A Spectrophotometric Study of the Carbohydrate Binding Site of Concanavalin A∗

WOLFGANG BESSLER, J. A. SHAFER, AND I. J. GOLDSTEIN

From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104

SUMMARY

The carbohydrate binding sites of the jack bean lectin, concanavalin A, were studied spectrophotometrically. A number of ligands were examined for their ability to displace the chromogenic ligand p-nitrophenyl a-D-mannopyranoside. It is shown that this technique provides a simple and convenient approach for obtaining association constants for a series of displacing ligands. The data can best be interpreted in terms of a single carbohydrate binding site per monomeric unit for the protein in solution.

The jack bean lectin, concanavalin A, is a carbohydrate binding protein of considerable biological interest (1, 2). In its interaction with carbohydrates it serves as a model for the antibody-antigen system (1, 3-6). This protein stimulates lymphocytes to divide (7, 8), interacts differentially with normal and malignant cells (9) and serves as a probe for investigating carbohydrate-containing components of cells (1, 2, 10). Extensive binding studies have demonstrated that the combining sites of concanavalin A are most complementary to a-D-mannopyranosyl residues although structurally related sugars, e.g. a-glucose, a-fructose, and a-arabinose (in the furanosyl ring form), will also bind to the protein (11-17).

The primary (18, 19) and three-dimensional x-ray structure (19, 20) of concanavalin A have been reported. The carbohydrate binding sites of the protein reportedly were located in difference electron density maps (21) employing a-iodophenyl a-D-glucopyranoside as the binding ligand, a glycoside reported by Poretz and Goldstein (22) to inhibit concanavalin A-polysaccharide interaction. The a-iodophenyl aglycone was shown by the latter workers (22) to bind to a hydrophobic region of the protein adjacent to the carbohydrate binding site. Edelman et al. (19, 21) and Hardman and Ainsworth (20) have reported that the aromatic aglycone-binding region of the protein is lined with apolar amino acyl residues. Alternatively, Brewer et al. (23) using 14C carbon magnetic resonance have presented evidence that the carbohydrate binding site may be located elsewhere in the protein.

Controversy has developed recently concerning the question of whether or not oligo- and polysaccharides bind to the same carbohydrate site as that occupied by the glycosyl moiety of a-iodophenyl a-D-glucopyranoside. In a recent communication, Hardman and Ainsworth (24) present new evidence suggesting that the a-iodophenyl a-D-glucopyranoside-binding site as observed by Becker et al. (21) may be due to the presence of a separate apolar site in concanavalin A crystals.

The present study was designed to explore the question of multiple concanavalin A binding sites and, at the same time, to investigate a convenient method for evaluating interactions between concanavalin A and its ligands. In this work, association constants for complexes formed between concanavalin A and several ligands were evaluated from the ability of these ligands to displace p-nitrophenyl a-D-mannopyranoside from concanavalin A. It will be shown that the data obtained can be interpreted best in terms of a single carbohydrate binding site.

MATERIALS AND METHODS

Concanavalin A was prepared from jack bean meal (Sigma Chemical Co., St. Louis, Mo.) by the method of Agrawal and Goldstein (25) and stored in 0.5 M NaCl (pH 5.65) at 4°C. Concanavalin A concentrations were determined by absorbance at 280 nm (ε1%1% = 11.4) (26). Molecular weight of a subunit was assumed to be 26,500.

All sugars used were chromatographically pure and available from previous studies. Additional a-iodophenyl a-D-glucopyranoside was prepared by a method communicated by C. F. Brewer (Albert Einstein College of Medicine, Bronx, N. Y.). Dr. Brewer also kindly provided a sample of a-iodophenyl a-D-galactopyranoside.

Ultrafilter difference spectra were recorded on a dual beam Cary 118 Recording Spectrophotometer (cf. Hassing and Goldstein (27)). Temperatures were maintained at ±0.3°C by a circulating pump (Bonnwill Scientific Division, Will Corp., Rochester, N. Y.). Most studies were conducted at 27°C, other runs were made at 4 and 8°C. Matched Yankeelov cuvettes (Pyrccell Manufacturing Co., Westwood, N. Y.) with an effective path length of 8.8 mm were employed.

All experiments were performed in phosphate-buffered saline at pH 7, 0.0875 M phosphate, 0.1 mM CaCl2, and 0.1 mM MnCl2, and adjusted to 1/2 0.5 with NaCl.

