Binding of monomeric and dimeric Concanavalin A to mannose-functionalized dendrimers†

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Because of the central role of Concanavalin A (Con A) in the study of protein–carbohydrate interactions, a thorough understanding of the multivalent functions of Con A is imperative. Here, the association of monomeric and dimeric derivatives of Con A with mannose-functionalized generation two through six PAMAM dendrimers is reported. Hemagglutination assay results indicate relatively low activity of the dendrimers for monomeric Con A, with small increases as the dendrimer generation increases. Isothermal titration microcalorimetry experiments indicate monovalent binding by the dendrimers with monomeric Con A and divalent binding by the dendrimers with dimeric Con A. Continuous (and comparable) but narrowing increases in enthalpy and entropy and the slight increase in association constants with monomeric Con A as the dendrimer generation increases suggest favorable proximity effects on binding. Both the hemagglutination assay and the calorimetry experiments suggest that statistical binding enhancements can be observed with monomeric Con A. The results described here should allow for a more quantitative evaluation of the enhancements that are often observed in protein–carbohydrate interactions for glycosylated frameworks binding to Con A.

Introduction

Many intracellular recognition events are mediated by protein–carbohydrate interactions.[1] Myraid biological communication processes such as infection by viral, parasitic, and bacterial pathogens rely on specific adhesion to cell surface carbohydrate epitopes.[2] Carbohydrate-based recognition facilitates cellular adhesion and growth in normal processes and in the progression of many cancers.[3] Because of the varied roles of protein–carbohydrate interactions, interest in the development of carbohydrate-based therapeutics has been the subject of intense research.[4]

Despite the enormous potential for carbohydrate-based pharmaceuticals, the construction of such compounds is hindered by the weak affinity of saccharides for their protein receptors; binding events typically proceed with millimolar to micromolar dissociation constants.[5] In order to increase affinity and confer selectivity, multivalent protein–carbohydrate interactions are widely used in nature.[6] Lectins are typically aggregated into oligomeric structures with higher order valencies that allow multivalent binding. Because lectin–carbohydrate adhesion generally involves multivalent interactions, a variety of glycopolymers that can span multiple lectin binding sites have been developed to decipher the mechanistic details of these binding processes. Glycoconjugates have been created using many frameworks, ranging from linear polymers[7] to dendrimers.[8]

Dendrimers have a regular branching pattern with (relatively) predictable physical properties.[9] By controlling the number and physical characteristics of the tethered functional groups, the solubility and avidity of the molecule can be attenuated.

Detailed investigations into the specificity of carbohydrate–protein interactions require not only relative binding affinity data, but also thermodynamic data to establish the energetics and functional valencies of the two interacting systems. Recently, isothermal titration microcalorimetry (ITC) has been used to study the binding interactions between multivalent carbohydrates and lectins.[10] ITC measurements provide direct determination of the number of binding sites, n, the enthalpy of binding, ΔH, and the association constant, Ka. The association constant can then be used to determine the free energy, ΔG, from which entropic contributions, ΔS, to binding can be calculated. Therefore, a complete thermodynamic profile for lectin–saccharide interactions can be obtained, providing quantitative information regarding the structural and functional valency of protein–carbohydrate interactions and probing for the effects of multivalent interactions.

Reports by Brewer *et al.* have suggested that multivalency effects for the binding of multivalent carbohydrates to Concanavalin A (Con A) and *Dioclea grandiflora* (DGL) arise from increasing positive entropy ($T\Delta S$) contributions relative to monovalent analogues. The enthalpy of binding, ΔH, was shown to be directly proportional to the number of binding epitopes.[10d]

Con A is a plant lectin isolated from the jackbean which exists as a homotetramer at pH 7. Each monomer unit has one sugar binding site. Con A has specificity for the α-pyranose forms of D-mannose and D-glucose, and the four sugar binding sites are 65 Å apart.[11] Con A has been used extensively as a model system with which to study protein–carbohydrate interactions. Because of the essential role that Con A serves in the study of multivalency, a

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thorough understanding of the multivalent binding properties of Con A is required.

Glycoside clustering has been previously defined as "affinity enhancement achieved by multivalent ligands over monovalent ones that is greater than would be expected from a simple effect of concentration increase."[12] For the discussion in this paper, we adopt this definition of glycoside clustering but apply it more specifically than it is sometimes used in the carbohydrate literature.[1b] Namely, we define multivalent binding (the ability of one dendrimer to bind to multiple lectin binding sites) and proximity/statistical effects (a ligand concentration effect) as two related but distinct terms. These definitions are shown pictorially in Fig. 1.

