Specificity of *C*-Glycoside Complexation by Mannose/Glucose Specific Lectins†

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ABSTRACT: The binding of the mannose/glucose specific lectins from *Canavalia ensiformis* (concanavalin A) and *Dioclea grandiflora* to a series of *C*-glucosides and mannosides was studied by titration microcalorimetry and fluorescence anisotropy titration. These closely related lectins share a specificity for the trimannoside methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside, and are a useful model system for addressing the feasibility of differentiating between lectins with overlapping carbohydrate specificities. The ligands were designed to address two issues: (1) how the recognition properties of non-hydrolyzable *C*-glycoside analogues compare with those of the corresponding *O*-glycosides and (2) the effect of presentation of more than one saccharide recognition epitope on both affinity and specificity. Both lectins bind the *C*-glycosides with affinities comparable to those of the *O*-glycoside analogues; however, the ability of both lectins to differentiate between *gluco* and *manno* diastereomers was diminished in the *C*-glycoside series. Bivalent norbornyl *C*-glycoside esters were bound by the lectin from *Canavalia* but only weakly by the lectin from *Dioclea*. In addition to binding the bivalent ligands, concanavalin A discriminated between C-2 epimers, with the *manno* configuration binding more tightly than the *gluco*. The stoichiometry of binding of the bivalent ligands to both di- and tetrameric lectin was two binding sites per ligand, rather than the expected 1:1 stoichiometry. Together, these results suggest that concanavalin A may possess more than one class of carbohydrate binding sites and that these additional sites show stereochemical discrimination similar to that of the previously identified monosaccharide binding site. The implications of these findings for possible *in vivo* roles of plant lectins and for the use of concanavalin A as a research tool are discussed.

Protein−carbohydrate interactions mediate critical biological recognition processes, such as those involved in cell signaling, organogenesis, fertilization, and inflammation (Lis & Sharon, 1986; Feizi & Childs, 1987; Sharon & Lis, 1989; Stoolman, 1989; Bertels et al., 1991; Drickamer & Taylor, 1993). Additionally, carbohydrate−protein interactions facilitate the initial attachment of pathogens to host cells (Karlsson, 1989; Manocha & Chen, 1989; Anderson et al., 1991; Ingram & Molyneux, 1991) and the metastasis and growth of malignant cancer cells (Takeichi, 1991; Liener et al., 1986; Itzkowitz et al., 1990; Gabius & Gabius, 1991; Hiraizumi et al., 1992; Muramatsu, 1993). The identification of protein−carbohydrate interactions in human disease raises the possibility of the development of carbohydrate-based therapeutic products, but fundamental barriers to the development of such agents remain. One of the most severe is specificity. Many lectins exhibit overlapping selectivities for particular carbohydrate structures, and the same carbohydrate determinant is often bound by multiple lectins. Because of the important roles of protein−carbohydrate interactions in normal human biology, the need for specificity in carbohydrate-based therapeutics is of paramount importance.

An impediment to the development of carbohydrate therapeutics is the susceptibility of *O*-glycosidic linkages to both chemical and enzymatic degradation. Advances in the synthesis of *C*-glycosides have resulted in the generation of a variety of hydrolytically stable saccharide analogs (Kessler et al., 1992; Potsema, 1992; Smoliakova et al., 1995; Wang & Gross, 1995; Wei et al., 1995; Potsema, 1995). Although initial reports suggest that *C*-glycosides behave similarly to *O*-glycosides in binding viral and bacterial lectins in adhesion assays (Bertozzi & Bednarski, 1992; Bertozzi et al., 1992; Nagy et al., 1992; Sparks et al., 1993), little information regarding the thermodynamics of *C*-glycoside binding has been reported. An understanding of the underlying structural and energetic features that facilitate the specificity of binding are necessary for the development of *C*-glycosides as effective modulators of cell−cell interactions.

