Thermodynamic, Kinetic, and Electron Microscopy Studies of
Concanavalin A and Dioclea grandiflora Lectin Cross-linked with
Synthetic Divalent Carbohydrates*

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The jack bean lectin concanavalin A (ConA) and the
Dioclea grandiflora lectin (DGL) are highly homologous
Man/Glc-specific members of the Diocleinae subtribe. Both lectins bind, cross-link, and precipitate with carbo-
hydrates possessing multiple terminal nonreducing Man residues. The present study investigates the binding and
cross-linking interactions of ConA and DGL with a series of synthetic divalent carbohydrates that possess spacer
groups with increasing flexibility and length between termi-
al α-mannopyranoside residues. Isothermal titration
microcalorimetry was used to determine the thermody-
namics of binding of the two lectins to the divalent ana-
logs, and kinetic light scattering and electron microscopy
studies were used to characterize the cross-linking inter-
actions of the lectins with the carbohydrates. The results
demonstrated that divalent analogs with flexible spacer
groups between the two terminal Man residues possess
higher affinities for the two lectins as compared with
those with inflexible spacer groups. Furthermore, despite
their high degree of homology, ConA and DGL exhibit
differences in their kinetics of cross-linking and precipi-
tation with the divalent analogs. Electron microscopy
shows the loss of organized cross-linked lattices of the two
lectins with analogs possessing increased distance be-
tween the terminal Man residues. The loss of lattice pat-
terns with the analogs is distinct for each lectin. These
results have important implications for the interactions
of lectins with multivalent carbohydrate receptors in bi-
ological systems.

Lectins are carbohydrate-binding proteins that are widely
conserved in nature, such as those in animals, plants, and
microorganisms (1). The biological activities of many animal
lectins have been determined, including receptor-mediated en-
docytosis of glycoproteins, cellular recognition and adhesion (cf.
Ref. 2), regulation of inflammation (3), and metastasis and
control of cell growth (4, 5). A common feature of lectins is their
multivalent binding properties (6, 7). As a consequence, lectin
binding to cells leads to cross-linking and aggregation of glyco-
protein and glycolipid receptors. In many cases, these interac-
tions are associated with signal transduction effects, including
the arrest of bulk transport in ganglion cell axons (8), molecu-
lar sorting of glycoproteins in the secretory pathways of cells
(9), apoptosis of human T cells (10, 11), regulation of the T cell
receptor (12, 13), and growth regulation of neuroblastoma cells
(14). Thus, the carbohydrate cross-linking properties of lectins
are a key feature of their biological activities.

The cross-linking properties of a variety of plant and animal
lectins with multivalent carbohydrates and glycoproteins have
recently been reviewed (15). Studies show that a number of
lectins form homogeneous cross-linked complexes with
branched chain oligosaccharides and glycoproteins. For exam-
ple, quantitative precipitation experiments with the Man/Glc-
specific lectin concanavalin A (ConA)* in the presence of binary
mixtures of a series of bivalent N-linked oligomannose glyco-
peptides indicate that each glycopeptide forms its own unique
cross-linked complex with the lectin (16). Subsequent x-ray
crystallographic studies have demonstrated different lattice
structures of crystalline cross-linked complexes of the soybean
agglutinin with four different divalent carbohydrates (17). The
different lattice structures are due to differences in the struc-
tures of the cross-linking carbohydrates (17). The ability to
form unique cross-linked complexes with glycoconjugates and
to separate different counter-receptors into homogeneous
cross-linked aggregates has recently been implicated in the
apoptotic activity of galectin-1, a member of the β-galactosid-
ase-specific animal lectin family (18).

Recently, galectin-3, another member of the galectin family,
has been shown to form disorganized, heterogeneous cross-
linked complexes with multivalent carbohydrates (19). The
biological properties of galectin-3, including its anti-apoptotic
activities (20) and ability to antagonize the growth inhibitory
activity of galectin-1 in neuroblastoma cells (21), may relate to
its ability to randomly cross-link glycoconjugates and prevent
separation of different receptors. Hence, the ability of lectins to
form organized or disorganized cross-linked complexes with
multivalent glycoconjugate receptors, such as galectin-1 and -3,
respectively, may relate to their biological activities.