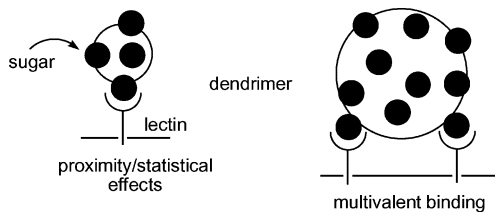

Fig. 1 Glycoside clustering/proximity effects *versus* multivalent binding.

Our motivation for these studies has foundations in the comparison of glucose- and mannose-functionalized linear polymers with Con A. Relative affinities for carbohydrate-functionalized polymers of varying lengths with tetrameric Con A were evaluated. Glycopolymers long enough to bind bivalently to Con A had affinities three orders of magnitude higher than methyl mannose. Polymers too short to bind to multiple binding sites on tetrameric Con A exhibited smaller binding enhancements, which were attributed to statistical effects.[7b] Similar observations with dendrimers functionalized with varying amounts of monosaccharide end-groups led us to hypothesize in previous work that both statistical and multivalent effects could be observed for dendrimer–Con A systems.[8p,q] Monomeric derivatives of Con A allow the evaluation of proximity/statistical effects on binding independent of multivalent binding, since no multivalent binding interactions are possible with the monovalent protein.

In the research reported here, we investigated the binding of a series of mannose-functionalized G(2) through G(6) PAMAM dendrimers **1–5** (Fig. 2) with divalent and monovalent derivatives of Con A. Results of hemagglutination assays and ITC measurements are reported. The results described here should allow for a more quantitative evaluation of the enhancements that are often observed in protein–carbohydrate interactions for glycosylated frameworks binding to Con A.

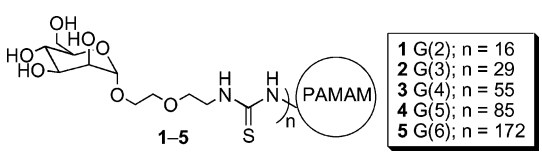

Fig. 2 Mannose-functionalized G(2) through G(6)-PAMAM dendrimers.

Results

Photochemical preparation and characterization of monomeric Con A

Chloroacetamide was used in the photochemical modification of Con A following the procedure of Tanaka *et al.*[13] Selective alkylation of one to two tryptophan residues per subunit of Con A causes the protein to dissociate into monovalent monomers.[13] The reaction was performed in the presence of a high concentration of methyl mannose so that the modified protein would still bind carbohydrates. The protein solution was then extensively dialyzed against 0.1 M Tris buffer at pH 7.3 to remove excess methyl mannose for subsequent affinity chromatography.

Fig. 3 (top) shows the elution chromatogram profile from a Sephedex G-100 column (10 mm × 60 cm), where three major peaks were eluted as a function of a linear gradient of D-glucose (0–100 mM glucose in 0.01 M Tris buffer, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.3). Fractions (2 mL) were collected at a flow rate of 0.5 mL min^{-1}. Following the literature precedent, the first peak was assigned as the monomeric form of Con A.[13] The second and third peaks were individually isolated and characterized with molecular weights of 60 000 and 105 000, which correspond to a dimeric fragment of Con A and tetrameric Con A, respectively (data not shown).

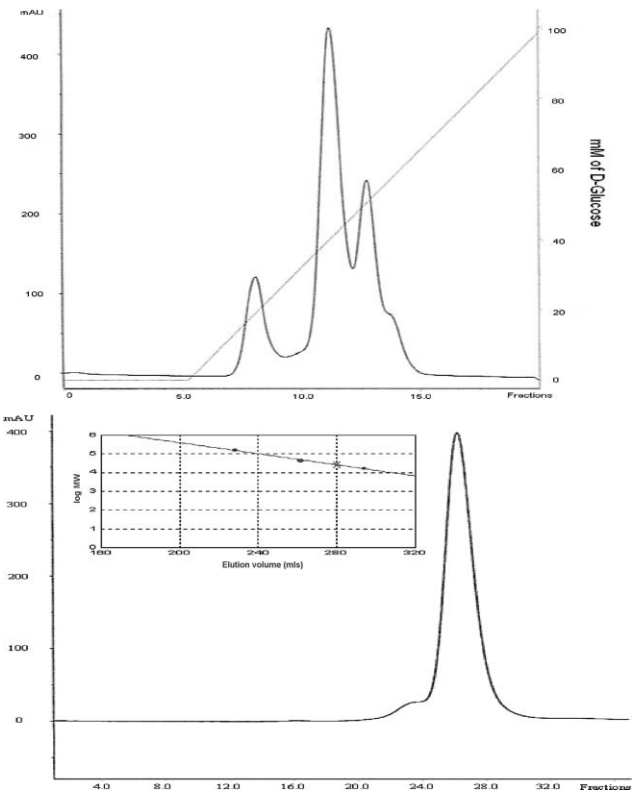

Fig. 3 *Top*: elution chromatogram profile of photoalkylated Concanavalin A on a Sephadex G-100 column. *Bottom*: Bio-Gel P-100 gel filtration profile of the first peak eluted from the Sephadex column. *Inset*: molecular weight calibration curve precalibrated with standard marker proteins γ-globulin, ovalbumin, and myoglobin with monomeric Con A shown as an X along the calibration line.