Recently, we have exploited a model system of plant lectins to explore the determinants of binding specificity (Chervenak & Toone, 1995; Mortell et al., 1994). To investigate the feasibility of developing highly specific ligands for a single lectin, we continue this work on the most challenging pair of lectins, the lectins from *Canavalia ensiformis* and from *Dioclea grandiflora*. The widely studied lectin from *Canavalia ensiformis*, or concanavalin A, is a 26 000 MW monomer that exists as a dimer at low (<5.5) pH and as a tetramer at high (>7) pH (Bittiger & Schnebli, 1976). Each subunit contains a binding site that binds ligands with unmodified hydroxyls at positions 3, 4, and 6 of an α-gluco- or α-mannopyranose, showing an approximate

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4-fold preference for the *manno* configuration at C-2 (Goldstein et al., 1965). In 1985, Brewer and co-workers reported that the trimannoside methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside binds approximately 100-fold more tightly than does methyl-α-D-mannopyranoside (Brewer et al., 1985).

The lectin from the Brazilian legume *Dioclea grandiflora* is highly homologous both in structure and carbohydrate binding specificity to concanavalin A. This lectin differs from concanavalin A in only 59 of 237 amino acid residues and shares a specificity for the trimannoside, yet it exists as a tetramer at all pH values (Moreira et al., 1983; Richardson et al., 1984). Previous studies have shown that although concanavalin A and the lectin from *Dioclea* display similar binding selectively for *O*-linked saccharides, the two lectins show significant differences in their mechanisms of carbohydrate binding (Chervenak & Toone, 1995). A comparison of the corresponding thermodynamics for the interaction of the lectin from *Dioclea* and concanavalin A with *C*-glycosides might provide additional insight into the differences in the modes by which these lectins bind their substrates.

Here we report the binding behavior of a group of mono- and bivalent *C*-glycosides to concanavalin A and the lectin from *Dioclea* using both titration microcalorimetry and fluorescence anisotropy. Our results show that although many aspects of the *C*-glycoside−lectin interaction are similar to those of the corresponding *O*-glycoside binding, there exist several useful, exploitable differences in the interaction. Secondly, we have used bivalent *C*-glycosides to differentiate between lectins with both overlapping binding specificities and structural similarities. Finally, we have potentially identified a second class of carbohydrate binding sites on the plant lectin concanavalin A. (See Figure 1.)

Materials and Methods. Concanavalin A and the lectin from *Dioclea grandiflora* were isolated from seed meal by affinity chromatography on Sephadex G75 as previously described. Succinyl concanavalin A, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, methyl 3-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside, methyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside, and methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside were either purchased from commercial sources and used without further purification or prepared *via* literature methods (Chervenak & Toone, 1995). All compounds were >95% pure as determined by ^{1}H NMR. The concentration of native concanavalin A was determined spectrophotometrically at 280 nm using $A^{1\%,1cm} = 13.7$ at pH 7.2 and expressed in terms of the monomer ($M_r = 26\,500$) (Goldstein & Poretz, 1986). The concentration of succinyl concanavalin A was determined spectrophotometrically at 280 nm using $A^{1\%,1cm} = 13.7$ at pH 7.2 and expressed in terms of the monomer ($M_r = 28\,000$) (Gunther et al., 1973). Ligands **1**, **5**, and **7** were prepared by literature protocols (Mortell et al., 1994), and compounds **2** and **6** were generated by methods analogous to those previously described. The syntheses of **3** and **4** (Weatherman & Kiessling, 1996) and of Fl-glc **8** and Fl-man **9** will be reported elsewhere (R. Weatherman and L. Kiessling, unpublished results). The fluorescein derivatives were generated as a mixture of regioisomers.

