On the Stringent Requirement of Mannosyl Substitution in Mannooligosaccharides for the Recognition by Garlic (Allium sativum) Lectin

A SURFACE PLASMON RESONANCE STUDY

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The kinetics of the binding of mannooligosaccharides to the heterodimeric lectin from garlic bulbs was studied using surface plasmon resonance. The interaction of the bound lectin immobilized on the sensor chip with a selected group of high mannose oligosaccharides was monitored in real time with the change in response units. This investigation corroborates our earlier study about the special preference of garlic lectin for terminal α-1,2-linked mannose residues. An increase in binding propensity can be directly correlated to the addition of α-1,2-linked mannose to the mannooligosaccharide at its nonreducing end. Mannosidase glycopeptide (Man₈GlcNAc₂Asn), the highest oligomer studied, exhibits its nonreducing end. Mannononase glycopeptide propensity can be directly correlated to the addition of α-1,2-linked terminal mannose on the α-1,6 arm is the critical determinant in the recognition of mannooligosaccharides by the lectin. The association (kₐ) and dissociation rate constants (k₋₁) for the binding of Man₈GlcNAc₂Asn to Allium sativum agglutinin I are 6.1 × 10⁴ M⁻¹ s⁻¹ and 4.9 × 10⁻² s⁻¹, respectively, at 25 °C. Whereas kₐ increases progressively from Man₉ to Man₉ derivatives, and more dramatically so for Man₉ and Man₈ derivatives, k₋₁ decreases relatively much less dramatically from Man₈ to Man₉ structures. An unprecedented increase in the association rate constant for interaction with Allium sativum agglutinin I with the structure of the oligosaccharide ligand constitutes a significant finding in protein-sugar recognition.

The structurally and evolutionarily related monocot mannose-binding proteins comprise a superfAMILY of mannose-specific lectins. Amaryllidaceae, Alliaceae, Araceae, Orchidaceae, Iridaceae, and Liliaceae families have been shown to possess these bulb lectins (1). Among the unique features that set them apart from the Glc/Mann/Gal-specific family of dicotyledonous legume lectins and the C-type mannose-binding animal lectins is their high degree of stereospecificity for mannose, so much so that they show no binding propensity even for its epimer, glucose, or the conformationally related analog, L-fucose. Their classification into the mannose-specific lectin family is corroborated by determination of the crystal structures of snowdrop (Galanthus nivalis) (2), daffodil (Narcissus pseudonarcissus) (3), bluebell (Scilla campanulata) (4), amaryllis (Hippeastrum hybrid) (5), and garlic (Allium sativum) lectin (6), representatives of the family of bulb lectins. Their subunits have been observed to possess a novel 3-fold symmetry having three four-stranded antiparallel β-sheets arranged as three sides of a triangular prism, forming a 12-stranded β-barrel referred to as the β-prism II fold. These 12 strands are positioned perpendicularly to the plane of symmetry, unlike the other known all-β-folds: β-prism I (e.g. Jacalin; (7)) and the β-trefoil (e.g. amaranthin; (8)) fold (6). The central region in the β-barrel is stacked with conserved hydrophobic side chains, which stabilize the subunit.

Bulbs of garlic are known to accumulate two types of mannose-binding lectins: the heterodimeric A. sativum agglutinin I (ASAI) (8) and the homodimeric ASAI (9). Interaction of ASAI and ASAI with mannooligosaccharides and glycoproteins has been investigated extensively by an enzyme-linked lectin adsorbent assay (10). Both of these lectins exhibit identical sugar specificities, although subtle differences between them cannot be ruled out in view of the qualitative nature of enzyme-linked lectin adsorbent assay. Enzyme-linked lectin adsorbent assay implicated α-1,2-linked mannose at the nonreducing end to determine the affinity for the higher order structures of mannose, evidenced by a sequential increase in potency with an increase in the number of α-1,2-linked mannose residues (10). In enzyme-linked lectin adsorbent assay, the recognition and adsorption properties of lectins with the specific sugar residues of glycoproteins are used for the assay purpose; therefore the method fails to provide information on the affinities of interaction or the elementary steps involved therein. On the other hand, kinetic studies using stopped flow and fluorescence titrations of the interaction of lectins with its ligands require large amounts of both the lectin and the saccharides as well as

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1 The abbreviations used are: ASA, Allium sativum agglutinin; SPR, surface plasmon resonance; RU, response unit; ITC, isothermal titra-
calorimetric.

