BBA 26013

PROTEIN–CARBOHYDRATE INTERACTION

XX. ON THE NUMBER OF COMBINING SITES ON CONCANAVALIN A, THE PHYTOHEMAGGLUTININ OF THE JACK BEAN

LUCY L. SO AND I. J. GOLDSTEIN*

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. (U.S.A.)

(Received May 27th, 1968)

SUMMARY

Equilibrium dialysis of concanavalin A against methyl α-D-mannopyranoside and methyl α-D-glucopyranoside conducted at 2° in the presence of 1 M NaCl showed that concanavalin A is bivalent. A SCATCHARD plot of the data obtained gave straight lines for both sugars with observed association constants (K') of $1.4 \cdot 10^4$ l/mole for methyl α-D-mannopyranoside and $0.3 \cdot 10^4$ l/mole for methyl α-D-glucopyranoside in the pH range 4.7–5.3. Binding studies carried out at various pH values (5, 6.2, 7.3) also indicated 2 binding sites on the concanavalin A molecule. Calculations were based on a molecular weight of 68000 for concanavalin A. The binding of methyl α-D-mannopyranoside to concanavalin A was maximal at pH 6.2 ($K' = 2.06 \cdot 10^4$ l/mole). The standard free energy change ($\Delta F°$) of this reaction was estimated to be −5.4 kcal/mole. No appreciable binding was observed when metal-free concanavalin A was employed in the dialysis experiment. The relative affinity of concanavalin A for methyl α-D-mannopyranoside and methyl α-D-glucopyranoside parallels the relative activity of these sugars in hapten inhibition experiments reported in previous studies.

INTRODUCTION

Previous work in this laboratory has been directed toward the study of the interaction of concanavalin A, an antibody-like protein from the jack bean, with various types of polysaccharides[1,2]. The stereochemical requirements for this specific interaction have been well defined with the use of hapten inhibition techniques[3,4]. Reactive polysaccharides which form a precipitate with concanavalin A contain terminal α-D-glucopyranosyl, α-D-mannopyranosyl, or β-D-fructofuranosyl residues[1–5].

The similarity of the concanavalin A–polysaccharide interaction to antibody–antigen precipitation has been suggested in our laboratory[1–6] and elsewhere[7,8]. We have employed various immunochemical methods in our studies of this protein–polysaccharide interaction[2–4,6].

A method of protein purification based on the specific adsorption of concanavalin A to crossed-linked dextran gel (Sephadex) was developed in our laboratory[9,10].

* Established Investigator of the American Heart Association.

Concanavalin A prepared according to this procedure is 95–98 % precipitable by a dextran (B-1355-S) using a quantitative precipitin technique[6]. Physical and chemical properties of this protein have already been reported[11–13]. At pH 2–5, concanavalin A sediments as a single peak in the ultracentrifuge with a molecular weight of 68000 (ref. 11).

One of the salient problems in our study of protein–carbohydrate interaction was to determine the number of binding sites on the concanavalin A molecule. The following communication describes equilibrium dialysis studies of concanavalin A employing methyl α-D-mannopyranoside and methyl α-D-glucopyranoside as binding haptens.

EXPERIMENTAL PROCEDURE

Materials

Dialysis tubing (20/100 ft) was obtained from Union Carbide Corporation (Chicago, Ill.). Methyl α-D-glucopyranoside and methyl α-D-mannopyranoside were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, Ill.). Concanavalin A was prepared by the Sephadex procedure of AGRAWAL AND GOLDSTEIN[10]. Metal-free concanavalin A was also prepared as described by AGRAWAL AND GOLDSTEIN[14]. Methyl α-D-galactopyranoside was prepared by Mr. G. HASSING of this laboratory.

Methods

The binding of sugars to concanavalin A was studied by the method of equilibrium dialysis described by MCMENAMY AND ONCLEY[15]. This may be summarized as follows: Dialysis tubing was washed with running tap water for 2 weeks and cut into strips (approx. 15 cm long). These were then rinsed several times with glass-distilled water, tied with a knot at one end, inflated with nitrogen, and clamped shut. After air drying, dialysis sacs were cut beneath the clamped end, filled with glass spacers, and placed in glass tubes (170 mm long, 20 mm inside diameter). These glass tubes (with stoppers) were specially drawn at the tip to accommodate the knot of the dialysis sac. The glass spacers were made such that when 3.0 ml of protein solution was placed inside the bag and 3.0 ml of a sugar solution outside, the solution level was the same inside and outside.

