

Reexamination of the Carbohydrate Binding Stoichiometry of Lima Bean Lectin^{1,2}

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The carbohydrate binding stoichiometry of lima bean lectin component III was reexamined using equilibrium dialysis and quantitative affinity chromatography following limited chemical modification. Equilibrium dialysis employing methyl[2-¹⁴C]benzamido-2-deoxy- α -D-galactopyranoside as ligand demonstrated that the lectin tetramer bound 4 mol of sugar with $K_{\text{assoc}} = 1.44 \pm 0.13 \times 10^3 \text{ M}^{-1}$ (T = 5°C, pH 7.0, ionic strength 0.1). The previous report of two sites/tetramer [Bessler, W. and Goldstein, I. J. (1974) *Arch. Biochem. Biophys.* 165, 444] appears to be the result of partial inactivation of the lectin due to oxidation of essential thiol groups. Following limited chemical modification of the thiol groups by methyl methanethiosulfonate, multiple intermediate forms with reduced affinity for Synsorb A were obtained. The number and hemagglutinating activities of these intermediates provided further support for the presence of four carbohydrate binding sites on lima bean lectin component III.

The lima bean lectin was one of the first plant seed agglutinins shown to be blood group specific (1). Isolated in pure form by two groups (2-4), the lectin was shown to be a glycoprotein composed of 31-KDa subunits joined by a disulfide bridge to form dimers ($M_r = 62$ KDa). Two noncovalently linked dimers comprise lima bean lectin component III, and four such dimers afford lima bean lectin component II (2-4). The stoichiometry of carbohydrate binding was determined by equilibrium dialysis to be two sites/component III tetramer and four/component II octamer (5).

Lima bean lectin (LBL)⁵ contains three classes of subunits (denoted α , β and α') which can be resolved by isoelectric focusing under denaturing conditions (6). The subunit types have very similar but non-identical peptide maps and amino acid compositions (6). At present there is no evidence for a difference in carbohydrate binding by the three subunit types.

LBL contains two cysteine residues/subunit (2, 3). One cysteine in each subunit forms an intersubunit disulfide bond; the sulfhydryl group of the second cysteine was found to be required for carbohydrate binding activity (7-8).

The demonstration that haptenic sugars protected the free cysteine sulfhydryl groups present on each subunit against chemical modification by Ellman's reagent ruled out a model in which only one LBL subunit class bound carbohydrate (8). Other explanations consistent with the

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⁵ Abbreviations used: benzamido-MeaGalN, methyl-2-benzamido-2-deoxy- α -D-galactopyranoside; LBL, lima bean lectin; MMTS, methyl methanethiosulfonate; GalNAc, N-acetylgalactosamine.

data include negative cooperative binding of GalNAc or a binding site shared between two subunits. However, the requirement for activity of a sulfhydryl group which is labile to air oxidation (8) suggested that nonstoichiometric binding of GalNAc could also result from partial inactivation of the lectin during isolation or equilibrium dialysis. In this case, tetrameric LBL component III containing 1.0 free SH/subunit would be expected to bind 4 mol of GalNAc.

As a basis for further characterization of the sugar binding site of LBL and for understanding its action as a mitogen, the stoichiometry of sugar binding to LBL was reexamined. We repeated the determination of LBL component III stoichiometry by equilibrium dialysis using LBL containing 1.0 SH/subunit, and examined the effect of partial inactivation by limited thiol modification of the properties of LBL.

