity values.

Examination of the calorimetry data for the derivatives in Table 2 shows that K_a for Glc(6) binding is about 3.5-fold lower than that of Man(3,6), which is consistent with the hemagglutination inhibition data in Table 1 and the results of Goldstein and co-workers (Kaku et al., 1991). The ΔH for Glc(6) binding is −12.5 kcal mol^{-1} compared to −14.4 kcal mol^{-1} for Man(3,6). These differences in the ΔH and affinity between Glc(6) and Man(3,6) are similar to the ~2 kcal mol^{-1} lower ΔH and ~4-fold lower affinity of MeαGlc relative to MeαMan (Mandal et al., 1994; Goldstein & Poretz, 1986; Munske et al., 1984). Since MeαGlc and MeαMan bind to the monosaccharide site in Con A, this suggests that the Glc moiety of Glc(6) binds to the monosaccharide site and that the α(1–3) arm binds to a secondary site. By analogy, the α(1–6) arm of Man(3,6) is also expected to preferentially bind to the monosaccharide site and the α(1–3) arm to a secondary site. Since MeαGlc and MeαMan induce the same conformational change in the protein (Brewer & Brown, 1979), the similar NMRD profiles and CD spectra for Glc(6) and Man(3,6) support these conclusions. These results help explain

the loss in binding affinity of Gal(6) since Gal is known not to bind well to the monosaccharide site of Con A (Brewer & Brown, 1979; Goldstein et al., 1965). These conclusions also agree with previous NMR studies of the binding of Man(3,6) to Co^{2+}-substituted Con A (Carver et al., 1985).

The K_a values determined by titration microcalorimetry for Glc(3), Gal(6), and Gal(3) (Table 2) are only 5–6-fold higher than that of MeαMan, which is consistent with the hemagglutination inhibition data in Table 1 and the results by Goldstein and co-workers (Kaku et al., 1991). The ΔH values for Glc(3) and Gal(3) are –11 kcal mol^{-1}, and the ΔH value is –10.5 kcal mol^{-1} for Gal(6) (Table 2). These results show that Glc(3) and Gal(3) bind with 10–12-fold lower affinity and ~3.4 kcal mol^{-1} lesser ΔH than Man(3,6), while Gal(6) binds with ~12-fold lower affinity and nearly 4 kcal mol^{-1} lesser ΔH. Since the only difference between Glc and Man is the configuration of the 2-hydroxyl group (equatorial for Glc, axial for Man), the results suggest that the 2-OH on the α(1–3) arm of Man(3,6) may be important for binding at the secondary site of Con A. The similar affinity and ΔH for Gal(3) is understandable since Gal possesses the same configuration at the 2-OH as well as a change in configuration at the 4-OH (axial rather than equatorial).

Binding of 2-dMan(3,6), 3-dMan(3,6), 4-dMan(3,6), and 6-dMan(3,6). In order to test the possibility that the axial 2-OH of the α(1–3)Man residue in Man(3,6) is involved in extended site binding to Con A, we have synthesized and determined the NMRD profile, CD spectrum, and thermodynamics of binding of the 2-deoxy α(1–3) derivative, 2-dMan-(3,6) (Figure 1), with Con A. The NMRD profile as well as the CD spectrum of the lectin in the presence of 2-dMan(3,6) are essentially the same as that of Man(3,6) (not shown). Titration microcalorimetry shows that ΔH is –14.5 kcal mol^{-1} and K_a is 5.7×10^5 M^{-1} for 2-dMan(3,6), which are thermodynamic values essentially identical to those for Man-(3,6) (Table 2). The relative K_a values are also consistent with the hemagglutination inhibition data in Table 1. These results demonstrate that the 2-OH of the α(1–3)Man residue of Man(3,6) is not involved in binding to Con A and that the equatorial 2-OH of the Glc residue of Glc(3) makes unfavorable interactions with the lectin which result in loss of affinity. This also explains the loss of binding of Gal(3). Since the 2-OH of the α(1–3) arm of Man(3,6) does not contribute to binding but the α(1–3)Man residue does in some manner, the 3-, 4-, and 6-deoxy derivatives of the α(1–3) residue were synthesized in order to evaluate the respective binding contributions of the other hydroxyl group of this residue.

