Carbohydrate Binding Properties of the *Dolichos biflorus* Lectin and Its Subunits

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Equilibrium dialysis studies on the binding of the *Dolichos biflorus* lectin with \(^{14}C\)methyl \(\alpha\)-D-GalNAc showed that the lectin has two combining sites/molecule and an intrinsic association constant at \(3^\circ C\) of \(4.2 \times 10^7\) liters mol\(^{-1}\). Binding studies on individual fractions (II, IV, and VII) of the lectin that differ in their chromatographic properties on concanavalin A-Sepharose gave association constants for methyl \(\alpha\)-D-GalNAc of 2.2 \(\times 10^7\) liters mol\(^{-1}\), 3.2 \(\times 10^7\) liters mol\(^{-1}\), and 3.2 \(\times 10^7\) liters mol\(^{-1}\), respectively.

Molecular exclusion chromatography of iodinated Subunits I and II of the lectin, as well as sedimentation velocity studies of the noniodinated subunits, showed that the isolated subunits form aggregates in aqueous solution. Aggregates of subunit I were capable of agglutinating blood type A erythrocytes, precipitating blood group A + H substance, and binding to blood group A + H substance in an affinity electrophoretic system. Aggregates of subunit II exhibited none of these binding properties and did not inhibit the ability of the intact lectin to agglutinate type A erythrocytes. Affinity electrophoresis of subunit I showed that it has an association constant for \(N\)-acetyl-\(d\)-galactosamine similar to that of the intact lectin. The results suggest that it is subunit I that is primarily responsible for the carbohydrate binding properties of the lectin.

All plant lectins characterized to this date have been found to be oligomers of peptide or glycopeptide chains. Although some lectins are composed of identical subunits, other lectins contain more than one type of subunit (for review see Ref. 1), and this subunit heterogeneity must be considered in studying the carbohydrate binding properties of these molecules.

The seeds of the *Dolichos biflorus* plant contain a lectin that is a tetramer composed of apparently equal amounts of two types of subunits (Subunits I and II) (2, 3). These subunits have similar amino acid compositions and give reactions of identity with one another in immunodiffusion using antisera made against either subunit (3). They have identical NH\(_2\)-terminal amino acid sequences (4) and their NH\(_2\)-terminal halves isolated after CNBr cleavage of the subunits appear to be identical (5). However, Subunit I is slightly larger than Subunit II, and these subunits have different COOH-terminal amino acids (3).

This lectin can selectively agglutinate type A erythrocytes (6) and precipitate blood group A substance (7) due to its specificity for terminal nonreducing \(\alpha\)-N-acetyl-\(d\)-galactosamine (8). Although the lectin is a tetramer, this paper presents evidence that it is bivalent and that it is Subunit I that is primarily responsible for the carbohydrate binding activity.

**EXPERIMENTAL PROCEDURES**

**Materials—** *Dolichos biflorus* seeds were obtained from the F. W. Schumacher Co., Sandwich, MA. Blood group A + H substance was isolated by ethanol precipitation (9) from hog gastric mucin (Wilson Laboratories, Chicago, IL). Carrier-free Na\(^{252}\)I (17 Ci/mg) was purchased from New England Nuclear. \(\text{C}^1\)-labeled methyl \(\alpha\)-D-GalNAc\(^{97.4\text{Ci/mg}}\) was kindly supplied by Dr. Irwin J. Goldstein, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI.

**Isolation of Lectin and Subunits—** The *D. biflorus* lectin was isolated by affinity chromatography on polyacrylam blood group A + H substance as previously described (8, 10). The lectin was separated into 7 fractions by chromatography on concanavalin A-Sepharose as reported earlier (2). The lectin was dissociated into its subunits by treatment with urea, and the subunits (IA and IIA) from the predominant form A of the lectin (2) were isolated by ion-exchange chromatography as described by Carter and Etzler (5). Protein concentration was determined either by absorption at 280 nm (\(E_{1%}^1 = 1.38\) (11) or by nitrogen analysis using a modified ninhydrin procedure (10, 12).