MATERIALS AND METHODS

Materials. Lima bean lectin was prepared from green lima beans (*Phaseolus lunatus* cv. Thorogreen) by affinity chromatography on Synsorb A (6). All buffers were degassed and saturated with N₂ and maintained under a N₂ atmosphere. Lectin prepared in this manner contained 1.06 ± 0.03 SH/subunit by titration with Ellman's reagent (9) and was stable to prolonged storage at 4°C. Components II and III were isolated from the purified lectin by gel filtration on Ultrogel AcA-34 (6, 10). Methyl methanethiosulfonate (MMTS) was provided by Dr. J. Shafer (University of Michigan). Methyl 2-acetamido-2-deoxy- α -D-galactopyranoside was prepared as described (11). [¹⁴C]Benzoyl chloride was prepared by refluxing [¹⁴C]benzoic acid (ICN, Irvine, Calif.) with a fivefold molar excess of thionyl chloride (Aldrich). The excess thionyl chloride was removed under vacuum (*Caution: volatile radioactivity*) and the residual [¹⁴C]benzoyl chloride used without further purification. Methyl [2-¹⁴C]benzamido-2-deoxy- α -D-galactopyranoside (benzamido-Me α GalN) was prepared by reaction of a slight excess of [¹⁴C]benzoyl chloride with the amino sugar in methanol in the presence of NaHCO₃. The product was recrystallized twice from methanol-ether and was of >98% radiochemical purity (sp act 1 Ci/mol). The chromatographic and spectral properties of the labeled compound were identical to those of the unlabeled compound prepared as described elsewhere.⁶

Equilibrium dialysis. Binding of [¹⁴C]benzamido-Me α GalN to LBL was conducted in microdialysis cells (Technilab Instruments, Inc., Pequannock, N. J.). Lectin and sugar were in N₂-saturated sodium phosphate buffer, pH 7.0, ionic strength 0.1. Following equilibration for 48 h with end-over-end mixing at 5°C, the concentration of sugar in each chamber was determined by scintillation counting in ACS (Amersham). Lectin concentrations were determined by absorbance at 280 nm using $E_{1\%}^{1\text{cm}} = 12.3$ (2).

Limited thiol modification. LBL was incubated under N₂ with substoichiometric concentrations of MMTS for 24 h. The reaction mixtures were then analyzed by affinity chromatography on a high-capacity Synsorb A column (0.7 × 14 cm). The lectin was eluted using a pH gradient formed by running a linear gradient from buffer A (20 mM glycine, 20 mM sodium formate, 20 mM sodium succinate, 20 mM sodium citrate, 0.1 mM CaCl₂, 0.1 mM MnCl₂, pH 6.8) to buffer B (70 mM HCl). The total gradient volume was 200 ml and was pumped at 77 ml/h. Eluent was monitored at 280 nm. Eluted lectin was dialyzed into sodium phosphate buffer, pH 7.0, ionic strength 0.1 for determination of thiol content. Activity was determined by hemagglutination of human Type A₁ red blood cells (4).

RESULTS AND DISCUSSION

The previous determination of the sugar binding stoichiometry of LBL by equilibrium dialysis employed [¹⁴C]methyl α -D-GalNAc (5). This sugar was found to bind with relatively low affinity ($K_{\text{assoc}} = 1 \times 10^3$ M⁻¹ at 2°C). Since the 2-*N*-benzamido analog bound with higher affinity (8) and could be prepared in radiolabeled form, it was used in place of methyl α -D-GalNAc in the present study. In order to ensure full activity of the lectin, the dialysis cells were flushed with nitrogen, and dialysis was limited to 48 h. Thiol content was determined before and after dialysis to correct for any inactivation of lectin during dialysis. Specificity of the labeled ligand for the carbohydrate sites was confirmed by complete inhibition of binding in the presence of excess unlabeled methyl α -D-GalNAc.

A Scatchard plot (12) for binding of [¹⁴C]benzamido-Me α GalN to LBL component III (Fig. 1) indicated that fully active component III can bind 4 mol of carbohydrate ($n = 1.07 \pm 0.06$ on a subunit basis assuming $M_r = 31,000$) with $K_{\text{assoc}} = 1.44 \pm 0.13 \times 10^3$ M⁻¹ (T = 5°C, pH 7.0, ionic

⁶ Kaifu, R. and Goldstein, I. J., submitted for publication.