Into one compartment of a Yankeelov cuvette was added 1.00 ml of the concanavalin A solution (2.02 mg per ml of phosphate-buffered saline; 38.1 μM in combining sites). p-Nitrophenyl a-D-mannopyranoside in phosphate-buffered saline (1.00 ml) was pipetted into the second compartment. A second cuvette was prepared in exactly the same manner. After a satisfactory baseline with the two cuvettes was obtained, the contents of the sample cuvette were mixed by inverting several times and the difference spectrum was recorded. Difference spectra remained constant for at least 30 min. Subsequently, the second cuvette was inverted and mixed in order to restore the baseline. Using the absorbance change at the maximum wavelength (317 nm), 1/ΔA

* This work was supported by grants AM-10171 and AM-09276 from the National Institutes of Health.
was plotted against the reciprocal of the total ligand concentration, \([D]\) for values of \([D]\) \(\gg [P,]\). The reciprocal of the intercept of these plots on the ordinate gave \(\Delta A_\alpha\), the change in absorbance occurring when all the protein becomes saturated with ligand.

Displacement experiments with the various sugars were done in the following manner: a series of dilutions of each sugar ligand, each dilution containing in addition a constant amount of reference sugar \((p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside})\), were mixed in the cuvettes individually with the concanavalin A solution to obtain a series of difference spectra and the absorption change at each dilution containing in addition a constant amount of reference sugar, \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\) displace the reference ligand, \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\), from concanavalin A. The upper spectrum shows the perturbation displayed by the reference sugar alone, the maximum absorbance difference being at 317 nm. The remaining spectra show the effect of adding increasing concentrations of \(p\text{-iodophenyl } \beta\text{-d-glucopyranoside}\) to the reference solutions. It is apparent that the perturbations become less intense with

\[
L + P + D = \frac{k}{K_D} PD + L
\]

The binding of certain ligands such as \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\) to concanavalin A is accompanied by pronounced changes in the ultraviolet spectrum (27). Since these spectral changes can be related to the amount of ligand bound, protein-ligand interactions can be determined conveniently and rapidly from spectral measurements. Unfortunately, the binding of most carbohydrate ligands to concanavalin A does not produce changes in the absorbance spectrum which are sufficiently large for accurately determining the amount of bound ligand (27-29). However, interactions between concanavalin A and such ligands can be evaluated by studying how these ligands displace a chromogenic ligand such as \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\) from concanavalin A. Such studies should yield data on the strength of the interaction of the second ligand with the protein, as well as information about the identity of the binding sites for the chromogenic ligand and the competitor ligand. The use of chromogenic ligands to characterize interactions between proteins and colorless ligands is not new. For example, Bernhard et al. (30) developed a system for studying interactions between chymotrypsin and its substrates which relied on the substrate's ability to displace the chromogenic inhibitor, proflavin.

Fig. 1 presents the series of difference spectra obtained when the competitor ligand, \(p\text{-iodophenyl } \beta\text{-d-glucopyranoside}\), displaces the reference ligand, \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\), from concanavalin A. The upper spectrum shows the perturbation displayed by the reference sugar alone, the maximum absorbance difference being at 317 nm. The remaining spectra show the effect of adding increasing concentrations of \(p\text{-iodophenyl } \beta\text{-d-glucopyranoside}\) to the reference solutions. It is apparent that the perturbations become less intense with an increase in the amount of competitor. The conditions of the assay are indicated in the legend.

Consider the equilibria depicted in Equation 1, where \(D, L, \) and \(P\) represent the chromogenic ligand, the second ligand, and the binding unit, respectively.

\[
L + P + D = \frac{k}{K_D} PD + L
\]

\[
\text{Setting } [P,] = [PD] + [PDL] + [P] + [PL] \Rightarrow [P,] = [PD] (1 + K_{DL} [L]) + \frac{1}{K_D[D]} \frac{[D]}{[D]}
\]

Assuming that binding of the second ligand to PD does not alter the molar absorptivity of bound chromogenic ligand,

\[
\frac{[P,] - [P]}{[PD] + [PDL]} = \frac{\Delta A_\alpha}{\Delta A}
\]

where \(\Delta A_\alpha\) is the change in absorbance when all the sites become saturated with \(D\), and \(\Delta A\) is the absorbance change observed when the protein is mixed with the ligands.

The concentration of free chromogenic ligand \([D]\) was calculated from the total concentration of this ligand \([D]\) using the relationship,

\[
[D] = [P,] - \frac{\Delta A}{\Delta A_{\alpha}} [P,] - [P,]
\]

where \([P,]\) was set equal to the concentration of monomeric units of concanavalin A.

It is apparent from Equation 4 that when \([L]\) = 0, a plot of \([P,]/[PD] + [PDL] - 1\) \([D]\) should yield a straight line with a slope of \(K_D^{-1}\). Such results are depicted in Fig. 2 for the interaction of \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\) with concanavalin A. A value for \(K_D\) of \(3.4 \times 10^4\) M\(^{-1}\) was determined for \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\) at 4°. This is in good agreement with a value of \(K_D\) of \(3.54 \times 10^4\) M\(^{-1}\) determined independently by equilibrium dialysis by Loontiens et al. (31).

