Synergistic Formation of Soluble Lectin Clusters by a Templated Multivalent Saccharide Ligand

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Protein−carbohydrate recognition events mediate significant biological processes including fertilization, pathogen-cell adhesion, and the inflammatory response.[1−4] Binding affinities for monovalent protein−carbohydrate interactions are often weak (i.e., $K_a = 10^{3-4}$ M^{-1}), yet the strength and specificity required for recognition in physiological settings is high.[5−7] Accordingly, carbohydrate epitopes often are presented in multivalent arrays at the cell membrane, and these arrays can serve as highly effective and specific ligands.[5−11] Despite many documented examples of enhanced activities for multivalent over monovalent ligands, an understanding of the molecular mechanisms that contribute to their potencies is only beginning to emerge.

The structural features of multivalent ligands influence their activities and the mechanisms by which they function.[12−15] In general, only ligands with widely spaced saccharide epitopes can interact with multiple binding sites within an oligomeric lectin (i.e., act by the chelate effect). Still, low molecular weight ligands that cannot span multiple binding sites within an oligomeric lectin often have increased activities relative to their monovalent counterparts.[16−19] To explore their mechanisms, we synthesized a preorganized trivalent mannose array and evaluated its ability to inhibit the mannose-binding lectin concanavalin A (Con A). We found that this ligand, which is more potent than the corresponding monovalent derivative, functions by clustering the lectin in solution. Although many multivalent ligands are known to function by forming lectin−ligand precipitates,[19−22] this is the first report of favorable clustering of a lectin in solution. Our results have ramifications for the design of inhibitors for receptors and for understanding the roles of carbohydrate−protein interactions in vivo.

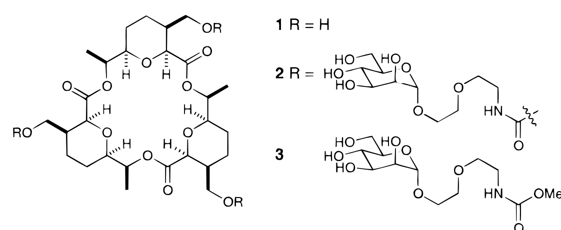

Figure 1. Structures of the unsubstituted macrocycle **1**, trivalent mannose derivative **2**, and the monovalent mannose control **3**.

The templated ligand array we investigated is based on conformationally defined macrocycle **1**, which belongs to a new class of rigid macrocyclic scaffolds (Figure 1).[23−25] The target ligand **2** was designed to display three mannose residues appended through a solubilizing linker to the macrocyclic core.[26a] This scaffold differs from many investigated previously for carbohydrate presentation in that the template is rigid and the saccharide residues are oriented such that they emanate from a single face. Molecular modeling studies indicate that the maximum separation between mannose residues is approximately 35 Å. Given that the binding sites within the Con A tetramer are separated by 65−70 Å,[27] trivalent ligand **2** (Figure 1) cannot simultaneously occupy two mannose binding sites within the tetrameric lectin. Therefore, this compound can be used to explore mechanisms of multivalent ligand binding in the absence of the chelate effect.

Our first objective was to compare the abilities of the trivalent ligand **2** and the corresponding monovalent control ligand **3** to bind to Con A. A surface plasmon resonance (SPR) competition binding assay has been developed to garner quantitative binding data on monovalent and multivalent lectin−ligand complexes.[27] The assay involves monitoring the ability of a substrate to inhibit the interaction of soluble Con A with a self-assembled monolayer containing a mannose-substituted lipid. To investigate whether the attachment of mannose to the template influences its interaction with Con A, the monomeric mannose derivative **3** was tested in the SPR assay. An inhibition curve (Figure 2A) was generated by measuring the binding responses for Con A in the presence of increasing concentrations of inhibitor. As the concentration of the inhibitor increases, the response units decrease as less protein binds to the surface. Analysis of these data reveals that the K_i value for **3** with Con A is 99 μM, similar to the K_i value for α-methyl mannoside (65 μM). These results indicate that the linker used for mannose attachment to the macrocyclic core does not perturb lectin binding significantly.

The interaction of tetrameric Con A with trivalent mannose derivative **2** is dramatically different from that of the monovalent ligand (Figure 2A). Surprisingly, as the concentration of **2** increased from 0.038 to 9.8 μM, the response units *increased*. Thus, compound **2** promotes rather than inhibits binding of Con A to the mannose-substituted surface. The data suggest that trivalent **2** binds two or three Con A tetramers simultaneously. The soluble clusters bind avidly to the immobilized ligand, presumably because the clustered Con A tetramers possess unoccupied saccharide binding sites that can interact with the

