A free sulfhydryl group previously has been shown to be required for carbohydrate binding to the lectin from lima bean (*Phaseolus lunatus*) (Gould, N. R. and Scheinberg, S. L. (1970) *Arch. Biochem. Biophys.* 141, 607-613). Modification of this group by sulfhydryl reagents was specifically inhibited by d-GalNAc. We have further examined the reactivity of sulfhydryl groups in lima bean lectin with 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs) as a probe for carbohydrate and metal ion binding.

The 4 thiol groups in tetrameric lima bean lectin component III gave identical kinetics for reaction with Nbs involving formation of a weak noncovalent complex between Nbs and the lectin. The pH-independent reactivity of the thiol groups at neutral pH suggested that the thiol may exist as an ion pair with a nearby ionized group.

Carbohydrate ligands were competitive inhibitors of thiol modification. The thiol groups on all 4 subunits of lima bean lectin were completely and reversibly protected by carbohydrate binding. The ability of carbohydrates to inhibit thiol modification correlated with their potency as inhibitors in a precipitin inhibition assay. The best inhibitors were the oligosaccharides α-D-GalNAc-(1→3)(α-1,2-fucose-(1→2))β-D-Gal (1→R) and α-D-GalNAc-(1→2)β-D-Gal(1→R). Apparent thermodynamic parameters for binding of several carbohydrates were determined by measuring the temperature dependence of thiol protection.

Removal of the bound metal ions Ca²⁺ and Mn²⁺ following dialysis into EDTA inactivated the lectin and increased the reactivity of the thiol groups 60-fold. This conversion was temperature-dependent and could be reversed upon addition of metal ions. The fast-reacting thiol groups were not protected by haptenic sugars from modifications by Nbs.

Extracts of lima beans (*Phaseolus lunatus*) contain blood group A agglutinating activity (1). The α-D-GalNAc¹-specific lectin was purified in two active forms, designated component II (Mᵣ = 247,000) and component III (Mᵣ = 124,000) (2-4). A single subunit of Mᵣ = 31,000 was identified, four or eight of which are combined to form components III and II, respectively (4, 5).

Whereas most legume lectins are poor in sulfur containing amino acids (6) and generally are devoid of cysteine, the LBL contains two cysteine residues/subunit (3, 4). One cysteine in each subunit forms an intersubunit disulfide bond (4); the sulfhydryl group of the second cysteine is free and was found to be required for carbohydrate binding activity (5). Modification of this residue by several sulfhydryl reagents inactivated LBL (3, 5). Reaction of the essential sulfhydryl group with Ellman's reagent (Nbs) and N-ethylmaleimide was inhibited in the presence of the haptenic sugar GalNAc, but not by the nonhaptenic sugar GlcNAc (5).

The stoichiometry of carbohydrate binding, as determined by equilibrium dialysis using [¹⁴C]methyl-α-D-GalNAc, was two binding sites/component III tetramer (7). Pandolfino and Magnuson (8) studied metal binding by equilibrium dialysis and reported two Mn²⁺-specific sites and two Ca²⁺/Mn²⁺ binding sites/component III tetramer. To account for the sugar and metal ion stoichiometries, three models were proposed: nonidentical subunits, negative cooperative sugar and/or metal ion binding, and sites shared between two subunits (8, 9). We described three subunit isoforms of LBL separable by isoelectric focusing under denaturing conditions (9). Whether this heterogeneity correlates with functional heterogeneity is not known.

To distinguish between the three models stated above, we extended the qualitative findings of Gould and Scheinberg indicating a role of the free sulfhydryl groups in carbohydrate binding. Since one free sulfhydryl group is present in each subunit (and in all three subunit forms (9)), the reactivity of these groups can be used to test predictions for the models. First, we wished to determine whether the four sulfhydryl groups on a component III tetramer are equally reactive and to define the kinetic parameters for modification. Inhibition of modification by haptenic sugars could then be used to determine whether all four sulfhydryl groups are protected by sugar binding, as predicted by the shared site and negative cooperativity models, or whether only two sulfhydryl groups are protected as predicted by the nonidentical subunit model.