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the labeling of the latter with a fluorophore or a chromophore. Therefore, in this study, the more sensitive, label-free, and fast response surface plasmon resonance (SPR) method took precedence as a means to delineate the affinities and the kinetic parameters for the interaction of ASAI with its complementary mannooligosaccharide ligands (11–13).

Barre et al. (1) used SPR to determine the carbohydrate specificity of a few monocot mannose-binding lectins. More recently, Van Damme et al. (14) characterized the carbohydrate binding propensity for Crocus vernus agglutinin using a biosensor. Both these investigations were carried out with large molecular weight glycoproteins, which are easier to monitor but fail to reveal the subtle features of carbohydrate moieties involved in these recognitions. In contrast, the study reported here with the individual purified mannooligosaccharide helps to unravel, in detail, the specificity invoked by garlic lectin for the recognition of its complementary ligands.

**EXPERIMENTAL PROCEDURES**

**Materials**—All the chemicals used, namely the components for phosphate-buffered saline buffer (pH 7.4), as well as N-ethyl-N'-{dimethylaminopropyl}carbodiimide hydrochloride and N-hydrorsuccinimide, were of the highest purity available. Certified grade CM5 sensor chips were purchased from Amersham Pharmacia Biotech.

**Saccharides**—Mannose, methyl-a-mannose, Man$_5$GlcNAc$_2$, Man$_6$GlcNAc$_2$, Man$_7$GlcNAc$_2$, Man$_{8}$GlcNAc$_2$ (I and II), and Man$_9$GlcNAc$_2$Asn were obtained from Sigma. Man$_3$ and Man$_5$ were procured from Dextra.

![Fig. 1. Structures of mannooligosaccharides.](image)

(1) Man$_3$  (2) Man$_3$GlcNAc$_2$  (3) Man$_5$

(4) Man$_5$GlcNAc$_2$  (5) Man$_5$GlcNAc$_2$  (6) Man$_5$GlcNAc$_2$Asn

(7) Man$_6$GlcNAc$_2$  (7') Man$_6$GlcNAc$_2$Asn  (8) Man$_7$GlcNAc$_2$ (I)

(9) Man$_7$GlcNAc$_2$ (II)  (10) Man$_8$GlcNAc$_2$  (11) Man$_9$GlcNAc$_2$Asn

![Mannose and GlcNAc](image)
Garlic Lectin-Mannooligosaccharide Recognition

Table I

Binding kinetics of ASAI-mannooligosaccharide interaction at 298 ± 0.1 K

| Oligosaccharide       | $k_1$ (M$^{-1}$ s$^{-1}$) | $k_2$ (M$^{-1}$ s$^{-1}$) | $K_a$ (M$^{-1}$) | $K_{v0}$ | $K_{ATC}$ |
|-----------------------|---------------------------|---------------------------|------------------|---------|---------|
| (1) Man$_5$          | 77.3                      | 0.61                      | 126.7            | 120.2   | 144     |
| (2) Man$_5$GlcNAc$_2$| 74.1                      | 0.59                      | 125.6            | 119.8   |         |
| (3) Man$_5$          | 98.3                      | 0.55                      | 169.5            | 163.4   | 162     |
| (4) Man$_5$GlcNAc$_2$| 102.2                     | 0.55                      | 185.8            | 181.5   |         |
| (5) Man$_5$GlcNAc$_2$| 97.4                      | 0.55                      | 167.9            | 165.8   |         |
| (6) Man$_5$GlcNAc$_2$| 105.2                     | 0.47                      | 223.8            | 218.2   |         |
| (7) Man$_5$GlcNAc$_2$| 105.2                     | 0.47                      | 223.8            | 218.2   |         |
| (8) Man$_5$GlcNAc$_2$| 5051                      | 0.060                     | No binding       | 8.4 $e^4$ | 8.5 $e^4$ |
| (9) Man$_5$GlcNAc$_2$| 105.2                     | 0.47                      | No binding       | 1.8 $e^5$ | 1.8 $e^5$ |
| (10) Man$_5$GlcNAc$_2$| 6.1 $e^4$                  | 0.049                     | 9.1 $e^5$        |         |         |