Concanavalin A in 1 M NaCl (3.0 ml, approx. 1 %) was placed inside the dialysis bag and a solution of sugar (3.0 ml) outside the bag. The concentration of the protein solution was determined spectrophotometrically using an extinction coefficient, $E^{1\%}_{1\,cm}$ at 280 mμ of 11.4 (ref. 11). Sugar solutions (20–500 μg/ml) were also prepared in 1 M NaCl. When buffer was used this was generally incorporated in the outside solution. Buffers employed were prepared according to the procedure of GOMORI[16]. In a typical dialysis experiment, 18–20 tubes were generally set up and placed on a multipurpose rotator (Scientific Industries, Inc., Queens Village, N.Y.) rotating at a rate of 5 rev./min. Equilibrium was attained overnight at 2°.

After 24 h at 2° aliquots were taken from the inside and the outside of the dialysis bag and the concentration of sugar present was determined by the phenol–H_2SO_4 method of DUBOIS *et al.*[17]. Duplicate tubes containing protein alone were always run with every set. The average values of the inside and the outside of the dialysis bag were used as blank corrections.

Since the amount of sugar present inside and outside the dialysis bag was determined for every tube in each experiment, corrections for binding of sugar to the dialysis membrane were not unnecessary.

All dialysis experiments were performed as described above except for the binding of methyl α-D-glucopyranoside to concanavalin A in 1 M NaCl in which uniformly ^{14}C-labeled methyl-α-D-glucopyranoside (Nuclear Chicago) was employed. Sugar solutions containing added unlabeled methyl α-D-glucopyranoside in the concentration range of 20–500 μg/ml were made in 1 M NaCl. After the regular dialysis period aliquots were taken and counted in a Packard Tri-Carb Scintillation Counter using a dioxane-based scintillation fluid[18]. The radioactivity was related to the concentration of methyl α-D-glucopyranoside by the phenol–H_2SO_4 method of DUBOIS *et al.*[17].

RESULTS

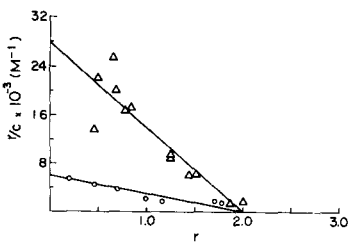
The binding of methyl α-D-glucopyranoside and methyl α-D-mannopyranoside to concanavalin A at 2° in the presence of 1 M NaCl is shown in Fig. 1 where r/c is plotted against r, according to the method of SCATCHARD ($r/c = Kn - Kr$) (ref. 19). The ratio of the molar concentration of bound sugar to that of the protein is represented by r, c is the molar concentration of free sugar, n, the number of binding sites, and K, the association constant. A molecular weight of 68000 for concanavalin A (ref. 11) was employed in the calculations. Linear plots were obtained for both sugars with values of n equal to 2. When extrapolated to the r/c axis values of binding constants of $1.4 \cdot 10^4$ l/mole and $0.3 \cdot 10^4$ l/mole were obtained for methyl α-D-mannopyranoside and methyl α-D-glucopyranoside, respectively. At the termination of the dialysis experiment the pH of the unbuffered solutions (1 M NaCl) ranged from 4.7 to 5.3.

The binding of methyl α-D-glucopyranoside and methyl α-D-mannopyranoside to concanavalin A was also studied at various pH values. Figs. 2 and 3 show studies conducted in the presence of 1 M NaCl at pH 5.0 and 7.3, respectively. The binding of methyl α-D-mannopyranoside to concanavalin A at pH 6.2 in the presence of 1 M NaCl is shown in Fig. 4. It appears that a change in pH does not alter the number of binding sites on the concanavalin A molecule. The binding of methyl α-D-mannopyranoside to concanavalin A was found to be maximum at pH 6.2. This can be seen better in Fig. 5 where the observed association constant, K', for methyl α-D-mannopyranoside is plotted against pH. At pH 6.2, K' reaches a maximum of $2.06 \cdot 10^4$ l/mole. This is in agreement with a previous study on the effect of pH on concanavalin A–mannan precipitation wherein maximum precipitation occurred in the pH range 5.7–6.7 (ref. 5). In this pH range about 95 % of the protein added was precipitated by a yeast mannan from *Saccharomyces cerevisiae*.

No detectable binding was observed with methyl α-D-galactopyranoside (1 M NaCl, pH range 4.7–5.3), a glycoside which does not interact with the combining sites of concanavalin A (refs. 3, 4).

The standard free energy change, $\Delta F°$, for the binding of methyl α-D-mannopyranoside to concanavalin A was estimated using the relationship $\Delta F° = -RT\ln K$. At pH 6.2 and 2°, $\Delta F° = -5.4$ kcal per mole.