When the product \((IP,)/(PD) + [PDL] - 1\) \([D]\) is plotted against the concentration of second ligand, one should obtain a straight line if the termolecular complex PDL does not form \((K_{DL} = 0, i.e. both ligands compete for the same site on the protein). As shown in Fig. 3, A and B, all ligands which displaced \(p\text{-nitrophenyl } \alpha\text{-d-mannopyranoside}\) gave linear relationships between \((IP,)/(PD) + [PDL] - 1\) \([D]\) and \([L]\). Values of \(K_{DL}\) determined from these plots, assuming \(K_{DL} = 0\), are listed in Table I.

The value of \(1.65 \times 10^4\) M\(^{-1}\) at 8° and pH 7.0 for \(K_D\) for the methyl \(\alpha\text{-d-mannopyranoside-concanavalin A complex obtained by these displacement studies is close to the value of \(K_D\) of 2.06 \times 10^4\) M\(^{-1}\) at 2° and pH 6.2 obtained by equilibrium dialysis (32). This agreement suggests that both methods are measuring the same interaction.

Although the observed linear relationships between \((IP,)/(PD) + [PDL] - 1\) \([D]\) and \([L]\) cannot absolutely exclude the possibility that concanavalin A can simultaneously bind the \(p\text{-nitrophenyl}glycoside and a second ligand such as methyl \(\alpha\text{-d-mannopyranoside}, the results of Fig. 3, A and B do indicate
FIG. 2 (left). Determination of the association constants, $K_D$, for the interaction of p-nitrophenyl α-D-mannopyranoside [D] with concanavalin A [P] at 4° (■) and 27° (□). The concentration of concanavalin A is 19.05 μM, corresponding to 38.1 μM binding sites.

FIG. 3 (center and right). Determination of the association constants, $K_L$, for the interaction of several ligands (L) with concanavalin A-carbohydrate interaction.

TABLE I

Binding data for concanavalin A-carbohydrate interaction

In each of the above experiments, concanavalin A was 38.1 μM with respect to binding sites and p-nitrophenyl α-D-mannopyranoside was 102.5 μM (both after tipping). The following substances (concentration in parentheses) were also examined for their capacity to displace the chromogen (all were active): methyl α-D-mannopyranoside (29 mM), p-nitrophenyl β-D-mannopyranoside (500 μM), o-hydroxymethylphenyl β-D-glucopyranoside (salicin, 415 μM), O-α-D-mannopyranosyl-(1→2)-O-α-D-mannopyranosyl-(1→2)-β-D-mannose (665 μM), and O-α-D-galactopyranosyl-(1→2)-O-α-D-mannopyranosyl-(1→2)-β-D-mannose (1830 μM). The following substances were inactive (did not abolish the ultraviolet difference spectrum): myo-inositol (316 mM), o-iodophenyl β-D-galactopyranoside (2985 μM), and o-nitrophenol (265 μM).

<table>
<thead>
<tr>
<th>Substance</th>
<th>$K_{ass}$ (M$^{-1}$)</th>
<th>Temperature (°C)</th>
<th>$K_{ass}$ (litature value)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenyl α-D-mannopyranoside</td>
<td>3.4 × 10$^2$</td>
<td>4</td>
<td>3.54 × 10$^2$ (31)$^a$</td>
<td>4</td>
</tr>
<tr>
<td>Methyl α-D-mannopyranoside</td>
<td>1.5 × 10$^4$</td>
<td>27</td>
<td>1.49 × 10$^4$ (31)$^a$</td>
<td>25</td>
</tr>
<tr>
<td>Methyl β-D-glucopyranoside</td>
<td>1.65 × 10$^6$</td>
<td>8</td>
<td>2.06 × 10$^6$ (32)$^a$</td>
<td>2</td>
</tr>
<tr>
<td>Methyl β-D-fructofuranoside</td>
<td>0.83 × 10$^3$</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>0.7 × 10$^2$</td>
<td>27</td>
<td>1.76 × 10$^2$ (32)$^a$</td>
<td>2</td>
</tr>
<tr>
<td>O-Iodophenyl β-D-glucopyranoside</td>
<td>0.53 × 10$^3$</td>
<td>27</td>
<td>2.20 × 10$^3$ (32)$^a$</td>
<td>2</td>
</tr>
<tr>
<td>Methyl α-sophoroside</td>
<td>1.33 × 10$^3$</td>
<td>27</td>
<td>2.88 × 10$^3$ (32)$^a$</td>
<td>2</td>
</tr>
<tr>
<td>Methyl α-glucopyranoside</td>
<td>1.14 × 10$^3$</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl α-fructofuranoside</td>
<td>3.3 × 10$^3$</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ At pH 5.35.