Laboratories. Man$_5$GlcNAc$_2$ was prepared in the laboratory from the Pronase digestion of soybean agglutinin (15, 16). Man$_5$GlcNAc$_2$ was obtained as a byproduct of the above preparation (15). Man$_5$GlcNAc$_2$ and Man$_5$GlcNAc$_2$ (Isosep, Tullings, Sweden) were kindly gifts from Prof. C. F. Brewer, Albert Einstein College of Medicine, New York and Prof. C. G. Gahmberg, Department of Biochemistry, University of Helsinki, Finland, respectively.

Protein Purification—ASAI was isolated and purified to homogeneity as described previously (10). The protein samples were prepared as required by the nature of the experiments described below.

Neutral Sugar Estimation—Concentrations of all the saccharides were determined by measuring their neutral sugar content by the phenol-sulfuric acid method (17). Mannose was used as the standard.

BIACore Biosensor Assays—Biospecific interaction analysis was performed using a BIACore 2000$\textsuperscript{TM}$ (Pharmacia Biosensor AB, Uppsala, Sweden) biosensor system based on the principle of SPR. Nearly 1500 response units (RU) of ASAI (0.1 mg/ml in 5 mM NaCl (pH 4.5)) were coupled to a certified grade CMS chip at a flow rate of 1 $\mu$l/min for 50 min using the amine coupling kit ($\text{N}^-$(ethyl-$\text{N}^-$(dimethylaminopropyl))-carbodiimide hydrochloride, $\text{N}$-hydroxysuccinimide) supplied by the manufacturer. Here, coupling of an RU corresponds to ~1 pg/mm$^2$ of immobilized protein. The unreacted species on the surface of the chip were blocked with ethanolamine. All measurements were done using 20 mM phosphate-buffered saline (pH 7.4). Prior to injection, protein samples were dialyzed extensively against the same buffer to avoid buffer effects.

Data Analysis—Association ($k_1$) and dissociation ($k_2$) rate constants were calculated from the basic equations of thermodynamics, where $K_a = k_1/k_2$ is the initial macromolecule concentration, and $K_a$ is the binding constant (21). The thermodynamic parameters were calculated from the basic equations of thermodynamics,

$$\Delta G = \Delta H - T \Delta S = -RT \ln K_a$$

RESULTS

Documented in Fig. 1 and Table I are the various mannose-containing carbohydrate ligands that were tested for their binding propensity to ASAI. The selection of the sugars was based on our previous study, wherein higher oligomers of manno- were implicated in binding to ASAI. Binding of saccharides shorter than mannitol could not be analyzed, because no appreciable change in RUs was observed upon their flow over the immobilized ASAI on the sensor chip, consistent with the sensitivity and specifications of the instrument. The use of a certified grade sensor chip in this study allowed satisfactory recording of RU changes corresponding to oligosaccharides having molecular masses greater than 400 Da. RU changes observed with the mannobioses (molecular mass 360 Da) provided sensorgrams that were not consistently reproducible.

A representative sensorgram for the interaction of varying amounts (5, 10, 20, 30, 40, 50, 60, 70, and 80 $\mu$l) of trimannoside (Man$_5$-3-3Man$_4$-Man$_2$) (1) passed over ASAI immobilized on a certified grade CM55 chip is shown in Fig. 2A. The curves, fitted by mass transport limited analyses at 25 °C, yielded values for $k_1$ and $k_1\cdot k_2$ of 77.3 s$^{-1}$ and 0.61 s$^{-1}$, respectively. An equal distribution of residuals for both phases of the reaction support the monoexponential nature of interaction (data not shown). The ratio $k_1/k_2$ provides an estimate of $K_a$ of 127 $M^{-1}$ at 25 °C (Table I). These parameters evaluated from SPR data are in good agreement with those obtained from isothermal titration calorimetric (ITC) measurements (Fig. 3).