To further substantiate the role of sulfhydryl groups in carbohydrate binding, we examined the correlation between inhibitory potency with respect to sulfhydryl protection and...
inhibitory potency in a precipitin inhibition assay. Temperature dependence of protection by sugars was also measured to allow comparison of the inhibition constants with binding constants for the carbohydrate measured by equilibrium di-
alysis.

MATERIALS AND METHODS

Materials—Lima beans (Thorogreen or Sieva varieties) were grown at the Matthei Botanical Gardens (University of Michigan), harvested as green beans, and stored at -20 °C. LBL was purified by affinity chromatography on Synsorb A (Chembiomed, Ltd., Edmonton, Alberta) as described previously (9), with slight modification. All buffers were degassed, saturated with N2, and maintained under a N2 atmosphere. Lectin prepared in this manner and stored under N2 contained 1.06 ± 0.05 SH/subunit by titration with Nbs2, and was stable during prolonged storage.

Nbs2 was purchased from Aldrich. Most sugars were purchased from Pfanzehl Laboratories (Waukegan, IL) or Sigma. Blood group analog using galactosamine HCl (10) (m.p. 104-108°C, aqueous methanol containing 0.5 M phosphate, or Tris-HCl buffers.

Nbs2 release was quantified using an atomic absorption analysis. Analyses were done by the Great Lakes Research Division, University of Michigan using a Perkin Elmer model 5000 atomic absorption spectrometer with a Perkin Elmer model 500 graphite furnace. The method of standard additions was used for manganese determinations. Corrections were made for background absorption and for metal contamination in buffer blanks.

RESULTS

Kinetics of Nbs2 Modification—Reaction of LBL at 20 °C with an excess of Nbs2 under pseudo-first order conditions resulted in the morphologic release of 4 eq of Nbs2, demonstrating identical reactivity of the 4 sulphydryl groups with Nbs2. When the concentration of Nbs2 was varied, it was found that the rate of modification deviated somewhat from that predicted for a second order reaction. A plot of kobs versus [Nbs2]−1 is linear (Fig. 1), indicating formation of a weak complex between Nbs2 and LBL as shown in Equation 1.

The deviation from 2 °C kinetics was more pronounced at higher ionic strength (Fig. 1). At I/2 = 0.1, the parameters for the reaction were Kc = 37 ± 10 mM and kc = 8.3 ± 1.4 min−1, with Kc = (k+s + k−)/k−. At I/2 = 1.0, Kc decreased to 11 ± 3 mM and kc decreased to 1.6 ± 0.3 min−1.

The effect of pH on the reaction was also examined (Fig. 2). At all pH values, the progress curves remained monophasic. At pH 7.5 and 8.5, the reactivity of the thiols on LBL was essentially pH-independent. At lower pH values, the reactivity increased to a maximum at pH 4.5 and then began to decrease. Due to weak complex formation between Nbs2 and LBL, it was difficult to separate pH dependence of Nbs2 binding from the pH dependence of kc, which reflects the reactivity of the thiols. It is clear, however, that the reactivity of the thiol is not consistent with a normal cysteine thiol pK, of 8-10 (15).

Protection by Sugars—As found by Gould and Scheinberg (6), increasing concentrations of GalNAc progressively inhibited the rate of modification of the thiols on LBL (Fig. 3). The progress curves again remained monophasic, indicating 7.0, from which trace metals were removed by passage through a column of Chelex 100 (Bio-Rad).

Ca2+ and Mn2+ in the lectin samples were analyzed by atomic absorption analysis. Analyses were done by the Great Lakes Research Division, University of Michigan using a Perkin Elmer model 5000 atomic absorption spectrometer with a Perkin Elmer model 500 graphite furnace. The method of standard additions was used for manganese determinations. Corrections were made for background absorption and for metal contamination in buffer blanks.

FIG. 1. Kinetics of modification of lima bean lectin by Nbs2.