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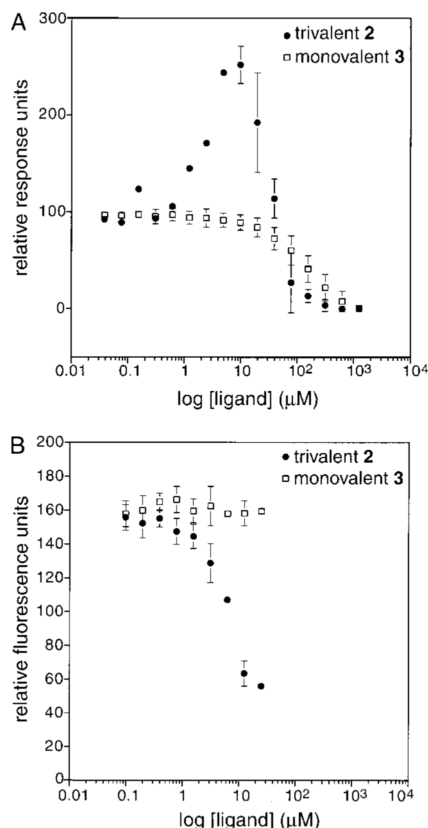

Figure 2. (A) Binding of Con A tetramer (1.0 μM) to the glycolipid surface in the presence of **2** (●) or **3** (□). All values are relative to those obtained for Con A in the absence of **2** (●) or **3** (□). Error bars represent ± one standard deviation around the mean relative response unit and are an indication of variations in experimental measurement. Data points with error bars represent three independent experiments; those without represent one experiment. (B) Quenching of fluorescein emission upon addition of **2** (●) or **3** (□). Samples contained F-Con A (4 μg/mL), TMR-ConA (4 μg/mL), and variable concentrations of added ligand (0, 0.098, 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 13.0, or 25 μM). Samples were excited at 492 nm and the emission was monitored at 517 nm. Error bars represent ± one standard deviation around the mean relative fluorescence intensity. Each data point is derived from three independent experiments.

surface. Only a single translational penalty must be paid for multiple binding interactions between the cluster and the carbohydrate-substituted surface to occur. The concentration of trivalent **2** needed to promote the interaction of Con A with the surface is 30−40-fold lower than that of monovalent **3** needed to inhibit. Thus, trivalent compound **2** appears to effectively bind and promote clustering of Con A in solution at concentrations where no inhibition by monovalent ligands is observed. As the concentration of **2** is increased further, inhibition of the binding of Con A and clustered Con A to the surface is observed, as expected.[28] Our results indicate that trivalent **2** promotes Con A clustering in solution, yet it is unclear from these data whether the formation of such clusters would be favorable in the absence of the mannose-substituted surface.

We used a fluorescence resonance energy transfer (FRET) assay to test whether trivalent ligand **2** or monovalent **3** can bring together differentially labeled Con A lectins such that donor and acceptor fluorophores can interact.[29] Specifically, fluorescein-labeled Con A (F-Con A) and tetramethylrhodamine-labeled Con A (TMR-Con A) were combined in the presence each of ligands **2** and **3**. Samples were prepared with constant concentrations of F-Con A and TMR-Con A and variable concentrations of either macrocycle **2** or monovalent **3**. Samples were irradiated individually at 492 nm and the fluorescein emission intensity was monitored at 517 nm. The data reveal that trivalent **2** is capable of quenching fluorescein emission at concentrations above 5 μM but monovalent **3** is not (Figure 2B). The activity is observed only for the trivalent macrocycle. When a solution containing **2**, F-Con A, and TMR-Con-A at concentrations known to result in fluorescence emission quenching was treated with increasing concentrations of α-methyl mannopyranoside, the intensity of fluorescence emission increased.[26b] This result indicates that the formation of soluble clusters is reversible. The concentration of **2** that results in 50% of the observed quenching in the FRET assay is 10 μM. Interestingly, the concentration of **2** needed to induce the maximum signal in the SPR assay is approximately 6 μM. Both assays indicate that the trivalent compound is at least 10-fold more potent than monovalent derivative **3**. No precipitation of the labeled Con A was detected in the presence of **2** or **3** under the assay conditions. These results indicate that clustering of Con A by macrocycle **2** does not depend on subsequent precipitation or cluster binding to a surface. The data also indicate that the interaction of trivalent **2** with multiple Con A proteins is synergistic; the formation of a Con A cluster is more favorable than the formation of a one-to-one complex of Con A and the trivalent ligand.

Multivalent ligands that cannot span the saccharide binding sites within the Con A tetramer are typically between 2- and 6-fold more potent than their monovalent counterparts. These ligands have been found to inhibit Con A by promoting its precipitation.[17−19,30] In contrast, trivalent ligand **2** facilitates the formation of soluble clusters in solution. The rigidity of the macrocyclic scaffold and/or the disposition of the saccharide epitopes likely play a critical role in the activity of the ligand **2**. Our results suggest that multivalent saccharide displays can act as superior ligands through their abilities to cluster target lectins. Intriguingly, complex carbohydrate side chains of glycoproteins may induce lectin clustering at the cell surface. This change in lectin organization could facilitate cellular processes, such as endocytosis[5,31] or cellular signaling.[32] Thus, synthetic multivalent ligand displays may be used not only as highly effective inhibitors but also to induce cellular responses[33,34] through receptor clustering.

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Supporting Information Available: A description of the surface plasmon resonance and fluorescence measurements and data analyses, experimental procedures for the synthesis of compounds **2** and **3**, and characterization data for all new compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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