Titration Microcalorimetry. Calorimetric titrations were performed using a Microcal Omega titration microcalorimeter; details of the instrument and data reduction have been

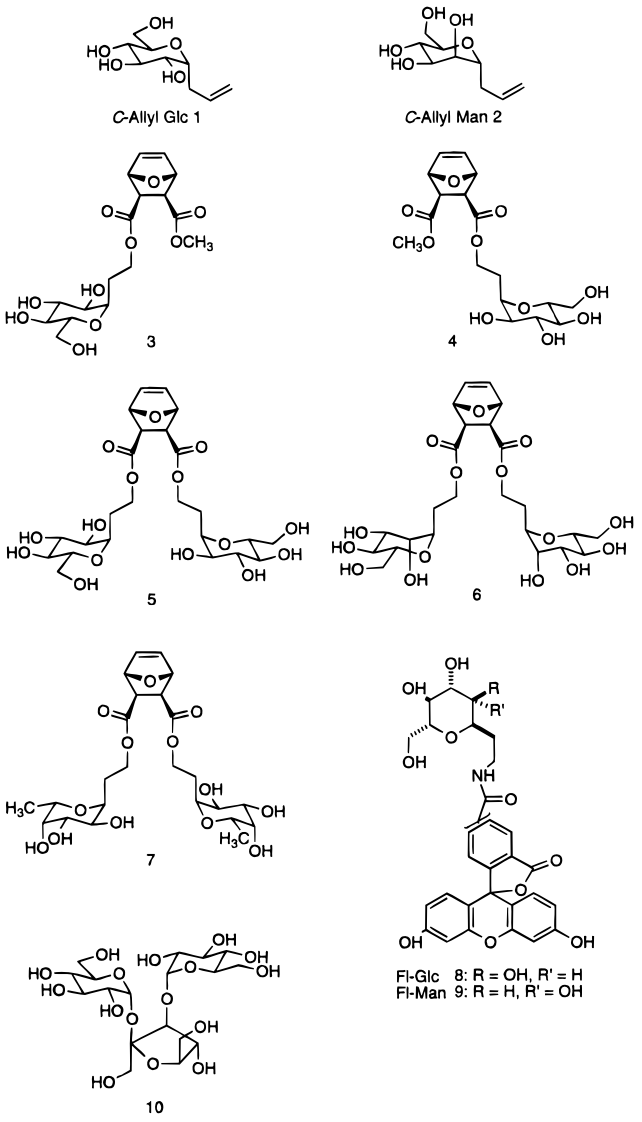

FIGURE 1: Ligands used in this study.

reported elsewhere (Wiseman et al., 1989). Specific details of the use of the instrument for studying protein−carbohydrate interactions have also been reported elsewhere (Chervenak & Toone, 1995). Titrations were carried out either at pH 5.2 in 3,3-dimethylglutarate buffer (50 mM) augmented with NaCl (250 mM), $CaCl_2$ (1 mM), and $MnCl_2$ (1 mM) or at pH 7.4 in phosphate buffer (50 mM) augmented with NaCl (500 mM), $CaCl_2$ (1 mM), and $MnCl_2$ (1 mM).

Protein concentrations in the titration calorimetry experiments ranged from 0.25 to 1.0 mM, as determined by the method of Edelhoch (1962), and the carbohydrate concentration used in each titration was 20 mM. In all cases, the product of the binding constant and the concentration of binding sites was in the range 10−100. Ligand concentrations were generally 25 times greater than protein concentrations. Ligand was added in 2.2 μL aliquots, and in all cases the final ligand concentration in the cell was at least $10K_D$.

Fluorescence Anisotropy Measurements. Detailed protocols for fluorescence anisotropy measurements will be reported elsewhere (Weatherman & Kiessling, 1996). Briefly, fluorescence anisotropy measurements were made of native concanavalin A binding to both Fl-glc and Fl-man and succinyl concanavalin A binding to Fl-man in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM $CaCl_2$, 1 mM

$MnCl_2$, and 200 nM ligand by varying the protein concentration from 1 to 480 μM. The binding energies were then determined using the BIOEQS curve-fitting program (Royer & Beechum, 1992).

Competition titration experiments of nonfluorescent ligands were performed using 480 μM concanavalin A (native or succinylated) in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 200 nM of Fl-man. The unlabeled ligand concentration was then gradually increased until further addition of ligand failed to significantly affect the anisotropy measurement. The dissociation constants of nonfluorescent ligands were obtained by fitting the competition curves as previously described (Weinhold & Knowles, 1992). A key asset of this assay is its ability to report on specific ligand binding at particular sites through the use of competition experiments.