The kinetics of modification of the free sulphydryl groups on LBL were determined at 20 °C in sodium phosphate buffer, pH 7.0. The observed rate constants (kobs) are plotted as a function of Nbs2 concentration at low (I/2 = 0.1, ○) and high (I/2 = 1.0, □) ionic strength.

2 R. Kaifu and I. J. Goldstein, manuscript submitted to Carbohydr. Res.
Reactivity of Thiol Groups in Lima Bean Lectin 905

FIG. 2. pH dependence of the reaction of Nbs2 with lima bean lectin. Kinetics of modification of the LBL sulfhydryl groups by Nbs2 were determined at 20 °C, I/2 = 0.1 over the pH range 4-8. Kinetics parameters were determined from double reciprocal plots done at each pH. The ratio k2/K is plotted as a function of pH.

FIG. 3. Inhibition of lima bean lectin sulfhydryl modification in the presence of d-GalNAc. Lima bean lectin (0.25 mg/ml) was equilibrated with increasing concentrations of d-GalNAc in PB, pH 7.0, I/2 = 0.1 at 20 °C. Kinetics of reaction with Nbs2 (1.8 mM) were determined. Progress curves are presented for LBL equilibrated without sugar (●) and with 10 mM (○), 20 mM (●), or 30 mM (□) d-GalNAc. Inset, double reciprocal plots were constructed by varying Nbs2 concentration at each fixed GalNAc concentration. The slope of the resulting plots is replotted as a function of the concentration of inhibitory sugar.

identical inhibition of the reactivity of all four sulfhydryl groups by GalNAc. Experiments where Nbs2 concentrations were varied at several fixed GalNAc concentrations demonstrated competitive inhibition of Nbs2 modification by the sugar. For a competitive inhibitor, the slope of the k_2/K vs [Nbs2]^-1 plot can be related to the inhibition constant, K_i.

slope = slope_0 (1 + [I]/K_i) \quad (2)

where [I] is the concentration of inhibitory sugar and slope_0 is the slope of the plot in the absence of inhibitor. K_i can be determined by reploting the slope of the primary plots versus the concentration of GalNAc (Fig. 3, inset), giving a K_i for GalNAc at 20 °C I/2 = 0.1 of 12 mM. Linearity of the secondary plot indicated an identical K_i for protection of each of the four sulfhydryl groups by GalNAc.

Inhibition of sulfhydryl modification by a number of GalNAc derivatives was determined. Estimates of K_i for each sugar were determined from measurements of the effect of at least three concentrations of inhibitory sugar on the slope of the primary plots. A summary of the K_i values measured by this method is presented in Table I.

The K_i for Me-α-2-D-GalNAc at 20 °C was 3.5 mM. Equilibrium dialysis experiments gave a K_i for Me-α-2-D-GalNAc at 2 °C of 1.0 mM (7). Since these constants were measured at different temperatures, a direct comparison was not possible. We therefore, determined the K_i for Me-α-2-D-GalNAc at 10 and 30 °C. By constructing a van't Hoff plot (Fig. 4), apparent thermodynamic parameters for the binding of Me-α-2-D-GalNAc were determined and the expected affinity at 2 °C was calculated to be 1.7 mM. Similar experiments were done for GalNAc and the type A trisaccharide. Inhibition constants and apparent