**Equilibrium Dialysis—** Equilibrium dialysis was performed in a rotating multichamber apparatus described by Furlong et al. (13) and manufactured by Riverside Scientific Enterprises, Riverside, CA. The dialysis tubing was pretreated by boiling with 3 changes of 1 mM EDTA, pH 5.0, for 5 min each change and washed with distilled H\(_2\)O. To one side of each dialysis chamber was added 300 \(\mu\)l of lectin at concentrations of 2.5 to 3.2 mg/ml of NaCl/P, and to the other side was added 300 \(\mu\)l of various concentrations (ranging from 1.25 to 17.1 \(\times 10^{-5}\) M) of \(\text{[}\text{I}^4\text{C}\text{]methyl }\alpha\text{-D-GalNAc} \text{NaCl/P,}\). Each experiment also included chambers containing 300 \(\mu\)l of NaCl/P instead of lectin on one side and 300 \(\mu\)l of radioactive hapten on the other side so that the attainment of equilibrium could be established. The chambers were rotated at 3°C for 24 h after which 200 \(\mu\)l from each side of the chambers were mixed with 10 ml of Bray's solution and counted for 20 min in a scintillation counter. The recovery of radioactivity ranged from 87 to 96% and was usually above 92%. The concentrations of free and bound hapten were calculated from the total amount of hapten added, the extent of binding to the membrane and the measurements of radioactivity on each side of the chamber.

**Physicochemical Studies—** Sedimentation velocity experiments were run in a Beckman model E analytical centrifuge monitored at 280 nm using a scanner accessory. Runs were made at 44,000 rpm at 10°C using protein concentrations of \(1 \text{mg/ml} \times 10^{-3}\) in 0.1 M Tris-HCl, pH 7.3. Molecular exclusion chromatography was conducted on a column (2.5 x 93 cm) packed with Bio-Gel P-200 and equilibrated with NaCl/P.

**Iodination of Subunits—** Subunits IA and IIA of the *D. biflorus* lectin were iodinated by the iodine monochloride method (14). Na\(^{252}\)I (5 mCi) was mixed with 1 ml of 1 M ICl in 0.1 M HCl followed by the addition of 3 ml of 1 M glycine, pH 8.5. Separate 0.5-ml aliquots of this mixture were then added to 4.0-ml solutions of the subunits at concentrations of \(1 \text{mg/ml} \times 10^{-3}\) in 0.2 M glycine, pH 8.5, containing 2 M NaCl/P.

The abbreviations used are: methyl \(\alpha\)-D-GalNAc, methyl \(\alpha\)-2-acet-ami-do-2-deoxy-\(\alpha\)-D-galactopyranoside; NaCl/P, 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.02% NaN\(_3\).
urea and 0.1 M N-acetyl-D-galactosamine. After mixing, the samples were kept on ice for 30 min and then dialyzed against 1 mM KI in NaCl/P, followed by exhaustive dialysis against H2O and lyophilized.

**Immunoelectrophoresis—** Affinity electrophoresis was conducted as previously described (15) on 5% polyacrylamide gels in the presence and absence of 0.5 mg/ml of entrapped blood group A + H substance. A discontinuous nondenaturing system was used with a running gel pH of 7.5. Association constants of the lectin and Subunit IA for N-acetyl-D-galactosamine were determined from electrophoretic mobility measurements made when various concentrations of the free sugar were included in the gels containing the entrapped blood group substance (15).

**RESULTS**

**Equilibrium Dialysis—** Equilibrium dialysis data for the binding of the D. biflorus lectin with [14C]methyl α-D-GalNAc were plotted according to Scatchard (16) (Fig. 1A) \( r/c = nK_o - nK_o + nK_o \) and according to Sipps (17, 18) (Fig. 1B) \( \log (r/n - r) = a \log c + a \log K_o \). In these equations, \( r \) is the number of moles of methyl α-D-GalNAc bound/mol of lectin, \( c \) is the concentration (m) of free hapten, \( K_o \) is the intrinsic association constant, \( n \) is the maximum value for \( r \) and represents the number of binding sites/molecule of lectin, and \( a \) is the index of heterogeneity. The slopes of the lines in both plots were determined by linear regression by the method of least squares. The molecular weight used for the calculation of lectin concentration was 110,000 (2).

The intercept (n) on the x axis in Fig. 1A is 1.88, indicating that there are two combining sites for methyl α-D-GalNAc/molecule of D. biflorus lectin. The intrinsic association constant, \( K_o \), at 3°C for the binding of the lectin to the hapten is 4.2 × 10⁻³ liter mol⁻¹. In Fig. 1B, the slope (a) of the line is 0.92, indicating a small amount of heterogeneity among the combining sites.

