Frontal Affinity Chromatography of Ovalbumin Glycoasparagines on a Concanavalin A-Sepharose Column

A QUANTITATIVE STUDY OF THE BINDING SPECIFICITY OF THE LECTIN*

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The interactions of Sepharose 4B-immobilized concanavalin A (ConA) with 10 glycoasparagines derived from ovalbumin were investigated quantitatively by frontal affinity chromatography. In this method, a carbohydrate solution is applied continuously to a ConA-Sepharose column and the retardation of the elution front is measured as a parameter of the strength of the interaction. The dissociation constant (Kd) for each saccharide with ConA can be determined. An analysis of the binding of p-nitrophenyl-α,β-mannoside has shown that the binding properties of ConA do not change essentially after immobilization on Sepharose 4B. Each of the ovalbumin glycoasparagines was labeled with tritium by the reductive methylation method for analysis. A comparison of the Kd values obtained showed that the binding of ConA varies considerably with very slight structural differences of the glycosyl chain. The results suggest that ConA recognizes a specific glycosyl chain structure, Manα1-6(Manα1-3)Man, in which at least one hydroxyl group at the C-3 position of C-6-linked mannose should be free. The glycoasparagines containing this structure bound strongly to ConA-Sepharose with dissociation constants below 3.4 x 10^-7 M.

Lectins are widely utilized as a tool in the field of carbohydrate biochemistry. Immobilized lectin columns are useful to separate the sugar compounds on the basis of slight structural differences. Furthermore, specific interactions between biomolecules can be investigated quantitatively by affinity chromatography. Kasai and Ishii (1, 2) have proved that frontal analysis in affinity chromatography is useful in evaluating the equilibrium constant. In this report, we present an application of this method to immobilized concanavalin A (ConA') and demonstrate its utility for the quantitative investigation of the binding specificity.

ConA is a lectin isolated from jack beans (Canavalia ensiformis). Its physical and biochemical properties have been investigated in detail (3-7). ConA binds to the membrane glycoproteins of a variety of cell types leading to various biological responses such as mitogenesis in lymphocytes (8). Since cell surface oligosaccharides linked to asparagine residues are thought to participate in a variety of specific interactions, the binding specificity of ConA toward these glycosyl chains is of great interest. Kornfeld and Ferris (9) and Baenziger and Fiete (10) studied the binding of ConA with several complex-type oligosaccharides derived from immunoglobulin. Their results had important implications, but their methods are not the most suitable for an accurate binding study. Frontal affinity chromatography has several advantages. Recently, Oda et al. (11) have reported a quantitative investigation of the interactions of these carbohydrates with ConA and various simple sugars by utilizing this method.

For a detailed study on the specificity of lectins, pure oligosaccharides of known structure are essential. Hen egg ovalbumin is an excellent source and yields large amounts of various glycosyl chains, the structures of which have been determined (12-15). It is also advantageous because various high mannose-type chains, which are suitable for the study of ConA can be obtained, as well as hybrid-type glycosyl chains.

In this report, the dissociation constants of 10 asparagine-linked oligosaccharides derived from ovalbumin are determined by the method of frontal affinity chromatography with a column of ConA immobilized on Sepharose 4B. The carbohydrate structural characteristics important for the binding of ConA are discussed.

MATERIALS AND METHODS

Preparation of ConA-Sepharose—ConA not containing split subunit was prepared from the commercial product (Sigma, Type IV) by absorption chromatography on Sephadex G-100 (16).

ConA-Sepharose 4B was prepared by the reported method (17). ConA was coupled at concentrations of 0.02, 0.1, 0.5, or 2.5 mg/ml of wet CNBr-activated gel to obtain gels having different ConA concentrations.

Preparation of Ovalbumin Glycoasparagines—Preparation of hen egg ovalbumin glycoasparagines was described before (12, 15, 18). Six major fractions obtained by exhaustive Pronase digestion were further purified by boronate chromatography on DEAE-Sepharose A-25. One of the fractions, GP-III, was separated into three subfractions, GP-III-A, -B, and -C. The purity and the correctness of the reported structure were confirmed by methylation analysis. Since GP-III, which contained two subfractions, was not separated by the above procedure, it was fractionated by ConA-Sepharose chromatography after being labeled with tritium as described below. The preparation of GP core (the structure shown in Fig. 4) by limited acid hydrolysis of ovalbumin glycoasparagine was described before (18).