Fluorescent Light Scattering Measurements. The relative aggregation state of concanvalin A in the presence of carbohydrate ligands was analyzed by observing fluorescent light scattering with emission and excitation at 350 nm (Rozema & Gellman, 1995). The experiments were performed using solutions composed of 0.23 mM concanavalin A in 50 mM phosphate buffer (pH 7.4) and 500 mM NaCl. The sample concentration of carbohydrate ligands was increased in 5 mM increments until further addition of ligand failed to significantly affect the intensity. All components of the solutions were filtered through a 0.22 micron cellulose acetate filter before use.

RESULTS AND DISCUSSION

Recognition Specificity of Monovalent C-Glycosides. Fluorescence anisotropy is a sensitive technique that is well-suited for monitoring carbohydrate−protein interactions, which are typically weak. By following the anisotropy changes of a fluorophore attached to a carbohydrate ligand in the presence of a lectin, the equilibrium binding constant for the interaction can be obtained (Weinhold & Knowles, 1992; Jacob et al., 1995; Weatherman & Kiessling, 1996). In addition, when applied to competition assays fluorescence anisotropy can provide information regarding the specificity of ligands for particular sites. Using this method, association constants for fluorescein-labeled glucose and mannose binding to tetrameric concanavalin A were determined (Table 1). Competition binding experiments with known ligands for concanavalin A showed that the fluorescent ligands were binding specifically to the lectin (Weatherman & Kiessling, 1996). Additionally, competition experiments were applied to determine the binding constants of various *C*- and *O*-glycosides, and the results from fluorescence anistropy were in good agreement with those from microcalorimetry (Table 1). In all cases where a binding constant could be determined, the unlabeled ligands competed directly for the binding site with the fluorescent ligand, indicating that all ligands were binding specifically to concanavalin A.

Thorough characterization of the thermodynamic parameters for *O*- and *C*-glycoside binding to glucose/mannose specific lectins was accomplished by microcalorimetry. Titration calorimetry is the only experimental technique that directly measures reaction enthalpies; consequently, it has been used recently to examine the energetics of protein−carbohydrate interactions (Ambrosino et al., 1987; Bain et al., 1992; Williams et al., 1992; Schwartz et al., 1991, 1993; Mandal et al., 1994; Chervenak & Toone, 1995). Binding constants, free energies, and enthalpies determined for binding of various ligands to concanavalin A or the lectin from *Dioclea* are shown in Tables 2 and 3, respectively.

Concanavalin A and the lectin from *Dioclea* both bind methyl α-D-glucopyranoside and methyl α-D-mannopyranoside with millimolar dissociation constants. Both lectins show a roughly fourfold preference for the *manno* configuration at C-2. The diastereotopic distinction between *gluco* and *manno* stereochemistry is lost on conversion to the analogous *C*-glycoside series. Although the *C*-allyl glucoside

Table 1: Equilibrium Binding Constants for Concanavalin A Complexation with *O*- and *C*-Glycosides Measured by Titration Microcalorimetry and Fluorescence Anisotropy Competition Assays

carboydrate	protein[a]	K_{eq} (M^{-1}) (calorimetry)	K_{eq} (M^{-1}) (fluorescence anisotropy)
Fl-Glc	tetramer	ND[b]	$(1.4 \pm 1.0) \times 10^3$
Fl-Man	tetramer	ND[b]	$(5.5 \pm 1.0) \times 10^3$
α-MeGlc	tetramer	$(2.4 \pm 0.1) \times 10^3$	$(1.7 \pm 1.0) \times 10^3$
α-MeMan	tetramer	$(7.6 \pm 0.2) \times 10^3$	$(5.5 \pm 1.0) \times 10^3$
1	tetramer	$(4.5 \pm 0.4) \times 10^3$	$(2.8 \pm 1.0) \times 10^3$
2	tetramer	$(5.2 \pm 0.4) \times 10^3$	$(3.9 \pm 1.0) \times 10^3$
3/4	tetramer	NB[c]	ND[b]
5	tetramer	$(4.7 \pm 0.4) \times 10^3$	precipitation
6	tetramer	$(2.9 \pm 0.4) \times 10^4$	precipitation
7	tetramer	NB[c]	ND[b]
5	dimer	$(3.6 \pm 0.1) \times 10^3$	$(3.9 \pm 1.0) \times 10^3$
6	dimer	$(5.0 \pm 0.1) \times 10^3$	$(7.5 \pm 1.0) \times 10^3$
10	dimer	$(6.2 \pm 0.3) \times 10^3$	ND[b]
10	tetramer	$(6.2 \pm 0.3) \times 10^3$	ND[b]