The D. biflorus lectin is heterogeneous with respect to its carbohydrate content and can be separated into several fractions by chromatography on concanavalin A-Sepharose (2). Fig. 2 shows the Scatchard plots obtained with equilibrium dialysis data for the binding of 3 of these fractions with [14C]methyl α-D-GalNAc. Fraction II, which consists of lectin that was not bound by concanavalin A and represents less than 12.5% of the total amount of lectin isolated from the seeds (2), has a \( K_o = 2.2 \times 10⁻³ \) liters mol⁻¹ and \( n = 2.51 \) (Fig. 2A). Fraction IV, which was bound to concanavalin A but eluted with relatively low concentrations of methyl α-D-glucoside (2) has a \( K_o = 3.2 \times 10⁻³ \) liters mol⁻¹ and \( n = 1.88 \) (Fig. 2B). Fraction VII, which was that portion of the lectin most tightly bound to concanavalin A (2), has a \( K_o \) of 3.2 × 10⁻³ liters mol⁻¹ and \( n = 2.02 \) (Fig. 2C). When the data for each of these fractions were plotted by the Sipps distribution, they gave slopes (a) of 1.00, 0.95, and 1.00, respectively. These results indicate that although the individual fractions may vary slightly in their association constants for the hapten, the combining sites within each fraction are homogeneous. Fractions IV and VII, which consist of Form A, the major portion of the lectin, have 2 combining sites/molecule. Due to a shortage of material, it was not possible to conduct further determinations on Fraction II to establish a more accurate value.

**Studies On Isolated Subunits—** Isolated Subunits I and IIA, which are Subunits I and II of the major form (Form A) of the D. biflorus lectin (2) can be dissolved in NaCl/P, or 40 mm Tris-HCl, pH 7.3, at concentrations up to 0.5 to 1 mg/ml. Although the subunits remain in solution at this concentration, sedimentation velocity experiments showed that a major portion of the subunits were aggregates with exclusion volumes equal to or greater than the exclusion volume of the intact lectin. This aggregation was also confirmed by chromatography of dilute solutions of iodinated subunits on Bio-Gel P-200. A major portion of Subunit IIA was eluted in a region corresponding to the elution volume of the intact lectin, whereas most of Subunit IIA and some of Subunit I were eluted in the exclusion volume of the column. Chromatography of the subunits after a longer time in solution showed that most of both subunit preparations were found in the void volume of the column.

Hemagglutination analyses of the subunits showed that Subunit IIA was capable of agglutinating type A1 erythrocytes, with solutions of ~1 mg/ml giving a titer of 4 as compared to a titer of 32 obtained with a similar concentration of intact lectin. Subunit IIA did not agglutinate type A1 erythrocytes and did not inhibit the agglutination of these cells by the intact lectin.

Quantitative precipitin analyses of the subunits showed that Subunit IIA had about three times the ability of the intact lectin to precipitate blood group A + H substance (Fig. 3). This precipitating ability was inhibited by N-acetyl-D-galactosa-
mine. Subunit IIA gave no substantial precipitation with blood group A + H substance (Fig. 3).

**Affinity Electrophoresis**—Because of the inability of the isolated subunits to stay in solution at concentrations necessary for equilibrium dialysis, affinity electrophoresis was chosen as a means of measuring their association constants. As previously described (15), the electrophoretic mobility of the *D. biflorus* lectin is reduced by interaction with entrapped blood group A + H substance in the polyacrylamide gels. The electrophoretic mobility of Subunit IA was also reduced by blood group substance, but no significant alteration was observed in the electrophoretic mobility of Subunit IIA.

Inclusion of various concentrations (0 to 40 mM) of N-acetyl-D-galactosamine in the running gels inhibits the binding of the lectin and Subunit IA to the blood group substance. The association constant of Subunit IA for N-acetyl-D-galactosamine was $2.5 \times 10^2$ liters mol$^{-1}$, which is identical within experimental error to the association constant obtained for the intact lectin (Fig. 4). The curvature of the plot obtained for Subunit IA indicates multivalent interactions between a protein and ligand (19, 20) and shows that this subunit is more aggregated than the native lectin.

**FIG. 2.** Scatchard plots of equilibrium dialysis data obtained with [14C]methyl a-D-GalNAc and Fractions II, IV, and VII obtained after chromatography of the *D. biflorus* lectin on concanavalin A-Sepharose (2). The lectin concentrations were: Fraction II (A), $1.85 \times 10^{-3}$ M; Fraction IV (B), $2.58 \times 10^{-3}$ M, and Fraction VII (C), $2.51 \times 10^{-3}$ M. The points in A are from individual determinations, whereas most of the points in B and C are averages of 2 separate determinations.