Fig. 3 (bottom) shows the elution chromatograph for the Bio-Gel P-100 gel filtration (2.6 cm × 80 cm) of the first peak eluted from the Sephadex column. Fractions (10 mL) were collected at a flow rate of 5 mL min^{-1}. The inset to Fig. 3 shows the molecular weight calibration curve. The small shoulder on the peak in Fig. 3 (bottom) corresponds to a dimeric impurity, which is present as less than 3% of the total isolated fraction.

SDS-PAGE and size exclusion chromatography indicate that the monomeric subunit has a MW of 27 000 g mol^{-1}, which agrees well with previously reported values.[13,14]

Hemagglutination assays

Hemagglutination assays were performed similarly to previously published procedures (ref. 15, see the Experimental section for additional details). Because a small amount of dimeric material was present in the samples of monomeric Con A, hemagglutination assays on dimeric material were performed as a control. Table 1 shows the relative activity of mannose-functionalized dendrimers **1–5** for dimeric and monomeric Con A (concentration adjusted) compared to methyl mannose.

Mannose-functionalized dendrimers **1** and **2** were bound to monomeric and dimeric Con A with comparable valence corrected affinities. Dendrimers **3–5** showed a four-fold higher affinity for dimeric Con A than for monomeric Con A.

Isothermal titration microcalorimetry experiments

Isothermal titration microcalorimetry (ITC) experiments were performed for compounds **1–5** with monomeric and dimeric Con A. Details are provided in the Experimental section. Concentrations of Con A ranged from 0.05–0.15 mM and glycodendrimer concentrations ranged from 0.80–10.0 mM. Titrations were done in 1 mM acetate buffer, pH 4.6 in the presence of 1 mM $MnCl_2$ and 1 mM $CaCl_2$ and at NaCl concentrations from 0–50 mM. Calorimetric data for the titration of monomeric Con A with **5** and for the titration of dimeric Con A with **5** are shown in Fig. 4 and 5, respectively.

Table 1 Hemagglutination assay results for dendrimers **1–5**

Monomeric Con A	Number of sugars	Rel. activity per sugar[a]
Me α-Man	1	1
1	16	3 ± 1.5
2	29	45 ± 0
3	55	45 ± 20
4	95	95 ± 50
5	172	195 ± 100
Dimeric Con A		
Me α-Man	1	1
1	16	3 ± 1.5
2	29	45 ± 25
3	55	180 ± 0
4	95	370 ± 185
5	172	770 ± 385

[a] Each reported value represents at least three assays.

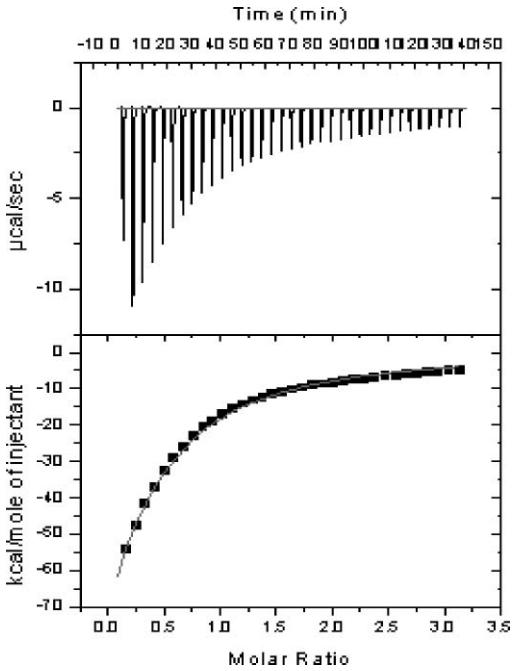

Fig. 4 Calorimetric data for the titration of monomeric Con A (0.051 mM), with **5** (1.1 mM). *Top*: raw data. *Bottom*: integrated curve showing experimental points and best fit using a one-site model.