Isothermal Titraton Calorimetry—Isothermal titration microcalorimetry was performed using an OMEGA microcalorimeter from Microcal Inc. (Northampton, MA). In individual titrations, injections of 5–6 $\mu$l of carbohydrate solution were made by a computer-controlled 250-$\mu$l syringe at an interval of 3 min into the lectin solution dissolved in the same buffer as the saccharide, while stirring at 396 rpm. Control experiments were performed by identical injections of saccharide into the cell containing only the buffer. The experimental data were fitted to a theoretical titration curve using the software provided by Microcal. All experiments performed were within $c$ values of $1 < c < 200$, where
Association rate constants (Table I). Extension of (3) with two α-1,2-linked mannosyl residues at its nonreducing end increases \( k_1 \) in a profound manner. Further extensions as in \( \text{Man}_9\text{GlcNAc}_2 \) (10) and \( \text{Man}_9\text{GlcNAc}_2\text{Asn} \) (11) do so even more dramatically (Fig. 2B). Although \( k_{-1} \) also decreases progressively for the extended structures as compared with \( \text{Man}_3 \) and \( \text{Man}_5 \), these changes are relatively less pronounced compared with the effects on the association rate constants (Table I).

**FIG. 2.** A, representative sensorgram depicting interaction of increasing amounts of trimannoside to the immobilized ASAI at 25 °C. The sugar, ranging in concentration from 5 \( \mu \text{M} \) (bottom trace) to 80 \( \mu \text{M} \) (top trace), was injected for 600 s at a flow rate of 5 \( \mu \text{l/min} \). The dissociation reaction was recorded by flowing buffer at 5 \( \mu \text{l/min} \). The surface of the chip was regenerated by a 10-s pulse of Me-α-mannopyranoside. Insert, Scatchard analysis of the sensorgram. B, the sensorgram depicts the binding of the higher order structures of mannose, \( \text{Man}_5\text{GlcNAc}_2 \) (8), \( \text{Man}_9\text{GlcNAc}_2 \) (10), and \( \text{Man}_9\text{GlcNAc}_2\text{Asn} \) (11) to ASAI immobilized on a CM5 sensor chip.
DISCUSSION

The binding kinetics for ASAI with the set of selected mannoooligosaccharides (Fig. 1) is seen to follow a single-step mechanism. The $k_1$ values observed here are in the range of $0.077 \times 10^3$ M$^{-1}$ s$^{-1}$ (for (1)) to $6.1 \times 10^4$ M$^{-1}$ s$^{-1}$ (for (11)). These second order rate constants are significantly lower than diffusion-controlled reactions (22, 24). Binding for such reactions is thought to involve an intermediate $PL_i$, which subsequently isomerizes to the final complex $PL^*$. 

\[
P + L \leftrightarrow PL_i \leftrightarrow PL^* \\
\text{Reaction 1}
\]

In such a situation,

\[k_{\text{on}} = k_{-1} + k_{+1} + k_{-2} + k_{+2} + k_{+3} + k_{-3}
\]

(Eq. 4)

where $K_{-1} = k_{-1}/k_{1}$.

The agreement between kinetically determined values of association constants ($k_1/k_{-1}$) and those determined by the Scatchard analyses of the SPR data suggest that the association and dissociation reactions are monoexponential in nature and describe faithfully the overall energetics of the system (Fig. 2A, inset). Moreover, the values of $K_s$ determined by SPR data are close to those estimated from ITC studies, thus ruling out an appreciable contribution from any unobserved binding process to the overall energetics of the system. These results thus imply a single step bimolecular association reaction between these saccharides and the lectin.