$^b$ Calculated value from Ref. 32.

that, at the very least, binding of one ligand greatly lowers the protein’s affinity for the second ligand, i.e. $K_L > K_{DL}$. A lower limit for the ratio ($K_L/K_{DL}$) can be obtained from the data. Consider the theoretical plots in Fig. 4 in which the product ($[P_D]/[P] + [PDL] - 1$) $[D] K_D$ is plotted versus $[L] × K_L$ for cases where $K_L/K_{DL}$ is 2.5, 5, and 10 and 20. Considerable deviation from linearity is seen when $[L] × K_D$ is 3 and $K_L/K_{DL}$ is 5 to 10. Since the plots of the observed experimental data in Fig. 2 were linear to values of $[L] × K_L$ of 3 or more we can safely say that $K_L/K_{DL} > 5$ to 10. The finding that binding of the second ligand severely impeded and probably prevented binding of the aromatic aglycone indicates that either all glycosides which displace the chromogenic ligand bind at overlapping loci or that binding of certain glycosides at a site removed from the chromogenic ligand causes a conformational change in the protein which abolishes the binding site for the chromogenic ligand.

In addition to the quantitative inhibition experiment cited, additional compounds were assayed qualitatively for their capacity to abolish the difference spectrum displayed by the reference sugar. We found inhibition with O-α-D-mannopyranosyl-(1→2)-O-α-D-mannopyranosyl-(1→2)-β-D-mannose, O-α-D-galactopyranosyl-(1→2)-O-α-D-mannopyranosyl-(1→2)-β-D-mannose, o-hydroxymethylphenyl β-D-glucopyranoside, p-nitrophenyl β-D-mannopyranoside, and methyl α-D-glucopyranoside, all inhibitors of the concanavalin A-polysaccharide interaction. No alter-
ation of the spectrum was observed upon the addition of myo-inositol and o-iodophenyl o-galactopyranoside, both noninhibitors of the concanavalin A-poly saccharide interaction. Furthermore, o-nitrophenol (965 μM) did not affect the difference spectrum.

The carbohydrates selected for the displacement experiments in this study were chosen so as to be broadly representative of the sugars which bind to concanavalin A. They included d-glucose and d-mannose-containing oligosaccharides, pyranosides, and furanosides (methyl β-d-fructofuranoside, methyl α- and β-glucosides, p-nitrophenyl α- and β-glycosides, and α- and β-mannosides which bind via "internal" glycosyl residues, e.g. methyl α-mannoside (33) and O-α-D-galactopyranosyl-(1→2)-O-α-D-mannopyranosyl(1→2)-d-mannose (34). All of these carbohydrates displaced the chromogenic ligand p-nitrophenyl α-d-mannopyranoside from concanavalin A leading to the conclusion that all of these substances bind to the same site.

These studies do not exclude the possibility that certain displacing ligands might also bind at a second site in addition to the site involved in the binding of p-nitrophenyl α-d-mannopyranoside. Indeed, Hardman and Ainsworth (24) have presented evidence that o-iodophenyl β-d-galactopyranoside binds to an apolar site separate from the carbohydrate binding site in crystalline concanavalin A. They also reported that o-iodophenyl β-d-galactopyranoside (a ligand which does not inhibit poly saccharide precipitation by concanavalin A) occupies this same apolar site on concanavalin A in the solid state. On soaking concanavalin A crystals in dilute solutions of o-iodophenyl β-d-galactopyranoside, Hardman and Ainsworth (24) noted that only the apolar site becomes occupied by the ligand; soaking concanavalin A crystals in more concentrated solutions of this glucose oxidized the crystals to crack.

If a separate apolar binding site (apart from the carbohydrate binding site) exists when concanavalin A is in the solution phase, then p-nitrophenyl α-d-mannopyranoside is excluded from this site. There is only one p-nitrophenyl α-d-mannopyranoside binding site per monomer unit of concanavalin A (31) and methyl α-d-mannopyranoside, a ligand which would not be expected to interact with an apolar binding site, completely displaces p-nitrophenyl α-d-mannopyranoside from concanavalin A, whereas o-iodophenyl β-d-galactopyranoside fails to displace p-nitrophenyl α-d-mannopyranoside from the protein.

It is conceivable that concanavalin A crystallizes from solution in a conformation in which the carbohydrate binding site is absent or nonfunctional and that soaking the protein crystals in solutions of certain apolar ligands results in the filling of an apolar crevice in the protein which is quite apart from the carbohydrate binding site. However, we could obtain no evidence for the existence of any separate apolar binding site for the protein in solution.

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