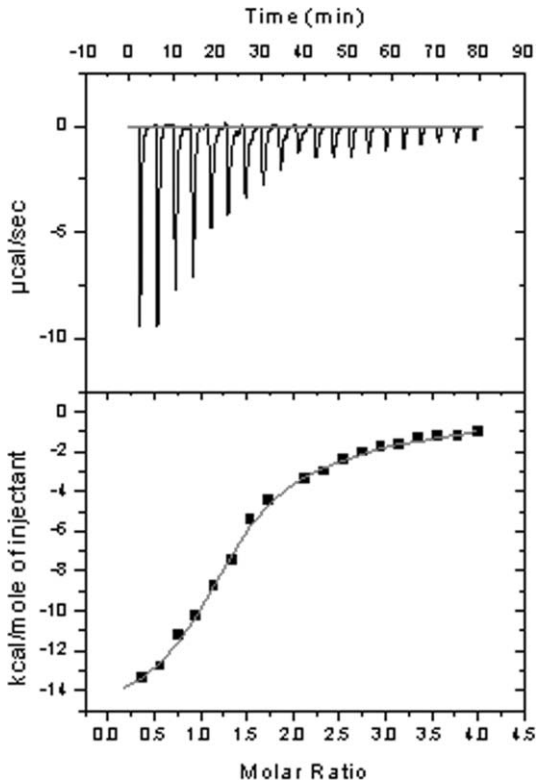

Fig. 5 Calorimetric data for the titration of dimeric Con A (0.10 mM) with **5** (3 mM). *Top*: raw data. *Bottom*: integrated curve showing experimental points and best fit using a one-site model.

Discussion

Con A has been used widely as a model protein for the study of physiologically relevant protein–carbohydrate interactions.[11] Because of its central role in multivalent research, a thorough understanding of its multivalent functions is imperative. Control of the oligomerization of monomeric subunits of Con A into dimers and tetramers has been demonstrated to be pH dependent.[11,16] Chemical modifications of Con A including succinylation, acetylation, partial hydrolysis, and covalent cross-linking have also been reported.[17] Con A derivatives have been used to study biological processes such as the rate of migration of lymphocytes[18] and lymphocyte activation.[19]

Succinylated Con A was crystallized in tetrameric form, even though the dimeric form was dominant in solution. This result strongly suggests that tetrameric and dimeric forms of succinylated Con A exist in equilibrium in solution.[20] The monomeric Con A that we are using could be in equilibrium with dimeric and tetrameric forms. Tetrameric Con A is not detected by dynamic light scattering or size exclusion chromatography. However, we are unable to remove all traces of dimeric Con A from our monomeric Con A samples (see the small shoulder peak in Fig. 3, bottom). Either the dimeric Con A co-elutes with monomeric Con A during the purification procedures, or an equilibrium between monomeric Con A and dimeric Con A (greatly favoring the monomeric Con A derivative) is established. Because our monomeric Con A samples contain a small amount of dimeric Con A, we performed control binding experiments using dimeric Con A. Results for monomeric and dimeric Con A are compared.

Characterization results for monomeric Con A

The results of the SDS-PAGE for monomeric Con A (Fig. S1 in the ESI†) were compared to SDS-PAGE results with tetrameric Con A. Since tetrameric Con A dissociates into monomers in the presence of SDS, similarities in band migration for the two proteins should be observed in the gel. Four bands were observed for the tetramer, corresponding to molecular weights of 27 000, 22 000, 15 000 and 10 100 g mol^{-1}. The last three bands demonstrate the heterogeneity of conventionally prepared Con A, which is due to the presence of naturally occurring low molecular weight fragments.[21,22] Comparison of SDS-PAGE results for monomeric and tetrameric Con A indicates that the isolated monomeric material has a subunit composition nearly identical to that of the native, tetrameric species and suggests that the monovalent Con A is intact.

Hemagglutination assay results

Theoretically, a monovalent protein should be incapable of simultaneously binding more than two epitopes on a dendrimer, precluding extensive cross-linking and aggregation. That the monovalent derivative was able to agglutinate cells has previously been reported[13,14,23] and was suggested to result from hydrophobic interactions between the lectin and the cell membrane.[24]

Hemagglutination assays (Table 1) revealed that mannose-functionalized G(2)-PAMAM **1** binds to monomeric Con A with an assay activity comparable to that of methyl mannose (on a per sugar basis), suggesting that **1** binds monovalently to monomeric Con A. Comparable results were observed for **1** with dimeric Con A. Mannose-functionalized G(3)-PAMAM **2** displays an activity in the assay that is one order of magnitude higher than that of methyl mannose (valence corrected), both for monomeric and dimeric Con A. Mannose-functionalized G(3)-PAMAM **2** is too small to bind to multiple binding sites on dimeric or tetrameric Con A, and we have attributed this one order of magnitude increase in assay activity to a statistical/proximity effect on binding.[8q,r] Even when dendrimers are too small to bind to multiple binding sites on a multivalent protein, having multiple ligands clustered around a binding site should increase the overall interaction between the dendrimer and the protein. As one weakly binding ligand dissociates from the protein, another is readily available to take its place.