<table>
<thead>
<tr>
<th>Sugar</th>
<th>K_i (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me-α-2-D-GalNAc</td>
<td>3.5</td>
</tr>
<tr>
<td>Me-α-2-D-GalNAc (I/2 = 1.0)</td>
<td>1.8</td>
</tr>
<tr>
<td>2,6-Di-O-acetyl-d-GalNAc</td>
<td>5.6</td>
</tr>
<tr>
<td>2-Nitrobenzenesulfonylamido-d-GalNAc</td>
<td>6.5</td>
</tr>
<tr>
<td>d-GalNAc</td>
<td>7.5</td>
</tr>
<tr>
<td>galactose</td>
<td>12.1</td>
</tr>
<tr>
<td>l-arabinose</td>
<td>13</td>
</tr>
<tr>
<td>N-acetyl-d-glucosamine</td>
<td>21</td>
</tr>
<tr>
<td>2-O-acetyl-d-GalNAc</td>
<td>&gt;30</td>
</tr>
<tr>
<td>2-O-benzyl-d-GalNAc</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-O-methyl-d-GalNAc</td>
<td>&gt;76</td>
</tr>
<tr>
<td>Me-β-2-D-GalNAc</td>
<td>89</td>
</tr>
<tr>
<td>Me-α-2-D-GalNAc</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2,6-Di-O-acetyl-2-deoxy-d-GalNAc</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

α-D-GalNAc(1→3)-[α-L-Fuc(1→2)]β-D-Gal(1→O)(CH2)3COCH3 | 0.28 |
| α-D-GalNAc(1→2)-β-D-Gal(1→O)(CH2)3COCH3 | 0.75 |
| α-D-GalNAc(1→3)-β-D-Gal(1→O)(CH2)3COCH3 | 4.3 |
| α-D-GalNAc(1→6)-d-Gal | 5.5 |
| α-D-GalNAc(1→9)-d-Gal | 5.7 |
| α-D-GalNAc(1→6)-d-Gal | 14 |
| Me-β-L-Fuc | 69 |
| galactose | 350 |

* Determined at pH 7.0, 20 °C, and except where indicated, at ionic strength 0.1.

FIG. 4. van't Hoff plot for inhibition of LBL sulfhydryl modification by methyl α-2-D-GalNAc
thermodynamic constants for binding of these ligands are listed in Table II.

**Metal Ion Dependence**—Initial experiments with Nb₃⁺ modifications were done in the presence of 1 mM EDTA to stabilize the Nb⁺⁺ released. In some experiments, biphasic reaction with Nbs was observed, where the fraction of a faster reacting form increased with time. This conversion was not seen when LBL was maintained at 4 °C in buffer containing 1 mM EDTA. To determine whether this change in reactivity was correlated with loss of bound metals, the rate of conversion from slow to fast reacting thiol and metal ion content of LBL were measured following dialysis into PB containing 1 mM EDTA at 2 and 24 °C. The rate of loss of slow reacting thiol at the two temperatures is shown in Fig. 5. In all experiments, the total thiol content did not change. At 24 °C, the loss of slow reacting thiol followed first order kinetics, with k = 0.11 h⁻¹. At 2 °C, the rate of conversion was very slow (k ~ 0.012 h⁻¹). However, after 2 weeks at 2 °C, only fast reacting thiol was observed. The fast reacting LBL thiol followed second order kinetics for reaction with Nb²⁺, with ΔT₀ = -0.012 h⁻¹. This form of LBL was inactive in a hemagglutination assay using EDTA washed red blood cells. The fast reacting thiol was not protected by sugars. Lectin, dialyzed into EDTA for 24 h at 2 °C, in contrast, retained agglutinating activity; the slow reacting thiol was still protected from modification by GalNAc. Atomic absorption analyses of the Ca and Mn content demonstrated correlation between the change in reactivity of the thiol and loss of bound metal ions. Native lectin contained 1.0 eq of Ca²⁺ and 0.22 eq of Mn²⁺/subunit; 97% of the thiol was slow reacting. Whereas Ca²⁺ was lost more rapidly than Mn²⁺ during demetalization, loss of slow reacting thiol correlated best with the total metal content. Partially inactivated lectin with 65% and 19% of the thiol remaining in the slow reacting state contained 0.75 and 0.08 eq/subunit, respectively, of bound metal ions (Ca + Mn). This correlation was strengthened by demonstration of reversibility of the change in thiol reactivity. Addition of Ca²⁺ and Mn²⁺ to the demetalized LBL restored the thiol to the slow reacting form (Fig. 5).