[a] Tetrameric protein was native concanavalin A, pH 7.4; dimeric protein was succinyl concanavalin A, pH 7.4. [b] Not determined. [c] No binding detected.

Table 2: Thermodynamic Parameters for Concanavalin A−*C*-Glycoside Complexation (kcal mol^{-1} at 25°C)

carboydrate	protein[a]	ΔG	ΔH	$T\Delta S$	n[b]
α-MeGlc	tetramer	-4.6 ± 0.03	-5.3 ± 0.4	-0.7	1.00
α-MeMan	tetramer	-5.3 ± 0.04	-6.8 ± 0.3	-1.5	1.02
1	tetramer	-5.0 ± 0.06	-5.9 ± 0.4	-0.9	1.08
2	tetramer	-5.1 ± 0.03	-5.9 ± 0.8	-0.8	1.00
3/4	tetramer	NB[c]			
5	tetramer	-5.0 ± 0.03	-8.5 ± 0.5	-3.5	0.50
6	tetramer	-6.1 ± 0.1	-11.1 ± 1.0	-5.0	0.51
7	tetramer	NB[c]			
5	dimer	-4.8 ± 0.04	-14.5 ± 0.9	-9.7	0.49
6	dimer	-5.1 ± 0.04	-13.8 ± 0.8	-8.7	0.52
10	dimer	-5.2 ± 0.03	-5.0 ± 0.7	$+0.2$	1.00
10	tetramer	-5.2 ± 0.01	-6.2 ± 0.9	-1.0	1.05

[a] Tetrameric protein was native concanavalin A, pH 7.4; dimeric protein was succinyl concanavalin A, pH 7.4. [b] Stoichiometry of binding: moles of ligand/moles of protein monomer. [c] No binding detected.

Table 3: Thermodynamic Parameters for *Dioclea* Lectin−*C*-Glycoside Complexation (kcal mol^{-1} at 25 °C)

ligand	K_{eq} (M^{-1})	ΔG	ΔH	$T\Delta S$	n[a]
α-MeGlc	1.2×10^3	-4.2 ± 0.02	-4.4 ± 0.3	-0.2	1.00
α-MeMan	3.6×10^3	-4.8 ± 0.03	-7.8 ± 0.7	-1.5	1.00
1	1.8×10^3	-4.4 ± 0.03	-2.4 ± 0.4	$+2.0$	1.10
2	2.5×10^3	-4.6 ± 0.03	-6.3 ± 0.8	-1.7	1.01
3/4	NB[b]				
5	NB[b]				
6	NB[b]				
7	NB[b]				

[a] Stoichiometry of binding: moles of ligand/moles of protein monomer. [b] No binding detected.