When metal-free concanavalin A was employed in the dialysis experiment with methyl α-D-mannopyranoside as the binding hapten, no detectable binding was observed as shown in Table I. This was performed in the presence of 0.1 M NaCl since



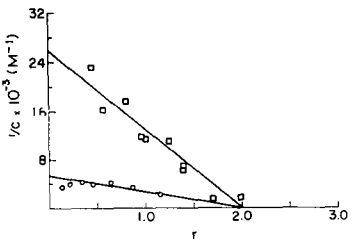
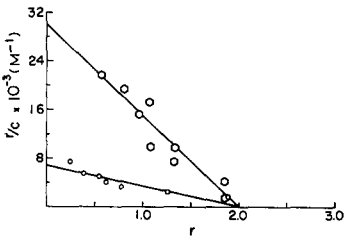


Fig. 1. Binding of methyl α-D-mannopyranoside (△) and methyl α-D-glucopyranoside (○) to concanavalin A, 1 M NaCl.

Fig. 2. Binding of methyl α-D-mannopyranoside (□) and methyl α-D-glucopyranoside (○) to concanavalin A, 1 M NaCl, 0.1 M acetate (pH 5).



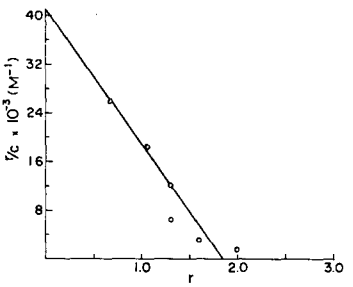


Fig. 3. Binding of methyl α-D-mannopyranoside (◇) and methyl α-D-glucopyranoside (○) to concanavalin A, 1 M NaCl, 0.05 M Tris–HCl (pH 7.3).

Fig. 4. Binding of methyl α-D-mannopyranoside to concanavalin A, 1 M NaCl, 0.025 M cacodylate (pH 6.2).

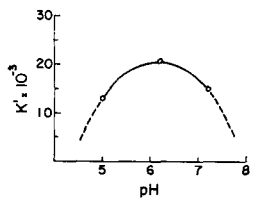


Fig. 5. pH dependence of binding of methyl α-D-mannopyranoside to concanavalin A.

TABLE I

BINDING OF METHYL α-D-MANNOPYRANOSIDE TO METAL-FREE CONCANAVALIN A AT 2° IN 0.1 M NaCl.

Metal-free concanavalin A, 25.3 mg. *i*, inside the dialysis sac. *o*, outside the dialysis sac.

Methyl α-D-mannopyranoside (i) (μg)	*Methyl α-D-mannopyranoside (o)* (μg)	*Δμg sugar* (*i* − *o*)
332	341	−9
167	172	−5
111	110	+1
83	82	+1
58	58	0
43	42	+1

metal-free concanavalin A tends to precipitate from solution at higher salt concentration. The various parameters affecting concanavalin A–polysaccharide interaction have been reported in a previous communication[6]. The concentration of NaCl does not affect the precipitation of concanavalin A by a dextran (B-1355-S). The metal-free concanavalin A employed in this experiment was quantitatively precipitated by dextran B-1355-S upon addition of Mn^{2+}.

DISCUSSION

In earlier studies the effect of sugars (haptens) on concanavalin A–polysaccharide interaction was examined by determining the extent to which these small molecules inhibited concanavalin A–polysaccharide precipitation (hapten inhibition). This involves a competition between the sugar and the polysaccharide for the active site(s) of the protein and is thus an indirect measure of concanavalin A–hapten interaction. Equilibrium dialysis studies described in this paper using methyl α-D-mannopyranoside as the binding hapten afford a direct measure of such an interaction. Methyl α-D-mannopyranoside was chosen for these experiments because it is one of the most potent inhibitors of concanavalin A–polysaccharide interaction examined. For comparison, the binding of methyl α-D-glucopyranoside was also studied.

The bivalency of precipitating antibodies to various antigens has been well documented in the literature[20,21]. Thus 1 antibody molecule can combine with 2 antigen molecules or with 2 haptens. Data of this type have been obtained from ultracentrifugation and electrophoresis[22–25] of soluble antibody–antigen complexes and from equilibrium dialysis studies[26,27].