The “core” structure of the mannoooligosaccharide recognized by the ASAI carbohydrate recognition domain appears to be the trimannoside (1) common to all the N-linked glycans ($K_a = 127$ M$^{-1}$ at 25 °C), which is only a marginally poorer ligand than (3) ($K_a = 169$ M$^{-1}$ at 25 °C). The equivalence of the observed binding parameters for the interaction of compounds (1)–(6) suggests that the Man$_3$ structure constitutes the minimal epitope recognized by the lectin and that its substitution at the reducing end with GlcNAc$_2$ Asn does not alter either the mechanism or the extent of binding.

As listed in Table I, the binding affinity for the mannose-containing oligosaccharides increases with increasing chain length, with a significant jump observed when extended with $\alpha$-1,2-linked mannose residues at their nonreducing ends. These observations corroborate our previous study on the elucidation of the carbohydrate specificity of ASAI (10).

Among the higher oligomers of mannose, the binding affinities for Man$_7$ GlcNAc$_2$ (I) (8), (10), and (11) are appreciably higher than those of the above compounds, indicating that the extension of the Man$_3$ structure with $\alpha$-1,2-mannosyl residues at its nonreducing end increases the binding potencies. However, a mere extension with a single mannosyl residue in the $\alpha$-1,2 linkage at the $\alpha$-1,3 branch, as in Man$_6$ GlcNAc$_2$ (7) or Man$_6$ GlcNAc$_2$ Asn (7*), not only is inadequate but also perhaps compromises the binding. Additionally, these data suggest that a mere extension of the Man$_3$ structure by an $\alpha$-1,2Man, although necessary, is not sufficient to drive the reaction. The high affinities of (8), (10), and (11) suggest that the substitution of the $\alpha$-1,6 branch by two $\alpha$-1,2-linked mannosyl residues is apparently necessary for the moderate to strong binding to ASAI. Absence of this branch in (7), (7*), and Man$_6$ GlcNAc$_2$ (II) (9), therefore, make them nonbinders. A significant, 500-fold increase in the binding potency over manno pentaose is observed with (8) as the carbohydrate ligand ($8.4 \times 10^4$ M$^{-1}$ at 25 °C), which has two $\alpha$-1,2-linked mannosyl residues, one on the $\alpha$-1,3 arm and the other on the $\alpha$-1,6 arm. This suggests that for the combining site of ASAI to establish stronger bond-
ing contacts, substitution with α-1,2-linked mannose residues on both the arms is sufficient. Moreover, extensions on the α-1,3 arm are recognized, subsequent to recognition of the α-1,2-linked mannose on the α-1,6 arm. These binding characteristics thereby implicate the α-1,2-linked mannose on the α-1,6 arm to play a crucial role in lodging the complementary manno oligosaccharide in the ASAI combining site. Moreover, although (7) and (8) are structurally similar, an additional α-1,2-linked terminal mannose residue on the α-1,6 arm of (8) confers on it an exceedingly high binding affinity. This provides further insight into the mode of recognition of carbohydrate ligands by ASAI, where in the Manα (1–2) Man at the α-1,6 arm seems to drive the binding interaction further.

In general, the binding constant \( K_a \) is determined by the ratio of \( k_1 \) and \( k_{-1} \), and its value can increase with either an enhancement in the former or a decrease in the latter. For most enzyme-substrate interactions and also for the lectin-sugar interactions studied so far, it is the decrease in the dissociation rate constants that has been shown to be responsible for the increased binding affinities (25). In these studies on ASAI-manno oligosaccharide interactions, the occurrence of both, i.e., an increase in \( k_1 \) and decrease in \( k_{-1} \), is implicated for the observed enhancement in affinities, although to a large extent it is the relatively more dramatic increase in \( k_1 \) that imparts to (10) and (11) their highest affinities. Thus, one of the interesting observations of this study is the significant increase in the association rate constants with the change in the structure of the manno oligosaccharide ligand.

In conclusion, this study provides a molecular basis for explaining the exquisite specificity of ASAI for the high manno oligosaccharides by demonstrating a stringent requirement for α-1,2-mannosyl substitution at their α-1,6 arms. Additionally, ASAI-manno oligosaccharide interactions constitute a unique example where the association rate constants vary dramatically by a factor of about 800 between the weakest and the strongest ligand.

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