**DISCUSSION**

The results presented here confirm and extend the findings of Gould and Scheinberg suggesting involvement of the free thiol group on each subunit of LBL in carbohydrate binding. In addition, we demonstrated for the first time linkage between the reactivity of this thiol and metal ion binding to LBL. Thus, the sulfhydryl groups in LBL, unique to this lectin, provide a convenient signal for probing the metal and carbohydrate binding properties of the lectin. Although our efforts have focused primarily on carbohydrate binding, it is likely that detailed analysis of metal ion binding and accompanying conformational changes could also be conducted using the same methods.

Protection by GalNAc of a sulfhydryl group on each of the four subunits of an LBL tetramer rules out models for LBL where only two of the four subunits bind carbohydrate. The present data cannot be used to distinguish negative cooperative carbohydrate binding from binding to sites shared between two subunits. If sites are shared between subunits, however, sulfhydryl groups from the two subunits must participate equally in the site to account for the uniform and complete protection of the sulfhydryl groups by carbohydrate. The present data are also consistent with binding of GalNAc to all four subunits of LBL. In view of the low affinity for Me-α-GalNAc binding and the sensitivity of thiol oxidation, it is possible that equilibrium dialysis yielded the incorrect stoichiometry. Previous studies with LBL were probably done using LBL with less than one —SH/subunit. LBL isolated without precautions to avoid thiol oxidation typically contained 0.85 —SH/subunit, decreasing to 0.5 to 0.6 —SH/subunit after two weeks of storage at 4 °C. Therefore, the low stoichiometry could reflect partial inactivation of the lectin during isolation.

Several experiments were done to validate the sulfhydryl group protection assay as a method for measuring carbohydrate binding. Binding constants for some of the sugars used in this study have been measured previously in a precipitin inhibition assay (3, 16). Correlation between Kᵣ values measured by the two methods is illustrated in Fig. 6. For the more potent inhibitors, a linear relationship was obtained. A similar comparison has been presented for concanavalin A comparing precipitin inhibition data with association constants determined by equilibrium dialysis (17). In both systems, precipitin inhibition gave somewhat higher Kᵣ values than the reference method. For weak inhibitors of LBL (Kᵣ > 50 mM), deviation from the linear relationship was observed. The increased potency of the weaker sugars in inhibiting precipitation of blood group A reactive glycoconjugates relative to potency in masking the sulfhydryl groups could have several bases. High sugar concentrations (greater than 0.1 M) could nonspecifically inhibit the precipitin reaction. These sugars could also inhibit precipitation by binding to portions of the carbohy-

**Table II**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>T</th>
<th>Kᵣ</th>
<th>ΔHᵣ</th>
<th>ASᵣ</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-GalNAc</td>
<td>10</td>
<td>8.9 ± 0.2</td>
<td>-5.5</td>
<td>-10.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>12.1 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>17.1 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Me-α-d-GalNAc</td>
<td>10</td>
<td>2.35 ± 0.07</td>
<td>-6.1</td>
<td>-9.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.47 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.78 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-Trisaccharide</td>
<td>10</td>
<td>0.211 ± 0.04</td>
<td>-6.2</td>
<td>-4.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.28 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.44 ± 0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 5.** Kinetics of loss of slow reacting form of lima bean lectin thiol in the presence of EDTA. LBL was made 1 mM in EDTA and dialyzed into PB, pH 7.0, containing 1 mM EDTA at 2 or 24 °C. Aliquots were removed at various times and the kinetics of modification of the essential thiol with Nbs was determined. Percent slow reacting thiol is plotted as a function of time at 2 °C (B) and 24 °C (C). After 26 h at 24 °C, an aliquot was made 1 mM in Ca²⁺ and Mn²⁺ and reanalyzed (Δ).

[D. D. Roberts and I. J. Goldstein, unpublished observations.](http://www.jbc.org/)

[^1]: Determination of sugar binding stoichiometry by equilibrium dialysis using lectin prepared under N₂ indicates that lima bean lectin component III does bind 4 eq of GalNAc (D. D. Roberts and I. J. Goldstein, manuscript in preparation).
Precipitation of an LBL blood... binding site generally...