Concanavalin A–polysaccharide interaction has been shown previously to be analogous to the antibody–antigen reaction in several respects[6]. The problems involved in studying antibody–antigen interaction are extremely complex due mainly to the heterogeneity of antibody preparations. SINGER[28] has classified antibody heterogeneity according to class, intrachain, and site. SCATCHARD plots (r/c *vs.* r) of antibody–hapten interaction generally give curved lines[26,27], an indication of heterogeneity of antibody combining sites and a distribution of K values[28]. Two methods for the treatment of equilibrium dialysis data have been described in the literature, one by KARUSH[29], another by NISONOFF AND PRESSMAN[30]. In the concanavalin A system no such treatment of data is necessary as will be discussed below.

The most striking feature of our equilibrium dialysis experiments is the finding that concanavalin A possesses 2 combining sites per 68000 g of protein. This result further supports the antibody-like character of concanavalin A. Although concanavalin A is biologically homogeneous, *i.e.* the protein is 95–98 % precipitable by a dextran (B-1355-S), it has been shown to exhibit physical heterogeneity[11]. The protein has a tendency to associate at pH 7 and above whereas between pH 2 and 5 it migrates as a single symmetrical boundary in the analytical ultracentrifuge[11]. We have therefore employed in our calculations a molecular weight of 68000 for concanavalin A, a value obtained by AGRAWAL AND GOLDSTEIN[11] at pH 5.

In spite of its physical heterogeneity concanavalin A appears to be homogeneous with respect to sites. Straight lines were obtained in all SCATCHARD plots whether methyl α-D-mannopyranoside or methyl α-D-glucopyranoside was employed as the binding hapten, all lines extrapolating to a value of $r(n) = 2$ at infinite hapten concen-

tration. A change in pH from 5 to 7.3 did not alter the number of binding sites on the concanavalin A molecule. Therefore, in the pH range studied as well as in 1 M NaCl alone, there are 2 combining sites on the protein molecule for every 68000 g. OLSON AND LIENER'S studies[13] indicated that the smallest subunit of concanavalin A has a molecular weight of 16500. If active concanavalin A exists as a tetramer of 4 such subunits, as has been suggested[11–14] it may very well be that 2 of these subunits constitute a sugar combining site. The fact that no binding of methyl α-D-mannopyranoside was observed when metal-free concanavalin A was employed in the equilibrium dialysis experiments suggests that Mn^{2+} is essential in the binding of sugar haptens to concanavalin A. This is in agreement with the observation of AGRAWAL AND GOLDSTEIN[14] on the requirement of Mn^{2+} in the precipitation reaction between concanavalin A and polysaccharides. Whether Mn^{2+} participates directly in the binding of specific sugars to concanavalin A or is involved in holding the subunits together has not yet established.

The observed association constants (K') of concanavalin A for methyl α-D-mannopyranoside and methyl α-D-glucopyranoside in 1 M NaCl were calculated to be $1.4 \cdot 10^4$ and $0.3 \cdot 10^4$ l/mole, respectively. Concanavalin A binds methyl α-D-mannopyranoside 4.5 times better than methyl α-D-glucopyranoside. At pH 5 and 7.3 the association constants for methyl α-D-mannopyranoside were also 4.5 times those for methyl α-D-glucopyranoside. The constancy of this ratio suggests that the same parameters are important in determining the binding of methyl α-D-glucopyranoside and methyl α-D-mannopyranoside to concanavalin A. It is interesting that this ratio reflects the inhibition potency of methyl α-D-mannopyranoside over methyl α-D-glucopyranoside in hapten inhibition experiments reported previously[4]. Thus, whereas it required 0.6 μmole of methyl α-D-mannopyranoside to effect 50 % inhibition of concanavalin A–dextran B–1355–S precipitation, it was necessary to add 2.5 μmoles of methyl α-D-glucopyranoside to obtain the same percentage inhibition. Assuming that the inhibition potency is approximately proportional to the association constant one

TABLE II

K' AND $\Delta F°$ VALUES FOR SOME TYPICAL INHIBITORS OF CONCANAVALIN A–POLYSACCHARIDE INTERACTION

Conditions: 0.025 M cacodylate, pH 6.2, 1 M NaCl, at 2°.