The NMRD profiles of Con A in the presence of 3-dMan-(3,6), 4-dMan(3,6), and 6-dMan(3,6) indicate similar conformational changes induced in the protein upon binding of the three carbohydrates. However, the calorimetry data clearly show that while the 4- and 6-deoxy analogs possess ΔH and K_a values similar to those of Man(3,6), the 3-deoxy derivative, 3-dMan(3,6), binds with a 10-fold lower K_a and 3.4 kcal mol^{-1} lesser ΔH. The relative K_a values are also consistent with the relative binding affinities determined by hemagglutination inhibition measurements (Table 1). This indicates that the 3-OH of the α(1–3)Man residue of Man-(3,6) is involved in binding to Con A at a secondary site in addition to the 3-, 4-, and 6-OH groups of the α(1–6)Man residue at the monosaccharide site [by analogy to the requirements for monosaccharide binding (Goldstein & Poretz, 1986)]. Because of the magnitude of the loss in the change of the binding enthalpy (3.4 kcal mol^{-1}) of 3-dMan(3,6) relative to Man(3,6), the extended binding site of Con A appears to include hydrogen bonding of a residue(s) of the lectin with

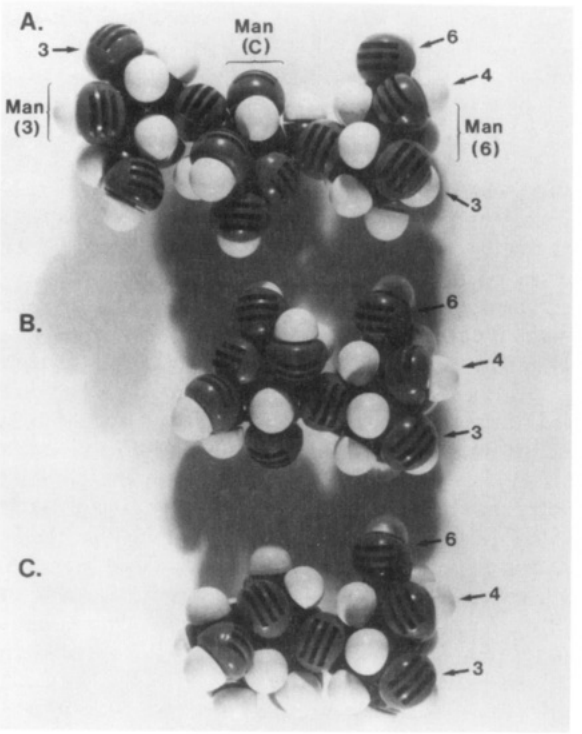

FIGURE 4: Corey–Pauling–Koltun space-filling model of (A) Man-(3,6) in the $\omega = 180°$ conformation (ω is the dihedral angle formed by H-5, C-5, C-6, and O-6 of the "core" Man residue); (B) the disaccharide α(1–3)mannobiose [**5** in the preceding paper (Mandal et al., 1994)]; and (C) the disaccharide α(1–2)mannobiose (**2** in the preceding paper). The "C" in panel A represents the "core" Man residue of Man(3,6).

the 3-OH of the α(1–3)Man residue of Man(3,6) (Wells & Fersht, 1986). This extended site interaction would also be expected to be present in the binding of larger N-linked type carbohydrates such as complex type and oligomannose type carbohydrates to Con A and responsible, in part, for their high-affinity interactions with the lectin.

Presence of an Additional Contact Site in Man(3,6) for Binding to Con A. The ΔH of –11 kcal mol^{-1} for 3-dMan-(3,6), although less than the –14.4 kcal mol^{-1} of Man(3,6), is still nearly 3 kcal mol^{-1} greater than that of MeαMan (–8.2 kcal mol^{-1}) (Mandal et al., 1994). This suggests that there is yet another site on Man(3,6) which is responsible for the extra 3 kcal mol^{-1} change in binding enthalpy. In fact, several oligosaccharides in the preceding study (Mandal et al., 1994) all showed ΔH values close to –10.5 to –11 kcal mol^{-1}, including the α(1–2)dimannoside, methyl α(1–2)mannopyranosyl-α-mannopyranoside (**2**); the α(1–2)trimannoside, methyl α(1–2)mannopyranosyl-α(1–2)mannopyranosyl-α-mannopyranoside (**3**); the α(1–3)dimannoside, methyl α(1–3)mannopyranosyl-α-mannopyranoside (**5**), and the biantennary complex type carbohydrate with terminal GlcNAc residues (**11**). In fact, the plot of ΔH versus $T\Delta S$ for many of the carbohydrates in the preceding paper (Figure 3 in that paper) shows a horizontal line through the data points for the above carbohydrates (**2**, **3**, **5**, and **11**) which intersects the ordinate at a ΔH value of ~10.5 kcal mol^{-1}. These results suggest a common mechanism of binding of the oligosaccharides involving extended site interactions with the lectin.