As discussed below, LBL may have an extended... inhibition constants determined by inhibition of thiol modification performed in buffer of ionic strength 0.35. At 20 °C, increasing... without blocking the essential thiol from reaction with Nbs, and recognized subterminal sugar residues in addition to the carbohydrate binding to LBL have not been determined.

Binding to concanavalin A, the relationship between... linkage. The slow rate of modification by Nbs2 (generally faster, with the respective dissociation constants, Kd, measured by equilibrium dialysis (1.0 mM).)

Rapid monophasic binding of monosaccharides to another legume lectin, concanavalin A, has been reported (18–20). However, some oligosaccharides have been reported to follow biphasic kinetics (19, 21). For our analyses, we assume fast binding whereby equilibrium is maintained and Kd = Kd. However, in view of the complex kinetics for oligosaccharide binding to concanavalin A, the relationship between Kd and Kd values for the oligosaccharides tested with LBL must be interpreted with caution. The calculated inhibition constant for Me-α-GalNAc at 2 °C, Kd = 0.17 mM, is somewhat higher than the Kd measured by equilibrium dialysis (1.0 mM). However, it should be noted that the equilibrium dialysis was performed in buffer of ionic strength 0.35. At 20 °C, increasing ionic strength from 0.1 to 0.2 decreased the Kd for Me-α-GalNAc by a factor of two. Thus, the lower Kd measured by equilibrium dialysis may be due to an ionic strength effect.

We have used the sulfhydryl group protection assay to examine the nature of the interaction between LBL and the C-2 N-acetyl group of N-acetyl-d-galactosamine. We have also tested a series of N-acetyl-D-galactosamine-terminated oligosaccharides for their ability to bind to LBL.

The nature of the substituent on the C-2 position plays a decisive role in the binding affinity. The 2-O-acetyl derivative of methyl α-D-galactopyranoside is 14 times more potent an inhibitor than the parent glycoside. Me-α-GalNAc is 25 times more potent than methyl-α-D-galactopyranoside, indicating a locus in the lectin’s combining site that interacts either with an N-acetyl or O-acetyl group. A somewhat greater preference is observed for an amide relative to an ester when methyl(2-N-p-nitrobenzamido)-α-D-GalN (Kr = 0.87 mM) and methyl (2-O-p-nitrobenzoyl)-α-D-Gal (Kr = 5.6 mM) are compared. On the other hand, a 2-O-methyl or 2-O-benzyl group, both lacking a carbonyl function, did not enhance the binding of methyl-α-D-Galp to lima bean lectin. An N-acetyl group is 4 times more effective than an N-formamido unit, suggesting the necessity for some bulk at this position. The most potent inhibitors were those containing an N-benzamidomethyl group or its derivatives. These compounds were bound 4 to 5 times more avidly than Me-α-GalNAc, suggesting the presence in the LBL binding site of a hydrophobic region complementary to this aromatic group. The fact that methyl-(2-N-p-nitrobenzenesulfonylaminomethyl)-α-D-GalN binds almost as well as methyl-(2-N-p-nitrobenzamidomethyl)-α-D-GalN indicates tolerance of a sulfonamido function at this position. Binding of 2-N-dansyl-GalN also is consistent with tolerance of a sulfonamide at the 2-N position.

The p-nitrophenyl α- and β-glycosides of GalNAc were no more potent than the corresponding methyl glycosides, indicating absence of a hydrophobic binding site adjacent to this portion of the carbohydrate binding site as is present in several other legume lectins such as concanavalin A (6, 22).