Dendrimers **3–5** display higher agglutination inhibition activity for dimeric Con A than for monomeric Con A. These dendrimers are all large enough to bind to two binding sites on tetrameric Con A simultaneously, and we have previously observed comparable differences between bivalent binding by **3–5** and monovalent binding by **2** with tetrameric Con A.[8q,r]

With both monomeric and dimeric Con A, a two-fold difference between valence corrected hemagglutination inhibition activity for **3** *vs.* **4** and **4** *vs.* **5** was observed. The sugars should be closer together on mannose-functionalized G(6)-PAMAM **5** than on lower generation dendrimers, and so proximity effects may be enhanced for larger generation dendrimers even when multivalent binding is precluded. The curvature of the dendrimers also changes, and shape complementarity of the dendrimer and the protein may cause small but observable changes in relative activities for the different generations of dendrimers. Certainly, that mannose-functionalized G(4)-PAMAM **3** binds to monovalent Con A with a relative activity comparable to that of mannose-functionalized G(3)-PAMAM **2** is strong evidence supporting a proximity enhancement on binding even in the absence of multivalent binding for protein–carbohydrate interactions.

Thermodynamics of binding

Thermodynamic binding equilibria of multivalent ligands provide insight into the physical mode of interaction between ligand and receptor. Although hemagglutination assays provide information regarding the relative activity of mannose-functionalized dendrimers to Con A, they do not provide association constants or energetics of binding. Isothermal titration calorimetry (ITC) can provide valuable information about the number of binding sites per monomer of protein, n, the enthalpy of binding, ΔH, and the association constant, Ka, from which the free energy, ΔG, and entropy of binding, ΔS, can be calculated. The binding of small mannose-functionalized multivalent frameworks to Con A has previously been reported,[10d–f] which led us to hypothesize that ITC would be useful for binding studies with dendrimers **1–5** as well.

Isothermal titration microcalorimetry studies of the dendrimers binding to monomeric Con A yielded curves indicative of simple reversible binding (Fig. 4). This is as expected, given that the monovalent derivative of Con A has only one binding site and therefore should be incapable of simultaneously binding more than one epitope. In the dimeric system, the dendrimers seem to bind bivalently, leading to cross-linking and minimal aggregation as revealed by the noise in the baseline (Fig. 5).

Table 2 ITC-Derived thermodynamic binding parameters for monomeric Concanavalin A with dendritic ligands **1–5**[a]

	$Ka^b/M^{-1} \times 10^3$	ΔH^c/kcal mol^{-1}	ΔG^d/kcal mol^{-1}	$T\Delta S^e$/kcal mol^{-1}	n^f/sites per monomer
Methyl α-mannose	6.8	−7.1	−5.2	−1.9	1.02
1	18.6	−20.5	−5.8	−14.7	1.01
2	32.0	−47.0	−6.1	−40.9	0.98
3	37.6	−54.6	−6.2	−48.4	0.96
4	46.4	−61.0	−6.3	−54.7	0.93
5	53.5	−65.2	−6.4	−58.8	0.95

[a] All calorimetric values are expressed in terms of mannose equivalents. [b] Errors in *K*a range from 1–9%. [c] Errors in ΔH are from 1–4%. [d] Errors in ΔG are less than 1%. [e] Errors in $T\Delta S$ are from 1–8%. [f] Errors in *n* are less than 2%.

Table 3 ITC-Derived thermodynamic binding parameters for dimeric Concanavalin A with dendritic ligands **1–5**[a]

	$Ka^b/M^{-1} \times 10^3$	ΔH^c/kcal mol^{-1}	ΔG^d/kcal mol^{-1}	$T\Delta S^e$/kcal mol^{-1}	n^f/sites per monomer
Methyl α-mannose	7.54	−7.9	−5.3	−2.6	0.98
1	27.0	−15.1	−6.0	−9.1	0.70
2	91.0	−54.2	−6.7	−47.5	0.61
3	96.0	−64.5	−6.8	−57.7	0.51
4	300	−79.5	−7.4	−72.0	0.47
5	240	−71.2	−7.3	−63.9	0.49

[a] All calorimetric values are expressed in terms of mannose equivalents. [b] Errors in *K*a range from 1–6%. [c] Errors in ΔH are from 1–3%. [d] Errors in ΔG are less than 1%. [e] Errors in $T\Delta S$ are from 1–5%. [f] Errors in *n* are less than 2%.