In the present study, we investigated the effects of varying
the flexibility and distance between terminal Man residues in
divalent carbohydrate analogs on their binding thermody-
namics and cross-linking interactions with ConA and the lectin
from Dioclea grandiflora (DGL). Isothermal titration calorim-
etry (ITC), kinetic light scattering, and electron microscopy
(EM) studies were used to characterize these interactions. The

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1 The abbreviations used are: ConA, concanavalin A; DGL, Dioclea
grandiflora lectin; αMDM, methyl α-D-mannopyranoside; ITC, iso-
thermal titration microcalorimetry; EM, electron microscopy.
results demonstrated that the thermodynamics of binding and cross-linking properties of the two lectins are sensitive to the flexibility and spacing between the carbohydrate epitopes of the analogs.

**EXPERIMENTAL PROCEDURES**

ConA was purchased from Sigma and/or prepared from jack bean (*Canavalia ensiformis*) seeds (Sigma) according to the method of Agrawal and Goldstein (22). The concentration of ConA was determined spectrophotometrically at 280 nm using $A_{280,1\text{cm}} = 13.7$ and 12.4 at pH 7.2 and 5.2, respectively (23), and expressed in terms of monomer ($M_r = 25,600$). DGL was isolated from *D. grandiflora* seeds obtained from northeastern Brazil (Albano Ferreira Martin Ltd., Sã o Paulo, Brazil) as described previously (24). The concentration of DGL was determined spectrophotometrically at 280 nm using $A_{280,1\text{cm}} = 12.0$ at pH 7.2 and expressed in terms of monomer ($M_r = 25,000$) (24). αMDM was purchased from Sigma. The synthesis of carbohydrate analogs 1, 2, 3, and 4 has been reported previously (25), as have analogs 9–13 (26). Synthesis of 5–8 will be reported elsewhere. The concentrations of carbohydrates were determined by modification of the Dubois phenolsulfuric acid method (27, 28) using appropriate monosaccharides as standards. Structures of these analogs are shown in Figs. 1 and 2.

**Isothermal Titration Microcalorimetry—I TC experiments were performed using an MCS ITC instrument from Microcal, Inc. (Northampton, MA). Injections of 4 ml of carbohydrate solution were added from a computer-controlled 250- or 100-μl microsyringe at an interval of 4 min into the sample solution of lectin (cell volume = 1.34 ml) with stirring of 350 revolutions/min. An example of an ITC experiment is shown in Fig. 3 for bivalent analog 11 with ConA at 27 °C. Control experiments performed by making identical injections of saccharide into a cell containing buffer without protein showed insignificant heats of dilution. The concentrations of lectins were 0.1–0.19 mM, and the sugars were 1.0–6.0 mM, respectively. Titrations showed insignificant heats of dilution. The concentrations of lectins were identical injections of saccharide into a cell containing buffer without protein.

**RESULTS**

**Thermodynamics of Binding of ConA and DGL to Analogs 1–13**—At pH 5.0 and low salt concentrations, both ConA and DGL are dimers and do not precipitate with analogs 1–13. Hence, ITC studies were performed under these conditions.

ITC data for ConA binding to 1–13 at 300 K are shown in Table I. $K_a$ values for 1–4 are very similar and nearly twice as great as that of the monosaccharide αMDM. $K_a$ values for 5–13 are nearly 4–6 times greater than that of αMDM. The $\Delta H$ values for ConA binding to 1–13 are greater than that of αMDM, with values for 1–4 generally lower than those of 6–13. The $\Delta S$ values for ConA binding to 1–13 are between 0.68 and 0.52, as compared with 1.0 for αMDM.

The ITC data for DGL binding to 1–13 are shown in Table II. $K_a$ values for DGL binding to 1–4 are similar and nearly four times as great as that of αMDM. $K_a$ values for 5–8 are 8–20-fold greater than that of αMDM, whereas the $K_a$ values of 9–13 are 3–9-fold greater than that of αMDM. The $\Delta H$ values for
DGL binding to 1–13 are generally greater than that of αMDM (Table II). The $n$ values for DGL binding to 1–13 are between 0.76 and 0.53, as compared with 1.0 for αMDM.

Time-dependent Light Scattering of ConA and DGL in the Presence of 1–13—Fig. 4 shows the time-dependent light-scattering profiles of 90 μM ConA with 45 μM of 1–4, respectively, at pH 7.2, room temperature. The kinetic profile is slowest and lowest for 1, fastest for 2, and greatest for 3, with an intermediate profile for 4. Fig. 4b shows the time-dependent light scattering of 90 μM DGL with 1–4 (45 μM each) at pH 7.2 at room temperature. The profiles for 1 and 2 appear the slowest of the four, with 3 nearly as fast as 4.