Tritium Labeling of Glycoasparagines—Each glycoasparagine was labeled with tritium by the reductive methylation method of Means and Feeney (19). Aliquots (1 μl each) of 0.2 M formaldehyde and...
NaB\(^{3H}\) (5.7 Ci/mmol, Amersham Corp. 0.05 M in 5 N NaOH) were added to 0.1 \(\mu\)mol of glycoasparagine in 10 \(\mu\)l of 0.2 M sodium borate buffer (pH 9.0), and the mixture was kept at 0°C for 1 h. After 30 min, the additions of formaldehyde and NaB\(^{3H}\) were repeated. The reaction was stopped by the addition of 200 \(\mu\)l of 0.2 M pyridine.

Low molecular weight substances were removed on a Sephadex G-25 column. Tritium-labeled glycoasparagines with specific activity between 2.5 and 4.0 \(\times\) 10\(^{9}\) dpm/\(\mu\)mol were obtained. KB\(^{3H}\) (581 mCi/mmol, Amersham Corp.) was also used instead of NaB\(^{3H}\), in which case the reaction was carried out at room temperature for 2 h.

Frontal Affinity Chromatography on ConA-Sepharose—Four columns, made of polystyrene, of the same size (0.6 x 18 cm; bed volume, 5 ml) were packed with ConA-Sepharose. All the chromatographic operations were carried out at 4 °C. For regeneration and re-equilibration, a column was washed with 50 ml of buffer I (20 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 0.1 mM CaCl\(_2\), 0.1 mM MnCl\(_2\), 0.2% Brij 35) containing 0.4 M \(\alpha\)-methylmannoside and then with more than 100 ml of buffer I without \(\alpha\)-methylmannoside. This wash was continued until just before the sample application. A sample carbohydrate solution was prepared in buffer I and applied to the column continuously at a flow rate of 6 ml/h. Collection of equal volume fractions was started at the beginning of the sample application.

The concentration of carbohydrate in each fraction was determined either spectrophotometrically or radiochemically. For the analysis of tritium-labeled glycoasparagines, the radioactivity of 0.5 ml of each fraction was measured with a liquid scintillation counter in a toluene-based emulsifying scintillator (20). The concentration of p-nitrophenyl-D-mannoside was determined by using the molecular absorption coefficient at 305 nm of 10,000 \(\textit{cm}^{-1}\) (21).

Sugar Concentration—The concentration of glycoasparagines before tritium labeling was determined by the phenol/sulfuric acid method (22). Quantitation of aspartic acid by amino acid analysis (Hitachi 835 amino acid analyzer) was also carried out for GP-III-A, -B, and -C after acid hydrolysis (6 N HCl, 110 °C, 24 h).

RESULTS

Frontal Affinity Chromatography of \(\alpha\)-Nitrophenylmannosides on ConA-Sepharose 4B—In the analysis by frontal affinity chromatography, a solution of one of the interacting substances, A, is applied continuously at constant concentration, \([A]_0\), to a column on which its counterpart, B, is immobilized. The elution volume of the front, \(V\), is measured. The relation between \([A]_0\) and \(V\) is given by the following equation,

\[
V = \frac{B_i}{K_d + [A]_0} + V_o
\]

where \(V_o\) is the elution volume of the front in the case without specific interaction, \(B_i\) is the total amount of B in the column that can interact with A, and \(K_d\) is the dissociation constant (1, 2). As each \(K_d\), \(B_i\), or \(V_o\) is constant, \(V\) varies depending on \([A]_0\) in the appropriate concentration range. Equation 1 can be rearranged as follows, so that a plot of \(1/[A]_0 (V - V_o)\) versus \(1/[A]_0\) should be linear.

\[
\frac{1}{[A]_0 (V - V_o)} = \frac{K_d}{B_i + [A]_0} + \frac{1}{V_o}
\]

The value of \(K_d\) can be determined from the intercept on the abscissa, which corresponds to \(-1/K_d\). The value of \(B_i\), the content of active ConA in the column, can also be obtained from the intercept on the ordinate of the same plot.