Sugar haptens	*μmole for 50 % inhibition*[4]	*K' (calc.) l/mole*	*ΔF° (calc.) kcal/mole*
1. Methyl α-D-mannopyranoside	0.60	$2.06 \cdot 10^4$	−5.4
2. Methyl β-D-fructopyranoside	0.85	$1.46 \cdot 10^4$	−5.2
3. Isomaltose	2.2	$5.61 \cdot 10^3$	−4.7
4. α,α-Trehalose	2.3	$5.38 \cdot 10^3$	−4.7
5. Methyl α-D-glucopyranoside	2.5	$4.94 \cdot 10^3$	−4.6
6. Maltose	4.3	$2.88 \cdot 10^3$	−4.3
7. Methyl β-D-fructofuranoside	5.6	$2.20 \cdot 10^3$	−4.2
8. D-Fructose	9.0	$1.37 \cdot 10^3$	−3.9
9. Methyl α-D-fructofuranoside	16.0	$7.71 \cdot 10^2$	−3.6
10. D-Glucose	21.0	$5.88 \cdot 10^2$	−3.5
11. Sucrose	23.0	$5.38 \cdot 10^2$	−3.4
12. Methyl β-D-glucopyranoside	70.0	$1.76 \cdot 10^2$	−2.8

can estimate the association constants for the various inhibitors examined in concanavalin A–polysaccharide interaction from previous inhibition data[4] based on a value of $K' = 2.06 \cdot 10^4$ for methyl α-D-mannopyranoside. Table II gives a tabulation of the computed values for $\Delta F°$ for some typical inhibitors of concanavalin A–dextran (B-1355-S) precipitation.

ACKNOWLEDGEMENT

This work was supported by U. S. Public Health Service Grants.

REFERENCES

1 I. J. GOLDSTEIN, C. E. HOLLERMAN AND J. M. MERRICK, *Biochim. Biophys. Acta*, 97 (1965) 68.
2 I. J. GOLDSTEIN AND L. L. SO, *Arch. Biochem. Biophys.*, 111 (1965) 407.
3 I. J. GOLDSTEIN, C. E. HOLLERMAN AND E. E. SMITH, *Biochemistry*, 4 (1965) 876.
4 L. L. SO AND I. J. GOLDSTEIN, *J. Immunol.*, 99 (1967) 158.
5 L. L. SO AND I. J. GOLDSTEIN, *J. Biol. Chem.*, 243 (1968) 2003.
6 L. L. SO AND I. J. GOLDSTEIN, *J. Biol. Chem.*, 242 (1967) 1617.
7 J. B. SUMNER AND S. F. HOWELL, *J. Bacteriol.*, 32 (1936) 227.
8 E. J. HEHRE, *Bull. Soc. Chim. Biol.*, 42 (1960) 1581.
9 B. B. L. AGRAWAL AND I. J. GOLDSTEIN, *Biochem. J.*, 96 (1965) 23c.
10 B. B. L. AGRAWAL AND I. J. GOLDSTEIN, *Biochim. Biophys. Acta*, 147 (1967) 262.
11 B. B. L. AGRAWAL AND I. J. GOLDSTEIN, *Arch. Biochem. Biophys.*, 124 (1968) 218.
12 M. O. J. OLSON AND I. LIENER, *Biochemistry*, 6 (1967) 105.
13 M. O. J. OLSON AND I. LIENER, *Biochemistry*, 6 (1967) 3801.
14 B. B. L. AGRAWAL AND I. J. GOLDSTEIN, *Can. J. Biochem.*, in the press.
15 R. MCMENAMY AND J. L. ONCLEY, *J. Biol. Chem.*, 233 (1958) 1436.
16 G. GOMORI, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 138.
17 M. DUBOIS, K. GILLES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
18 C. F. GORDON AND A. L. WOLFE, *Anal. Chem.*, 32 (1949) 660.
19 G. SCATCHARD, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660.
20 H. N. EISEN AND F. KARUSH, *J. Am. Chem. Soc.*, 71 (1949) 363.
21 S. J. SINGER AND D. H. CAMPBELL, *J. Am. Chem. Soc.*, 75 (1953) 5577.
22 M. HEIDELBERGER AND K. O. PEDERSON, *J. Exptl. Med.*, 65 (1937) 393.
23 A. M. PAPPENHEIMER, H. P. LUNDGREN AND J. W. WILLIAMS, *J. Exptl. Med.*, 71 (1940) 247.
24 S. J. SINGER AND D. H. CAMPBELL, *J. Am. Chem. Soc.*, 74 (1952) 1794.
25 J. R. MARRACK, H. HOCH AND R. G. S. JOHNS, *Brit. J. Exptl. Pathol.*, 32 (1951) 212.
26 F. KARUSH, *J. Am. Chem. Soc.*, 78 (1956) 5519.
27 F. KARUSH, *J. Am. Chem. Soc.*, 79 (1957) 3380.
28 S. J. SINGER, in H. NEURATH, *The Proteins*, Vol. III, Academic Press, New York, 1965, p. 346.
29 F. KARUSH, *Advan. Immunol.*, 2 (1962) 1.
30 A. NISONOFF AND D. PRESSMAN, *J. Immunol.*, 80 (1958) 417.