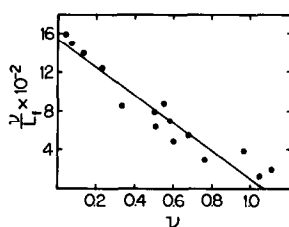


FIG. 1. Binding of [^{14}C]benzamido-MeaGalN to lima bean lectin component III by equilibrium dialysis. Lima bean lectin ($2\text{--}3 \times 10^{-4}$ M subunits) in sodium phosphate buffer, pH 7.0, ionic strength 0.1 was equilibrated with 0.025 to 5.7 mM [^{14}C]benzamido-MeaGalN at 5°C for 48 h under an N_2 atmosphere. Sugar concentrations in each chamber were determined by counting quadruplicate 50- μl aliquots. Stoichiometry of binding (ν) was calculated on a subunit basis using $M_r = 31,000$.

strength 0.1). Determination of benzamido-MeaGalN binding by a thiol protection assay (8) under the same conditions ($T = 5^\circ\text{C}$, pH 7.0, ionic strength 0.1) gave an apparent K_{assoc} (K_T^{-1}) of $1.51 \times 10^3 \text{ M}^{-1}$. Thus there is excellent agreement between the two methods for determining carbohydrate binding. The basis for the difference in stoichiometry from that reported previously is probably partial inactivation of the lectin prior to equilibrium dialysis. LBL isolated without precautions to minimize exposure to air typically contained 0.7 to 0.9 SH. Within 2 weeks the thiol content of purified lectin decreased to 0.5 to 0.6 SH/subunit. This instability was not seen with LBL prepared and stored under N_2 . The low binding affinity ($K_{\text{assoc}} = 10^3$) also probably contributed to the difficulty of obtaining an accurate value for the number of carbohydrate binding sites on the lectin.

Complete and reversible protection from chemical modification of a single free thiol group on each subunit of LBL by haptenic sugars is consistent with the stoichiometry determined using equilibrium dialysis. These results indicate the presence of a carbohydrate binding site on each subunit and a requirement of one thiol group for binding of each carbohydrate ligand. Therefore the measured stoichiometry should decrease linearly with modification of the thiols.

To confirm the sugar binding stoichiometry, we examined the properties of partially modified LBL. Affinity chromatography on Synsorb A was used to separate forms of LBL differing in valence. A similar approach has been utilized previously for separation of *Griffonia simplicifolia* isolectins (13, 14) and chemically modified concanavalin A (15, 16) on the basis of valence for an immobilized ligand. If LBL component III is divalent, partial modification should produce a monovalent derivative capable of being separated by affinity chromatography on Synsorb A. This intermediate would be inactive as a hemagglutinin. However, if component III is tetravalent, several intermediates which could be tri-, di-, and monovalent would be obtained. All except the latter intermediate would be expected to agglutinate erythrocytes. Chromatography of a natural mixture of LBL components on a Synsorb A column eluted with a pH gradient gave two peaks (Fig. 2a), representing components III and II as verified by electrophoresis at pH 4.3 (17). Chromatography of the isolated components, prepared by gel filtration, gave single peaks on the Synsorb column (Fig. 2b) with the same retention times as observed in the mixture.

Treatment of purified component III with 0.5 equivalents of MMTS eliminated the component III peak. A broad, trailing peak followed by a peak at 80 min was obtained (Fig. 2c). The early, middle, and late portions of this profile were dialyzed against pH 6.8 buffer and rechromatographed (Fig. 2d). Multiple peaks obtained in this experiment suggested that two to three distinct intermediates were present in partially modified LBL. The peak eluting at 80 min was stable to rechromatography and distinct from fully active component III. A second intermediate eluted as a broad peak at 30 min, and the third was only weakly retarded by the column. All intermediates gave a single component III band on pH 4.3 gel electrophoresis.