Fig. 5a shows the time-dependent light-scattering profiles of 90 μM ConA with 45 μM each of 9–12, respectively, at pH 7.2, room temperature. Analog 11 showed the slowest precipitation kinetics, with 9, 12, and 10 showing increasing rates of precipitation. Fig. 5b shows the time-dependent light-scattering profile of 90 μM DGL with 45 μM each of 9–12 at pH 7.2, room temperature. The kinetic precipitation profile of 10 is slowest, with 9 somewhat faster. Analogs 11 and 12 precipitate much faster with DGL as compared with 9 and 10. Analogs 5–8 also showed unique kinetics of precipitation with the two lectins under the above conditions (data not shown).

Electron Micrographs of the Precipitates of ConA and DGL with 1–13—Negative stain electron micrographs of the precipitates of ConA and DGL with 1, 2, and 3 are shown in Fig. 6. Patterns are observed for all three precipitates of ConA, whereas patterns are observed for the precipitates of DGL with 1 and 3 but not for 2. ConA and DGL precipitates with 4 failed to show patterns. Fig. 7 shows negative stain electron micrographs of the precipitates of ConA with 5–7 and DGL with 5 and 6. The precipitates of ConA with 5–7 all show observable lattice patterns, whereas the precipitates of DGL with 5 and 6 also show lattice patterns. The precipitates of DGL with 7 failed to show a pattern. The precipitates of ConA and DGL with 8 also failed to show a pattern. The precipitates of ConA and DGL with 9–13 also showed lattice patterns.

DISCUSSION

ConA and DGL are highly homologous lectins with 81% similarities in amino acid sequences and identical quaternary structures and subunit composition (cf. Ref. 31). Both are tet
Thermodynamics of ConA and DGL Binding to 1–13—For comparison, the thermodynamics of ConA and DGL binding to αMDM as well as to 3, 4, 8, 9, 10, and 12 are shown in Tables I and II, respectively (32). The results for αMDM show n values for both lectins close to 1.0, demonstrating that the monosaccharide binds as a monovalent ligand to both proteins. The ΔH values of −8.4 and −8.2 kcal/mol for ConA and DGL, respectively, are similar, as are the $K_a$ values of 1.2 × 10^9 M⁻¹ and 0.46 × 10^4 M⁻¹, respectively.

Analogs 1–13 show enhanced affinities relative to αMDM for ConA and DGL and different ΔH and n values. Previous ITC studies have shown that the value of n is inversely proportional to the functional valency of carbohydrate ligands for lectins (32). Values of n of 0.5 have been observed for higher affinity carbohydrate ligands binding to ConA and DGL, with values between 0.5 and 0.8 for lower affinity bivalent ligands due to incomplete binding of the second epitope (32). The n values for ConA and DGL in Tables I and II, respectively, are consistent with lower affinity divalent carbohydrates binding to both lectins. The greater −ΔH values for 1–13 binding to both lectins,
relative to αMDM, are also consistent with divalent binding of the analogs (32).

Interestingly, the enhanced $K_a$ values of both lectins for 1–13 appear to cluster into two groups for each lectin. Analogs 1–4 show approximate 2-fold enhanced affinities for ConA, and 3–4-fold enhanced affinities for DGL, relative to αMDM. On the other hand, analogs 5–13 show enhanced affinities of 4–6-fold for ConA, and 5–20-fold for DGL, relative to αMDM (the exception is 9, which possesses ~3-fold higher affinity). Thus, 1–4 possess smaller enhanced affinities for both lectins as compared with 5–13. The most obvious structural differences between these two groups of bivalent analogs is the flexibility of the linker regions between the outer two Man residues in each molecule. Analogs 1–4 possess relatively ridged linkers consisting of one or two acetylenic groups with or without a phenyl group, whereas 5–8 and 10–13 possess relatively flexible methylene groups. 9 is absent a methylene group between the two aryl glycoside moieties, which may be part of the reason for its relatively modest enhanced affinity for DGL in that group. Thus, the degree of flexibility of the spacer groups of the analogs appears to modulate their enhanced affinities for ConA and DGL.