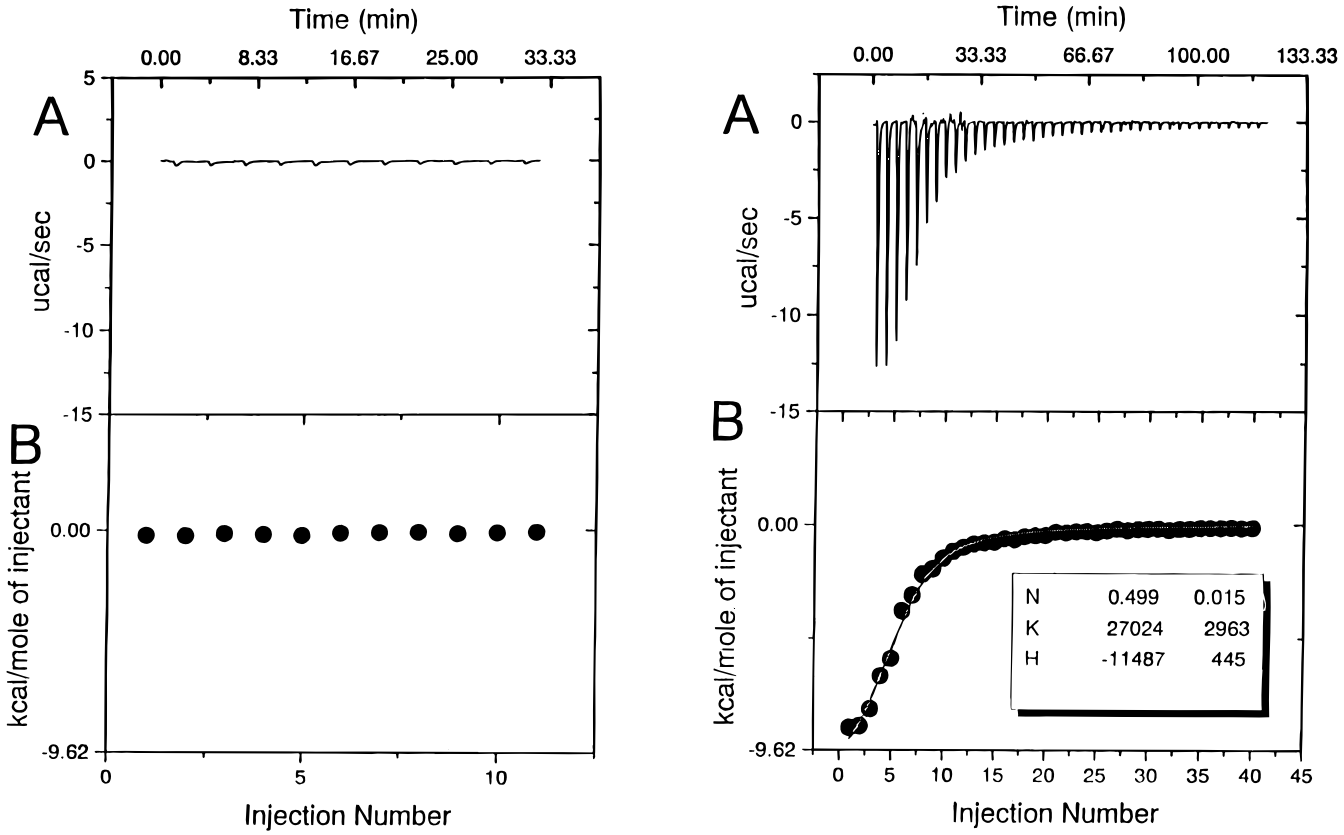

FIGURE 2: Raw (A) and integrated (B) data for calorimetric titration of concanavalin A with monovalent glucose oxanorbornene (**3**/**4**, left) and bivalent mannose oxanorbornene (**6**). The fit for the mannose oxanorbornene ligand is to a single-set-of-sites model.

1 bound more tightly than the corresponding *O*-linked saccharide, *C*-allyl mannoside **2** is bound by both lectins with similar affinities to those of methyl α-D-mannopyranoside (Table 1). Thus, both lectins display the same specificities for the monosaccharide *C*-glycoside derivatives.

Conversion of the *C*-allyl moiety to a norbornyl aglycon (**3**/**4**) completely abolished binding to both concanavalin A and the lectin from *Dioclea*. No exothermicities greater than heats of dilution were observed at protein concentrations as high as 1 mM (Figure 2). We note that the calorimetric titration curve shape is dependent only on the product of the binding constant and the concentration of binding sites within the cell. If only one diastereomer of the **3**/**4** mixture was recognized by the protein with an interaction energy of greater than approximately 2.5 kcal mol^{-1}, then binding would have been observed. Because the *C*-allyl glycosides bind with affinities comparable to those of methyl α-D-mannopyranoside, the origin of the decrease in affinity for the norbornyl *C*-glycosides can only reasonably be due to an unfavorable interaction of the oxanorbornene template on which the sugar substituents are displayed with the protein. (See Figure 2).