Tables 2 and 3 show the results of binding studies for **1–5** to monomeric and dimeric Con A averaged over the total number of mannose units on each dendrimer. For comparison, data for the binding of methyl mannose, which represents the monovalent binding epitope of the mannose-functionalized dendrimers, are also shown. For each thermodynamically derived parameter, a statistically superior fit using the one-site model was observed for the monomer. This model was used for analysis of the dimer, too, so that the relative thermodynamic contributions for independent, non-cooperative binding sites could be analyzed. This methodology is routinely used by Brewer and co-workers to compare associations.[10d–g]

Five-fold and twelve-fold enhancements in binding affinity, relative to methyl mannose, were observed for the binding of mannose-functionalized G(4)-PAMAM **3** with monomeric and dimeric Con A, respectively. This suggests an enhancement in affinity beyond the number of available epitopes, which we ascribe to a statistical/proximity effect. Interestingly, the *n* value, which determines the number of binding sites, was observed to be different in the monomeric and dimeric systems. We observed an *n* value of 0.96 in the monomeric system, which is close to the theoretical value of 1 seen for monovalent binding. In contrast, an *n* value of 0.51 was observed in the dimeric system. Since the theoretical value for divalent binding of a multivalent carbohydrate ligand is $n = 0.5$ (one binding interaction per monomer of a divalent protein[10d]), our results suggest that complete binding of the second carbohydrate residue occurs.

Differences in the fundamental binding mechanism for mannose-functionalized dendrimers with monomeric and dimeric Con A were observed as a function of both the relative affinity and the *n* values. Small increases in the association constant and approximately equivalent *n* values were observed for the binding of monomeric Con A to **1–5**, suggesting that (as expected) monovalent binding occurs between monomeric Con A and all of the dendrimers, regardless of dendrimer size or the number of epitopes.

For the dimeric system, larger association constants relative to those for binding of dendrimers to monovalent Con A imply a lower dissociation rate for the dendrimer–dimeric Con A conjugates. Thirteen-fold and forty-fold increases in the binding affinity of dimeric Con A for **3** and **4**, respectively, were observed. These values suggest effective bivalent binding of **3** and **4** to dimeric Con A. The *n* values of 0.51 and 0.47 for **3** and **4**, respectively also suggest predominantly bivalent interactions. Dendrimer **5** also appears to be involved in divalent interactions, with an *n* value of 0.49. Thus, under the experimental conditions, higher generation dendrimers appear to bind bivalently to dimeric Con A, precluding extensive aggregation and precipitation.

Interestingly, dendrimer **5** has a lower binding affinity for dimeric Con A than **4** does. Dendrimer **4** may have better shape complementarity for the protein, or the area available to the end groups of **4** may be larger, allowing for more flexibility and for optimization of the binding interaction. The approximate area available to the end groups on **5** is smaller than on **4**.[8q] Perhaps, although the theoretical number of epitopes is greater in **5**, **4** may be more efficient at bivalent binding. Of course, the decreased *K*a of **5** affects the ΔG, ΔH, and $T\Delta S$ values as well.

Since $Ka = kon/koff$, the ratio of the forward and reverse rate constants for binding, increasing *k*on, decreasing *k*off, or both together would result in increased *K*a values.[10d] Considering the binding of **1–5** to Con A, *k*on would not be expected to change significantly, while *k*off could be slowed if proximity effects occurred. As one sugar would move out of the binding site, another could take its place. Rather than complex dissociation, the dendrimer would remain bound, and *k*off would be slowed.[10d] The significant difference in *K*a for methyl mannose *versus* all mannose-functionalized dendrimers may occur because *k*on, *k*off, or both are too different for dendrimers and monosaccharides for

comparison. Nonspecific interactions may be responsible for the elevated Ka in the dendrimer–protein studies.

Brewer and co-workers have noted incremental increases in the enthalpy change, ΔH, for Con A and DGL binding by small mannose clusters. Mannose dimers, trimers, and tetramers had ΔH values two-, three-, and four-fold larger than mannose monomers, respectively.[10d] The increases observed here in ΔH for monomeric Con A binding to dendrimers are not so linearly related. This is as expected, since n approaches 1 for all the studies with monomeric Con A. Since n does not change in the monomeric Con A binding studies, the relative trends in ΔH that were observed by Brewer and co-workers should not be present here. In the binding studies with dimeric Con A, n approaches 0.5 for all larger generation dendrimers and is between 1 and 0.5 for smaller generation dendrimers. Without the orderly progression in n, an orderly increase in ΔH is not expected.

In general, as the generation of the mannose-functionalized dendrimers increased, the relative magnitude of ΔH increased as well. Correlating the $\Delta\Delta H$ values for each generation, increases of 26.5 and 39.1 kcal mol^{-1} from **1** to **2** were observed for monomeric and dimeric Con A, respectively. This is in comparison to $\Delta\Delta H$ from **2** to **3**, which shows more modest increases of 7.6 and 10.3 kcal mol^{-1} for monomeric and dimeric Con A, respectively. This suggests that significant statistical/proximity binding enhancements are occurring for dendrimers **3–5** (although enthalpic contributions to solvent reorganization could also be responsible for at least part of the observed enthalpy changes).