Fig. 1 shows the frontal chromatography of \(\beta\)-nitrophenyl-\(\alpha\)-mannosides (PNM) on a ConA-Sepharose column, Column A, which immobilizes ConA at 2.5 mg/ml. As shown in Panel A, the position of the elution front of \(\beta\)-nitrophenyl-\(\alpha\)-mannoside (\(\alpha\)-PNM) changed depending on the concentration. The retardation of the front increased with decrease of the concentration. The closed circles of Panel B show the \(1/[A]_0 (V - V_o)\) versus \(1/[A]_0\) plot for Panel A. In this plot, \(V_o\) obtained from the elution volume of \(\alpha\)-PNM in the presence of 0.4 M \(\alpha\)-methylmannoside was used. Each point lay on a straight line, and a \(K_d\) value of \(1.8 \times 10^{-6}\) M was obtained.
The amount of active ConA in the column was also obtained as $3.2 \times 10^{-7} \text{ mol}$ from the plot. The open circles in Fig. 1, Panel B, are the plots for a series of runs with $\alpha$-PNM on a different ConA-Sepharose column. The value of $K_d$ obtained in this case, $1.7 \times 10^{-6} \text{ M}$, was in good agreement with that obtained from the closed circles. Thus, the method is highly reproducible. A $B_i$ value of $3.7 \times 10^{-7}$ was also obtained.

Once $B_i$ of the column has been determined, dissociation constants for other saccharides can be determined by a single measurement of $V$. The following equation, which is derived from Equation 1, can be applied.

$$K_d = \frac{B_i}{V - V_o - [A]_0}$$

The $K_d$ for $\beta$-PNM was obtained as $1.0 \times 10^{-4} \text{ M}$, because $V = 9.0 \text{ ml}$. The figure indicates that two concentrations of $\beta$-PNM, 2.5 and $5.0 \mu\text{M}$, gave almost the same $V$, which presumably occurs because $[A]_0$ is negligibly small compared with $K_d$. In such cases, it is not necessary to know the exact value of $[A]_0$ for the determination of $K_d$. $K_d$ for $\beta$-PNM is about five times higher than that for $\alpha$-PNM.

The interactions studied here are those between ConA immobilized on Sepharose and the saccharides in free solution. However, the interaction between free ConA and saccharides can be also studied by frontal affinity chromatography. In this case, a solution of ConA is applied to a column on which one of the specific ligands is immobilized, in the presence of free saccharide. The effect of the saccharide on the elution volume of ConA makes it possible to calculate the dissociation constant, $K_d$. Oda et al. (11) reported the dissociation constant between $\alpha$-PNM and free ConA obtained by this indirect method, employing a column of aminophenyl-$\beta$,p-d-glucopyranoside. The value obtained ($1.4 \times 10^{-8}$) agreed well with that obtained above. Thus, the binding properties of ConA do not seem to be much affected by the immobilizing procedure, and the values obtained for ConA immobilized on Sepharose should reflect the binding of intact ConA.

**Frontal Affinity Chromatography of Ovalbumin Glycoasparagines—**Each ovalbumin glycoasparagine was labeled with tritium by converting the $\alpha$-amino group of the asparagine residue to a tritium-containing dimethylamino group. The volume occupied by the methyl group is relatively small, and the $pK$ value of the dimethylamino group is only 0.5 unit lower than that of the $\alpha$-amino group (20). Thus, the influence of the labeling on the interaction with ConA should be quite small.

Fig. 2 shows the result of frontal chromatography of GP-III-A. Since the binding of GP-III-A to ConA was much stronger than that of PNM, Column C (ConA, 0.1 mg/ml) was employed. The retardation of the elution front depended on the concentration of saccharide, and the $1/[A]_0(V - V_o)$ versus $1/[A]_0$ plot gave a straight line (inset of Fig. 2); a $K_d$ of $3.2 \times 10^{-7} \text{ M}$ and a $B_i$ of $1.04 \times 10^{-8} \text{ mol}$ were obtained.

Fig. 3 shows the frontal chromatography of GP-III-B on Column D (ConA, 0.02 mg/ml). The rise of the front was less sharp. In general, the sharpness of the elution front tends to be lost as the amount of immobilized ConA in the column decreases. This may be due to the decrease of the number of theoretical plates. Even in this case, the results gave a linear plot, and a $K_d$ of $2.9 \times 10^{-8} \text{ M}$ and a $B_i$ of $2.5 \times 10^{-8} \text{ mol}$ were obtained.

Table I summarizes the results of frontal affinity chromatographies of other ovalbumin glycosidic chains. $B_i$ values of the columns had been determined previously. Chromatography was carried out on a column of appropriate ConA concentration in order to obtain reasonable $V$ values. For GP-IV and GP-V, dissociation constants were measured on both Column C and Column D. Although the ConA contents of the columns differed, the same $K_d$ values were obtained on both columns for each sugar. For GP-I, Column B (ConA, 0.5 mg/ml) was also used and the concentration dependency was studied. A $K_d$ of $3.4 \times 10^{-7} \text{ M}$ was obtained (data not shown). This agrees well with the value of $3.5 \times 10^{-7} \text{ M}$ given in Table I.