Consequences of Bivalent Presentation of Carbohydrate Ligands. Bivalent glucose and mannose derivatives **5** and **6** were also tested as ligands for each lectin with both calorimetric titrations and fluorescence anisotropy competition experiments, to determine the effect of multivalency on *C*-glycoside binding. The bisglycosylated glucose (**5**) and mannose (**6**) oxanorbornene derivatives are immediate precursors to neoglycopolymers that have previously been shown to inhibit concanavalin A-induced hemagglutination of erythrocytes (Mortell et al., 1994). Binding to bisglycosylated norbornyl ligands was investigated with both di- and tetrameric concanavalin A. The bivalent derivatives exhibit remarkably different recognition properties compared to the corresponding monovalent norbornyl *C*-glycosides. With dimeric concanavalin A, either succinylated protein at pH 7.4 or native protein at pH 5.2, the bivalent ligands are bound with affinities similar to the monovalent *C*-allyl glycosides **1** and **2** (Table 1). The decrease in the specificity for *manno* over *gluco* configuration at C-2 noted for the *C*-allyl glycosides was also observed with the oxanorbornene derivatives **5** and **6** binding to succinyl concanavalin A, although the effect was not as pronounced.

Both bivalent glucose derivative **5** and mannose derivative **6** behave differently with tetrameric concanavalin A than with dimeric protein. With the tetrameric form, attempts to apply the competition fluorescence anisotropy assay resulted in precipitation of the protein. The energetics of binding could, however, be determined by titration microcalorimetry, which allowed analysis of the interaction at lower protein concentrations. Concanavalin A binds both bivalent ligands tightly, and exhibited higher selectivity for the *manno* derivative relative to the *gluco* analogue than for the monovalent *O*-linked sugars, binding the C-2-axial compound **6** roughly six-fold more tightly than the C-2-equatorial ligand **5**. This difference in binding constants translates to a free energy difference of approximately 1.0 kcal mol^{-1} at room temperature.

Most importantly, bivalent compounds **5** and **6** afford complete recognition specificity for concanavalin A over the lectin from *Dioclea grandiflora*. In our previous studies comparing the binding properties of the two lectins with seventeen carbohydrate ligands, differences in binding free energies were in all cases less than 1 kcal mol^{-1} (Chervenak & Toone, 1995). The lectin from *D. grandiflora* binds both

bivalent *C*-glycosides, but with binding constants too small to deconvolute. This behavior places an upper limit on K_A for both ligands of 500 M^{-1}, or a free energy of binding of less than 3.5 kcal mol^{-1} at room temperature. *C*-Glycosides **5** and **6** thus provide a differential free energy of binding between the two lectins of at least 2.5 kcal mol^{-1}.

There are some clues as to the origin of the selectivity between the lectin from *Dioclea* and concanavalin A. The bivalent ligands **5** and **6** interact with concanavalin A in a very different manner than has been generally observed with low molecular weight ligands. A striking result from the calorimetric analysis of binding interactions is that *the stoichiometry of binding of concanavalin A as either the dimer or tetramer to dimeric ligands **5** and **6** is two concanavalin A binding sites per ligand*. This finding is in contrast to the binding of most other ligands examined to date, for which the binding stoichiometry is one concanavalin A binding site per ligand. To exclude the possibility of nonspecific binding of the norbornyl aglycon, we examined binding of the bivalent fucose norbornyl ligand **7**. No binding was observed to either lectins, confirming stereospecific recognition of the *C*-glycoside domain of ligands **5** and **6**.

The stoichiometry observed for the interaction of unmodified concanavalin A with bivalent ligands **5** and **6** has previously been reported for only one other low molecular weight ligand, melezitose (Ambrosino et al., 1987). Like bivalent ligand **5**, melezitose displays two glucose residues. In our hands, melezitose yields a binding stoichiometry of 1:1 both at high and low pH (Table 1).