Kinetics of Precipitation of ConA and DGL with Analogs 1–13—At pH 7.0 and high salt concentration, ConA and DGL are tetramers and precipitate with analogs 1–13. The kinetics of precipitation of both lectins with the analogs is shown in the time-dependent light-scattering profiles in Figs. 4 and 5. Because the concentrations of the two lectins and the concentrations of the analogs are the same, a comparison of the precipitation rate profiles of different analogs with the two lectins can be made. Differences in the precipitation rates are due to several factors, including the affinities of the analogs, the rates of formation of soluble cross-linked complexes, and the solubility constants of their cross-linked lattices.

Fig. 4a shows the kinetics of cross-linking and precipitation of ConA with analogs 1–4. Analog 1 shows the slowest rate of precipitation with ConA followed by 4, 3, and 2, respectively. Analog 3, however, shows a greater degree of precipitation with ConA than 2. Thus, the effects of different spacer groups of the
analogs are observed in their kinetics and extent of precipitation with ConA.

Fig. 4b shows the time-dependent light-scattering profiles of DGL with 1–4. Although analog 1 is the slowest to precipitate with DGL, similar to ConA, the order of kinetics and extent of precipitation of DGL with 2–4 is different from that with ConA. Analogs 3 and 4 show the fastest and largest degree of precipitation of DGL, whereas 2 and 3 are fastest with ConA. Thus, the different spacer groups of analogs 1–4 exhibit different kinetics and extents of precipitation with ConA and DGL, even though the structures of the two proteins are very similar (31).

Fig. 5 shows the time-dependent light-scattering profiles of ConA and DGL with analogs 9–12, respectively. Similar to 1–4, analogs 9–12 show differential kinetics and the extent of precipitation with ConA and DGL. Furthermore, the relative kinetics and extent of precipitation of the analogs differs for the two lectins. These results are similar to those of analogs 1–4 with the two lectins. These results demonstrate that differences in the structures of the two lectins affect their kinetics of cross-linking interactions with 1–4 and 9–12 (Figs. 4 and 5).

**Electron Microscopy of the Cross-linked Lattices of ConA and DGL with 1–13**—We have previously used negative stain EM to observe the presence of organized lattices in cross-linked complexes of lectins with multivalent carbohydrates (cf. Ref. 15). In the present study, differences in the spacer groups in 1–13 are observed to affect the structures of their cross-linked complexes with ConA and DGL (15). For example, ConA shows EM patterns for precipitates with 1–3, 5–7, and 9–11. No patterns are observed for the precipitates of 4, 8, 12, and 13. The lack of patterns observed for these precipitates correlates with the increased distance and flexibility separating the Man residues in the analogs, which, in turn, prevents the formation of organized cross-linked complexes.

DGL also shows a pattern of structures for its precipitates with 1–13. EM patterns for precipitates are observed with 1, 3, 5, 6, and 9–13. No patterns are observed for 2, 4, 7, and 8. With the exception of 2, the lack of patterns observed with 4, 7, and 8 correlates with the increased distance and flexibility between the Man residues in the molecules, a finding similar to that for ConA.

Although the structures and binding specificities of ConA and DGL are very similar, both lectins show differences in their patterns of lattice structures with 1–13. DGL shows patterns with 12 and 13, unlike ConA, which shows no patterns with these two analogs. ConA shows a pattern for precipitates with 2, whereas DGL shows no pattern. ConA also shows a pattern for precipitates with 7, whereas DGL shows no pattern. Thus, two highly homologous lectins show differences in the observed patterns of their precipitates with 1–13. The detailed lattice structures of the cross-linked complexes of the two lectins with the analogs in Figs. 6 and 7 will await x-ray fiber diffraction and image reconstruction of the EMs (cf. Ref. 38) or x-crystallographic analysis of crystals of the respective cross-linked complexes (cf. Ref. 17).

**CONCLUSIONS**

The results demonstrated that bivalent Man analogs with flexible spacer groups exhibit higher affinities for ConA and DGL than analogs with rigid spacer groups. ConA and DGL also showed differences in their kinetics of precipitation with the bivalent analogs and differences in the EM patterns of their precipitates with 1–13. The present findings indicated that the spacing and flexibility of carbohydrate epitopes in divalent carbohydrates affects their thermodynamics of binding, kinetics of precipitation, and structures of their cross-linked complexes with different lectins. These results have important implications for the interaction of lectins with multivalent carbohydrate receptors in biological systems (39).

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