Entropy–enthalpy compensation plots of $-\Delta H$ *versus* $-T\Delta S$ are shown in Fig. 6 for binding of **1–5** with monomeric and dimeric Con A. The fit is linear in both cases ($R = 1$ for monomeric Con A and $R = 0.99$ for dimeric Con A). These plots show that the trends observed for ΔH also follow for $T\Delta S$ for our system; the slope is 1 since $T\Delta S$ varies by the same amount as ΔH for each value.

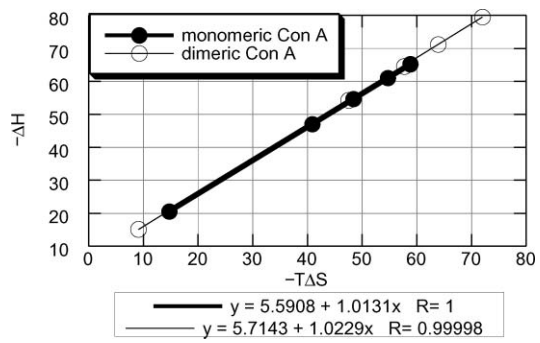

Fig. 6 Entropy–enthalpy compensation plots.

The continuous but narrowing increases in enthalpy and entropy and the slight increase in Ka for **1–5** with monomeric Con A are suggestive of favorable proximity effects on binding. In all cases, $n = 1$, so proximity enhancements are the simplest explanation for the observed trends. The same trends but with larger values of Ka, ΔH, and $T\Delta S$ for dimeric Con A are likely a result of both bivalent binding (by larger dendrimers) and proximity effects.

To ensure that the thermodynamic parameters were not a function of aggregation of monomeric Con A into oligomeric Con A, dynamic light scattering experiments were conducted. Measurement of the hydrodynamic diameters indicated that tetrameric Con A had a diameter of approximately 8 nm, while the diameter of the monomer was close to 5 nm (Fig. S2 in the ESI†). No aggregation was observed over two hours, which is the length of a normal ITC experiment. Thus, the ITC results were attributed to monomeric Con A rather than to Con A aggregates.

Conclusion

Because of the central role held by Con A in multivalent research, a thorough understanding of its multivalent functions is imperative. Here, hemagglutination assay and isothermal titration microcalorimetry results are reported for mannose-functionalized G(2)- through G(6)-PAMAM dendrimers **1–5** with monomeric and dimeric Con A derivatives.

In the hemagglutination assay, generally low activity was observed for dendrimers with monomeric Con A. Small increases in activity as the generation of the dendrimer increased suggest that only statistical increases in binding occur (as expected). Four-fold higher relative (per sugar) activities were observed for hemagglutination assays with larger generation dendrimers binding to dimeric Con A than to monomeric Con A, since bivalent associations could occur for dimeric Con A.

ITC studies also indicated that monovalent binding with monomeric Con A and divalent binding with dimeric Con A for larger generation dendrimers were occurring; 1 : 1 and 1 : 2 binding ratios were determined from the n values for the monomer and dimer, respectively. The observed 1 : 2 complex for the dimer suggests that, under experimental conditions, the dimer binds bivalently, precluding extensive aggregation and precipitation.

The binding enhancement for the binding of dendrimers **1–5** with monovalent Con A may be ascribed to a clustering/proximity effect, and multivalent interactions were observed with dimeric Con A for large dendrimers, as suggested by the forty-fold increases in the association constant (per mannose) for **4** and **5**.

Unlike previous reports with tetrameric Con A, enthalpy changes were not incremental. This was as expected, however, since enthalpy changes were previously linked to changes in n. Changes in $T\Delta S$ for our system were comparable to changes in ΔH, and enthalpy–entropy compensation plots had highly linear fits.

Overall, the studies reported here highlight differences in binding of glycopolymers with monomeric and dimeric Con A proteins.

Experimental

General protocols

Mannose-functionalized dendrimers **1–5** were synthesized as previously described in ref. 8*r*. Concanavalin A was purchased from CalBioChem and used without further purification. Sephedex G-100 was purchased from Sigma, and Bio-Gel P-100 and protein standards were purchased from Bio-Rad laboratories. All other chemicals were of reagent grade.