**Required Glycosyl Chain Structure for High Affinity Binding with Concanavalin A—**Fig. 4 shows the structures of 10 glycoasparagines and their dissociation constants. Dissociation constants, which have a definite physical meaning, should give precise information on the specificity of concanavalin A.

It is known that the $\alpha$-mannosyl residue of complex carbohydrates is very important for the binding of ConA. However, our results indicate that the binding strength of ConA is not determined merely by the number of $\alpha$-mannosyl residues. This can be easily seen by a comparison among 5 high mannose-type glycoasparagines. The number of $\alpha$-mannosyl residues of these increases successively, but the binding strength of ConA does not increase in the same order. In particular, the dissociation constant for GP-VI was nearly 10 times larger than that for the GP core, whereas GP-V only added 1 additional $\alpha$-mannosyl residue as compared with the GP core. The addition of 1 $\alpha$-mannosyl residue to GP-VI, which gives rise to GP-V, makes the binding of ConA much stronger, but the addition to GP-V, resulting in GP-IV, rather weakened the binding. These data completely agree with the qualitative observation reported by Harpaz and Schachter (23).

Goldstein et al. (24) have established that a monosaccharide is required to have D-mannos- or D-glucopyranose configuration with unmodified hydroxyl groups at the C-3, C-4, and C-6 positions to bind strongly to ConA. On the basis of this assumption, $\alpha$-mannose located at a nonreducing terminal or in the internal chain and linked to the next residue at its C-2 position should be important. However, our data showed that the binding strength of ConA is not determined by the number of such mannosyl residues (e.g., poor binding with GP-VI).

Our results strongly suggest that ConA recognizes and binds at high affinity only to some specific structure. The trimannose structure, illustrated in Fig. 5, is a good candidate for such a specific structure.

Seven glycoasparagines which strongly bind to ConA with dissociation constants of the order of $10^{-7} \text{ M}$ or below contain this structure. Its importance is clear from a comparison of the 5 glycoasparagines of so-called hybrid type. GP-I, GP-II-B, and GP-III-A, which all contain the trimannose structure, have small and nearly equal dissociation constants in spite of their structural differences. GP-II-A and GP-III-C that lack the C-6-linked mannose have nearly 1000 times larger dissociation constants.

It is not probable that ConA recognizes only the C-6-linked mannose. Ogata et al. (25) showed qualitatively that Man$_{1}$-6Man$_{1}$-1 mannitol was not absorbed on ConA-Sepharose, while oligosaccharides containing the trimannose structure were adsorbed. In addition, Kruisius et al. (26) and Narashimhan et al. (27) reported that ConA strongly binds to biantennary type but not to triantennary type of complex glycosyl chains. In the biantennary type, two chains of NeuAc $\alpha$2-6Gal$\beta$1-4GlcNAc$\beta$1- unit that are linked to the C-2 position of both the C-3- and C-6-linked chains. However, in the triantennary type another glycosyl chain is linked to the C-4 position of the C-3-linked mannose (28). The resultant triantennary type should become a weak binding species. Thus,
both C-3-linked and C-6-linked α-mannosyl residues are essential for strong binding. The concept that ConA requires 2 mannose residues attached to a common residue has been also described by Narasimhan et al. (27) before.

So and Goldstein (29) and Kornfeld and Ferris (9) also suggested multiple binding sites of ConA. On the other hand, as regards monosaccharide binding, equilibrium dialysis showed that only 1 mannoside molecule was bound per subunit (3, 4). The binding of the second residue might thus occur only when it is included in an oligosaccharide. The concept of subsites could be applied. Interaction of monosaccharide with binding sites other than first binding site should be too weak to be detectable. CD spectroscopic studies showed that the conformation of the ConA molecule changed considerably when 1 molecule of α-methylmannoside was bound (30, 31). Such a conformational change might be necessary for the binding of the second residue.

As for the structure required for strong binding with ConA, the following three conclusions can be derived from the results obtained here: 1) the anomeric configuration of the central mannose of the trimannose structure is not critical since the dissociation constant for the GP core, in which the central mannose has \( \beta \) configuration, did not differ much from those for GP-I, GP-II-B, and GP-III-A, in which the central mannose has \( \alpha \) configuration; 2) the hydroxyl group at the C-3 position of the C-6-linked mannose of the trimannose should be free since GP-VI has a very low binding ability; 3) linkage of another α-mannose to the C-2 hydroxyl group of the C-6-linked mannose strengthens the binding (that of GP-III-B is about seven times stronger than that of GP-IV).
It is interesting that the dissociation constant for GP-VI is about 10 times smaller than that for GP-II-A and GP-III-C (Table I). Probably ConA may interact with 2 mannos residues attached to β-linked mannose in GP-VI although only weakly for the linkage of α-mannose to C-3 position of C-6-linked mannose. This interaction should be weakened even more by the attachment of GlcNAc at C-4 position(s) of β-linked mannose and/or of C-3-linked mannose for GP-II-A and -III-C. The studies by Harpaz and Schachter (23) and Narasimhan (32) have supported this idea.