These results are clearly consistent with two hypotheses: (1) concanavalin A but not the lectin from *Dioclea* is cross-linked by the carbohydrate-substituted bivalent oxanorbornenes **5** and **6**, and (2) concanavalin A contains a second class of binding sites not found on the lectin from *Dioclea* that accommodates glucose- and mannose-derived saccharide residues. While we cannot unambiguously differentiate the two theses, our data most faithfully support the latter.

First, we find little evidence of protein aggregation under the conditions used in the calorimetry experiments (<0.5 mM concanavalin A). At sufficiently high protein concentrations, the bivalent glycoside ligands cross-link concanavalin A, leading to aggregation and precipitation. The large aggregates that formed at high protein concentration were easily detected by physical light scattering in fluorescence anisotropy experiments. Fluorescent light scattering experiments were employed to determine the relative extent of aggregation at protein concentrations identical to those used in the microcalorimetry experiments. The addition of the bivalent ligand **6** to a solution of concanavalin A caused a modest (1.2-fold) increase in the amount of light scattering (for comparison, methyl α-mannopyranoside afforded no increase under similar conditions); however, this increase is far less than the change observed for other protein aggregates (Wiech et al., 1992). The small change detected argues against the formation of large cross-linked aggregates under the conditions of the calorimetry experiments.

Second, the calorimetric data are best fitted to a single set of sites model and give linear Scatchard plots. If cross-linking is occurring, this finding requires that binding constants and enthalpies of binding are not functions of protein aggregation state. Another observation that is not easily rationalized by the cross-linking model is that the monovalent ligands are not bound by concanavalin A, and none of the norbornyl ligands is bound by the lectin from *D. grandiflora*. If the observed stoichiometry is a result of cross-linking, then concanavalin A presumably binds only a single *C*-glycoside residue: the monovalent ligands should thus be bound with affinities roughly equal to those of the bivalent ligands. The complete lack of interaction with monovalent ligands **3**/**4** strongly argues against this manner of interaction.

Finally, the binding site of the lectin from *Dioclea* is highly homologous to that of concanavalin A. While the two lectins differ in 59 of 237 amino acid residues, only one modification is within 10 Å of the crystallographically identified monosaccharide binding site. Given that the lectin from *Dioclea* effectively binds *C*-allyl glycosides, it is difficult to rationalize the inability of the same lectin to bind the norbornyl *C*-glycosides in terms of the monosaccharide binding site.

Although these arguments tend to support the alternative binding site model, we cannot rule out the possibility that bivalent **5** and **6** promote concanavalin A cross-linking. If this process is occurring, then our results raise several issues. For example, it is unknown what features distinguish low molecular weight ligands that may be cross-linking, such the bivalent ligands **5** and **6**, from those that do not, such as the trimannoside. Additionally, the origins of the differences in recognition by concanavalin A and the lectin from *Dioclea* are unclear. It is noteworthy that the specificity of concanavalin A binding for **6** over **5** is consistent with the enhanced sugar recognition specificity that Brewer has observed with polyvalent oligosaccharide−lectin cross-linked complexes (Bhattacharyya et al., 1988).

It has long been recognized that concanavalin A possesses more than one class of binding sites. A hydrophobic binding site has been well described both through binding studies and X-ray crystallography (Becker et al., 1971). The site identified in this work is clearly distinct. Our postulated new site (or sites) specifically binds glucose/mannose-containing residues and discriminates between the two in much the same way the previously identified monosaccharide site does. A crystal structure of concanavalin A bound to *myo*-inositol has also been reported: the relevance of that site to the one identified here is unclear (Hardman & Ainsworth, 1972).

This work emphasizes the need for detailed biophysical studies of protein−carbohydrate interactions. The required elements for carbohydrate binding specificity remain enigmatic. Assays such as titration microcalorimetry and fluorescence anisotropy provide a true binding constant for reversible complexation and allow direct analysis of the protein−carbohydrate interaction. This report indicates that changes in carbohydrate presentation, such as switching from a monovalent to bivalent ligand, can have major effects on lectin binding specificity. Alternate binding sites may also be exploitable in the creation of highly specific ligands capable of distinguishing between multiple proteins with overlapping binding specificities.

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