Examination of Corey–Pauling–Koltun (CPK) space-filling models of Man(3,6), the α(1–3)dimannoside (**5**), and α(1–2)dimannoside (**2**) are shown in Figure 4. The nonreducing terminal Man residues of disaccharides **2** and **5** were aligned with the α(1–6)Man residue of Man(3,6) since evidence suggests that these residues in the disaccharides bind to the

monosaccharide site (Bhattacharyya et al., 1987a). Although the conformations of the disaccharides are relatively rigid (cf. Brisson & Carver, 1983a), the α(1–6) arm of Man(3,6) is flexible and was assigned the $\omega = 180°$ conformation [ω is the dihedral angle formed by H-5, C-5, C-6, and O-6 (Brisson & Carver, 1983b)], based on our previous studies of the binding of the trimannoside to Con A (Bhattacharyya et al., 1987b). Of interest are the relative locations of the hydroxyl groups and ring oxygen atom of the central "core" Man residue of Man(3,6) (Figure 4A) and the hydroxyl groups or ring oxygen atom on the reducing Man residues of both the α(1–3)-dimannoside (**5**) and α(1–2)dimannoside (**2**) (Figure 4, panels B and C, respectively). It is possible that one of these groups represents a common locus of binding to Con A and that this third site in Man(3,6) could be responsible for the ~3 kcal mol^{-1} difference in ΔH between 3-dMan(3,6) and MeαMan. This site in **2** and **5** (and **3**) could be responsible for their greater change in binding enthalpies (~3 kcal mol^{-1}) relative to MeαMan. Preliminary evidence suggests that the 2- and 4-hydroxyl groups of the "core" Man residue of Man(3,6) are not involved in extended site interactions (S. Oscarson and C. F. Brewer, unpublished results). However, determination of this apparent additional site of binding will have to await the synthesis of the entire set of deoxy analogs of the "core" residue of Man(3,6), as well deoxy analogs of the reducing residues of **2** and **5**. Nevertheless, the high-affinity binding site on Con A for Man(3,6) appears to be comprised of at least three subsites: one higher affinity primary binding site (the "monosaccharide" site) and two weaker secondary binding sites. It may be noted that a "three subsite" structural model has been proposed for high-affinity binding of *N*-acetylglucosamine oligomers to the wheat germ agglutinin (Allen et al., 1973; Bains et al., 1992).

Finally, although the monosaccharide binding site in Con A has been identified by X-ray crystallographic studies (Derewenda et al., 1989), the secondary binding sites that interact with the α(1–3)Man residue (and presumably the core Man) of Man(3,6) and related N-linked carbohydrates have not been similarly identified. Interestingly, while the backbone amide –NH of Arg 228 appears to bind to the O3 oxygen atom of an α-mannopyranosyl residue at the monosaccharide site, the side chain of Arg 228 is not involved in such interactions (Derewenda et al., 1989). Furthermore, this residue is absent in other lectins such as the pea and lentil lectins which have similar monosaccharide binding specificities as Con A but do not bind the trimannoside with high affinity (Strosberg et al., 1986). The results of the present study may help determine the location of these additional interactions.

Summary. X-ray crystallographic studies (Derewenda et al., 1989; Hardman et al., 1982) together with data on the binding of a variety of mono- and oligosaccharides (cf. Goldstein et al., 1965) demonstrate that binding at the monosaccharide site in Con A involves strong hydrogen-bonding interactions with the 3-, 4-, and 6-hydroxyl groups of Glc and Man residues. Present findings indicate that secondary binding sites exist on the lectin that provide strong hydrogen-bonding interactions with the 3-hydroxyl of the α-(1–3)Man residue and possibly another site on the "core" Man residue of Man(3,6). These additional interactions are largely responsible for the high-affinity binding of N-linked carbohydrates to Con A.

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