**DISCUSSION**

The equilibrium dialysis data presented above show that *D. biflorus* lectin has two combining sites/molecule for methyl a-D-GalNAc. The intrinsic association constant at 3°C for the binding of the lectin to this ligand is $4.2 \times 10^2$ liter mol$^{-1}$. This value is higher than the association constants for this ligand of $1.01 \times 10^3$ and $0.93 \times 10^3$ reported for the lima bean isolectins (21), but lower than the association constant of $3 \times 10^4$ liters mol$^{-1}$ reported for the binding of soybean lectin with N-acetyl-D-galactosamine (22). These three plant lectins all have combining sites corresponding to the size of a single monosaccharide unit and have a predominant specificity for terminal non-reducing N-acetyl-D-galactosamine, with the *D. biflorus* lectin exhibiting the greatest specificity for this sugar (23).

Although the *D. biflorus* lectin has been shown to be homogeneous by a number of criteria (2, 8), there is a small degree of heterogeneity with respect to its carbohydrate composition and it is possible to separate the lectin into several fractions by chromatography on concanavalin A-Sepharose...
(2). The equilibrium dialysis data presented for three of these fractions indicate that there is no difference in the intrinsic association constants for the lectin fractions (IV and VII) that are bound to concanavalin A; these fractions have been classified as Form I of the lectin on the basis of electrophoretic mobility (2). However the fraction (Fraction II) of the lectin that does not bind to concanavalin A and which contains electrophoretic Form B of the lectin has a slightly lower association constant and an n value of 2.51. This fraction constitutes only a small percentage of the total amount of seed lectin, and it is difficult to obtain sufficient material for more extensive equilibrium dialysis studies. However, comparison of this fraction with Fractions IV and VII by affinity electrophoresis showed that all three fractions have identical association constants for N-acetyl-D-galactosamine (15). The lower association constants obtained for the lectin with affinity electrophoresis as compared to the constant obtained with equilibrium dialysis are most probably due to the use of N-acetyl-D-galactosamine instead of the methyl a-glycoside and the fact that the affinity electrophoresis was done at room temperature.

The finding that the D. biflorus lectin is bivalent is of particular interest when one considers that this molecule is a tetramer composed of apparently equal amounts of two types of subunits (2, 3). In a previous paper (3), we reported that preliminary attempts to renature these subunits resulted in no restoration of carbohydrate binding activity. The results presented above show that aggregates of Subunit I could agglutinate blood type A erythrocytes and specifically precipitate blood group A + H substance, whereas aggregates of subunit II did not exhibit this activity nor did they inhibit the ability of intact lectin to agglutinate blood type A cells. These results, plus the finding that the intrinsic association constant of Subunit I is identical to the constant of the intact lectin as measured by affinity electrophoresis, suggest that it is Subunit I that is primarily responsible for the carbohydrate binding activity of the lectin.

Previous studies have shown that Subunits I and II of the D. biflorus lectin are extremely similar to one another with differences found only at their COOH-terminal ends (3-5). These subunits may thus represent different degrees of completion or modification of a common polypeptide chain. The present finding of the difference in carbohydrate binding activity between these two subunits indicates that the combining sites may either be close to the COOH-terminal end or that its conformation may be greatly affected by the COOH-terminal sequence of the peptide chain. Further studies on these two subunits should contribute to our understanding of structure-function relationships in proteins.

The soybean lectin is a tetramer composed of two very similar types of subunits (24). This lectin has also been found to have two carbohydrate combining sites/molecule (22). In light of the data presented above for the D. biflorus lectin, it would be of interest to determine whether only one of the soybean lectin subunits may be primarily responsible for the carbohydrate binding activity of the lectin.

A number of lectins have been described that contain more than one type of subunit (for review see Ref. 1). Although not much is known about the individual activities of these subunits, there is evidence in the case of the Bandeirea simplicifolia I lectin that the individual subunits differ in their carbohydrate specificities (25). There is also evidence that the erythroagglutinating and mitogenic activities of the Phaseolus vulgaris lectin are associated with different subunits (26). It will be of interest to determine whether Subunit II of the D. biflorus lectin possesses some other biological activity.

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REFERENCES