Photochemical preparation of monomeric Con A

Monomeric Concanavalin A was prepared and purified according to the procedure reported by Tanaka *et al.* with minor modifications.[13] A 150 mL solution comprising 10 mM Tris buffer,

pH 7.3, with 1 mM $MnCl_2$, 1 mM $CaCl_2$, 10 mM methyl-α-D-mannopyranoside, 100 mM chloroacetamide and 1 M NaCl was deoxygenated under argon for 60 minutes. Lyophilized Con A was dissolved to a final concentration of 1.5 mg mL^{-1}. After 30 minutes of stirring, the protein solution was irradiated with a cylindrical high pressure UV lamp (450 W) for 90 minutes. The protein solution was extensively dialyzed against 0.1 M Tris buffer, pH 7.3, in order to remove methyl mannose and was concentrated by ultrafiltration to 10 mg mL^{-1} (Amicon, MW cutoff 5000 g mol^{-1}). The concentrated solution was applied to a column of Sephadex G-100 (10 mm × 60 cm) preequilibrated with 1 column volume of TBS. Adsorbed proteins were eluted with a linear concentration gradient of D-glucose (0–100 mM). The monovalent, chemically modified Con A eluted first. Two other major peaks eluted, corresponding to molecular weights of 60 000 and 105 000 g mol^{-1} (dimeric and tetrameric Con A).[13] The first peak was concentrated by ultrafiltration (Amicon) and was purified by gel filtration through a column of Bio-Gel P-100 (2.6 cm × 80 cm) eluted with TBS (to eliminate residual dimeric and tetrameric forms). Approximately 4% of the eluted protein from the first peak was dimeric protein; therefore the purified fraction of monomeric Con A was concentrated and chromatographed again with the same Bio-Gel column, giving a single peak with a dimeric impurity makeup of less than 3%. SDS-PAGE and size exclusion chromatography results indicate that the monomeric subunit has a MW of 27 000 g mol^{-1}, which agrees well with previously reported values.[13,15] Comparison of SDS-PAGE results for monomeric and tetrameric Con A suggests that the isolated monomeric material has a subunit composition analogous to that of the native, tetrameric species (See Fig. S1 of the ESI for SDS-PAGE results†). The purified monomeric Con A was concentrated to 10 mg mL^{-1} and dialyzed against 1 mM acetate buffer, pH 4.6 in the presence of 1 mM $MnCl_2$ and 1 mM $CaCl_2$ and was used for subsequent analysis and calorimetric experiments.

Protein concentrations of monomeric Con A solutions were measured spectrophotometrically at 280 nm using $A^{1\%,1\text{ cm}} = 12.4$[13] at pH 4.6 and are expressed in terms of the monomer (MW = 25 600 g mol^{-1}).

Hemagglutination inhibition assays

Hemagglutination assays were performed similarly to previously published procedures.[15] The assay buffer consisted of 0.5% w/v BSA in 10 mM phosphate buffer saline (PBS), pH 5.0. Monomeric Con A was added to serial dilutions of a 30 mg mL^{-1} glycodendrimer stock solution, while dimeric Con A was added to serial dilutions of a 20 mg mL^{-1} glycodendrimer solution. The solutions were incubated for 3 hours at 25 °C. Rabbit erythrocytes (3% v/v in assay buffer (0.5% w/v BSA in PBS)) were added and the lowest amount of dendrimer to cause inhibition was determined.

Dynamic light scattering

DLS experiments were performed on a Brookhaven 90Plus Particle Size analyzer with a photomultiplier detector angle at 90° using a 661 nm diode laser. The autocorrelation function was fit using a non-negatively constrained least-squares analysis. Tetrameric and monomeric samples were prepared in filtered (0.2 μM Pall syringe filter) PBS, pH 7.3, and sodium acetate buffer, pH 4.6, respectively.

Isothermal titration calorimetry

ITC experiments were performed using a VP ITC instrument from Microcal, Inc (Northampton, MA). Injections of 4 μL of glycodendrimer solution were added from a computer controlled 250 μL syringe at an interval of 4 min into the sample solution of lectin (cell volume = 1.435 mL) with stirring at 310 rpm. Data from control experiments performed by making identical injections of glycodendrimer into a cell containing buffer without protein were subtracted from the raw data. Concentrations of Con A ranged from 0.05–0.15 mM and glycodendrimer concentrations ranged from 0.80–10.0 mM. Titrations were done in 1 mM acetate buffer, pH 4.6 in the presence of 1 mM $MnCl_2$ and 1 mM $CaCl_2$ and at NaCl concentrations from 0–50 mM. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (enthalpy change in kcal mol^{-1}), Ka (association constant in M^{-1}), and n (number of binding sites per monomer) as adjustable parameters. Thermodynamic parameters were calculated from the equation $\Delta G = \Delta H - T\Delta S = -RT\ln K$a, where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively, T is the absolute temperature, and $R = 1.98$ cal mol^{-1} K^{-1}.

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