Gleeson and Schachter (28) showed qualitatively that linkage of β-GlcNAc to the C-2 position of the C-6-linked mannos also tightens the binding of ConA. Thus, the effect of the substitution at this position in increasing the affinity of ConA should not be restricted to that by α-mannose.

In conclusion, ConA specifically binds at high affinity to oligosaccharides which contain the trimannose structure, Manα1-6(Manα1-3)Man. The C-3 hydroxyl group of the C-6-linked mannose and the C-4 hydroxyl group of the C-3-linked mannose should be unmodified. The C-2 position of both the C-3- and C-6-linked mannose need not necessarily be free. Rather, the substitution of C-6-linked mannose by α-mannose or β-GlcNAc tightens the binding of ConA.

### DISCUSSION

Frontal affinity chromatography has several advantages. For example, the interaction can be measured without significantly altering the equilibrium. Furthermore, the introduction of some reporter groups, such as fluorescent or chromogenic groups, is not necessary nor is specially devised equipment required. The theoretical basis is very simple because the concentration of free solute is maintained at the initial concentration, [A]₀. After equilibrium is attained, the amount of binding complex is given by [A]₀(V - V₀).

Kornfeld and Ferris (9) studied the specificity of ConA by comparing the carbohydrate concentrations that gave 50% inhibition of the binding of ConA to guinea pig erythrocytes. Their experiment was quantitative, but the values obtained are not equilibrium constants. Baenziger and Fiete (10) reported the association constants between ConA and 125I-labeled oligosaccharides. Their values were based on the quan-

### TABLE I

Frontal affinity chromatography of several glycoasparagines on columns whose ConA contents had previously been determined

<table>
<thead>
<tr>
<th>Glycopeptide</th>
<th>Conc</th>
<th>Column</th>
<th>ConA content</th>
<th>V</th>
<th>Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>Column</td>
<td></td>
<td></td>
<td>(10^{-6})</td>
</tr>
<tr>
<td>GP-I</td>
<td>2.0</td>
<td>C</td>
<td>10.4</td>
<td>36.08</td>
<td>3.5 \times 10^{-7}</td>
</tr>
<tr>
<td>GP-II-A</td>
<td>2.4</td>
<td>A</td>
<td>320</td>
<td>7.60</td>
<td>3.0 \times 10^{-4}</td>
</tr>
<tr>
<td>GP-II-B</td>
<td>3.3</td>
<td>C</td>
<td>10.4</td>
<td>39.12</td>
<td>3.2 \times 10^{-7}</td>
</tr>
<tr>
<td>GP-III-C</td>
<td>7.1</td>
<td>A*</td>
<td>370</td>
<td>8.10</td>
<td>2.7 \times 10^{-4}</td>
</tr>
<tr>
<td>GP-IV</td>
<td>2.0</td>
<td>C</td>
<td>10.4</td>
<td>58.22</td>
<td>3.5 \times 10^{-7}</td>
</tr>
<tr>
<td>GP-V</td>
<td>1.3</td>
<td>D</td>
<td>2.5</td>
<td>18.86</td>
<td>2.0 \times 10^{-7}</td>
</tr>
<tr>
<td>GP-VI</td>
<td>3.3</td>
<td>C</td>
<td>10.4</td>
<td>37.0</td>
<td>2.7 \times 10^{-4}</td>
</tr>
<tr>
<td>GP core</td>
<td>2.5</td>
<td>C</td>
<td>10.4</td>
<td>49.61</td>
<td>2.4 \times 10^{-7}</td>
</tr>
</tbody>
</table>

**Fig. 4.** Structures of ovalbumin glycoasparagines and their binding strengths with ConA. The numbers in parentheses indicate the relative binding strengths calculated as the ratio of dissociation constants to that of GP-II-A, which showed the largest value among the glycoasparagines tested. A single bar (—) linking monosaccharides represents an α-glycoside bond and a double bar (=) represents a β-bond. The position of linkage represented by these corresponds to the number illustrated in Fig. 5.
Frontal Affinity Chromatography on ConA-Sepharose