Following dialysis into sodium phosphate buffer, pH 7.0, ionic strength 0.1, thiol content and hemagglutination titers of the intermediates were determined (Table I). A progressive decrease in both thiol

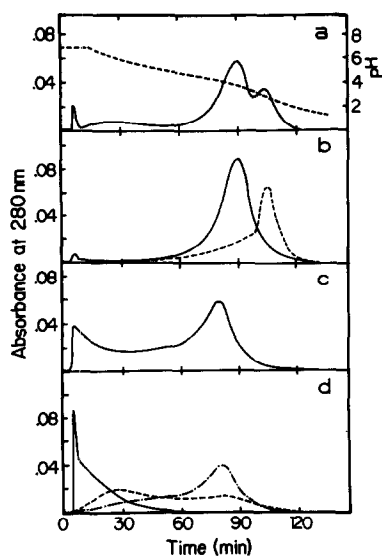


FIG. 2. Affinity chromatography of lima bean lectin on Synsorb A. Affinity chromatography was performed with a pH gradient elution as described under Materials and Methods. The pH gradient, presented in (a) was identical for all elution profiles. (a) 1.5 mg natural mixture of lima bean lectin components; (b) LBL components isolated by gel filtration on Ultrogel AcA 34, (—) 1.5 mg component III, (---) 1 mg component II; and (c) 1.5 mg LBL component III modified with 0.5 equivalents of MMTS. (d) Pooled fractions from each region of the elution profile in (c) were dialyzed against pH 6.8 buffer and reanalyzed: pool 1, 5–26 min (—); pool 2, 26–50 min (---); pool 3, 60–80 min (— · —).

content and specific titer with earlier retention time demonstrated correlation between decreasing valence and increasing degree of modification. Weak hemagglutination activity in peak 1 would not be expected for monovalent LBL and probably results from contamination with divalent lectin.

The presence of multiple intermediates with hemagglutinating activity in partially modified LBL is consistent with the presence of four binding sites on component III, and confirms the stoichiometry determined by equilibrium dialysis. These results cannot be reconciled with a model wherein component III is divalent. The behavior of component II on affinity chromatography indicates that it has a higher valence than component III. Although it

would be expected to be octavalent, this has not yet been confirmed by equilibrium dialysis.

Determination of the correct binding stoichiometry is crucial for interpreting the effects of lectins on cells. The affinity of lectin binding to cell surface carbohydrates and the mitogenic effect of lectins on lymphocytes are dependent on lectin valence. Derivatives of concanavalin A with reduced valence have been prepared and found to differ in their mitogenic properties from the native tetravalent lectin (15, 18).

The effect of valence on mitogenicity of LBL has also been studied. LBL component III, previously reported to be divalent (5), was a very weak mitogen (19, 20). Component II, having twice the valence of component III, was a potent mitogen. Natural polymers of LBL (21) or chemically cross-linked component III (22) also displayed enhanced potency as mitogens. Chemical crosslinkage of native soybean agglutinin into dimers and higher oligomers greatly enhanced its hemagglutinating and lymphocyte-transforming activities (23). Lymphophilization of the soybean lectin also enhanced its mitogenic activity (24). The present demonstration that LBL component III has four binding sites warrants its reexamination as a mitogen under conditions which protect its thiol groups from oxidation.

In conclusion, the results of two independent methods are consistent with LBL

TABLE I
HEMAGGLUTINATING ACTIVITY AND THIOL CONTENT
OF MODIFIED LIMA BEAN LECTIN

Lectin	Specific titer (mg ml ⁻¹)	SH/ subunit
Component II	3700	1.0
Component III	420	1.0
Modified component III—pool 1	5	0.26
Modified component III—pool 2	30	0.37
Modified component III—pool 3	180	0.56

component III being tetravalent for carbohydrate. In addition to defining the stoichiometry of LBL, the procedures presented here provide a method for preparing LBL component III with a variable number of carbohydrate binding sites. These forms will be of interest in further examining the effects of valence on the biological properties of LBL.

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