Four D-GalNAc-D-Gal disaccharides containing nonreducing GalNAc termini were tested. Of these, the α-(1→2)-linked saccharide was the most potent, being approximately 7 times more active than either the α-(1→3) or α-(1→6) disaccharide. Nor was the Forssman disaccharide composed of two GalNAc units linked α-(1→3) much different from α-D-GalNAc-(1→3)-D-Gal. Surprisingly, the β-(1→6)-linked disaccharide [β-D-GalNAc-(1→6)-D-Gal] was only 2½ times less active than the α-(1→6)-linked saccharide. This is probably due to the fact that GalNAc, linked to the C-6 primary alcoholic group of galactose, has greater conformational freedom of movement than if linked to a secondary alcoholic group (cf. gentiobiose versus isomaltose binding to concanavalin A). Melibiose, containing a terminal α-linked D-Gal unit, was bound 5 times more avidly than lactose.

Of the oligosaccharides tested, the type A trisaccharide with a linker arm (α-D-GalNAc-(1→3)-[α-L-Fuc-(1→2)]-β-D-Gal-(1→0)(CH2)3COOCH3) was the most potent, being approximately 15-fold more active than the corresponding type A disaccharide (α-D-GalNAc-(1→3)-D-Gal).

Hydrophobic forces have been inferred to be important for the binding of fucose containing oligosaccharides to antibodies and lectins (23). The interaction of fucose on the A-trisaccharide with LBL may also have hydrophobic character. The apparent entropy for binding of the trisaccharide at 20 °C was −4.9 cal deg⁻¹ mol⁻¹, which is 5·7 entropy units more positive than the apparent entropy of GalNAc binding (ΔS° = −10.2 cal deg⁻¹ mol⁻¹). The enthalpies of binding for both ligands are similar, indicating that the large difference in affinities of the two ligands is due primarily to the difference in entropy of binding. This is consistent with hydrophobic binding which is driven by an increase in solvent entropy (24). The high affinity of the 2-N-benzamidomethyl derivatives could also be the result of hydrophobic interactions, suggesting that the fucose on the A-trisaccharide might be topologically equivalent to a nonpolar aryl substituent at the C-2 position of GalNAc. In contrast, the decrease in entropy for binding of GalNAc to LBL indicates that hydrophobic interactions are not important for binding of the monosaccharide. Specific hydrogen bonds or dipole interactions involving the C-4 hydroxyl and C-2 carbonyl or sulfonyl oxygens are likely to be important for stabilizing the bound sugar.

The oligosaccharide specificity of LBL is somewhat differ-
vent from that reported for other GalNAc binding lectins. Only a slight preference (40%) for A-active saccharides containing an α-1→2 fucose over those lacking fucose was observed for the Dolichos biflorus lectin (25). The soybean agglutinin did not tolerate an α-1→2 fucose on the penultimate sugar (26). A fucose-containing A-active pentasaccharide was found to be approximately 24-fold weaker than the disaccharide α-D-GalNAc(1→3)Gal. Similar specificity was reported for Griffonia simplicifolia I-A (27). Fucose-containing oligosaccharides were 26- to 40-fold weaker than those lacking fucose. Thus, the lima bean lectin is the most specific of the characterized GalNAc-binding lectins for fucose-containing type-A blood group substance.

The kinetic parameters for the reaction between LBL and Nbs₂ may also provide some insight into the nature of the carbohydrate binding site. The pH-independent reactivity of the essential thiol near neutral pH is not expected for a simple thiol of pKₐ 8–10. Only the ionized form of the thiol can react with Nbs₂ (28, 29). Below the pKₐ of the thiol, the reactivity should decrease logarithmically with decreasing pH. Therefore, it is proposed that the thiol exists as an ion pair with a nearby positive charged group at neutral pH. In the following paper, we identify an imidazole of a histidine adjacent to the essential cysteine as a likely candidate for this base (30). Ion pair formation between cysteine and histidine is also observed in papain and may account for some of the unusual reactivity of the thiol in that enzyme (31–33). Abnormal pH dependence for thiol reactivity with Nbs₂ was also reported for metallothionein (34). In this protein, the thiols are ligands for bound metal ions and have some characteristics of ion pairs. In both metallothionein (34) and LBL, removal of metals leads to a marked increase in reactivity of the thiols with Nbs₂. Thus, it might be proposed that the essential cysteine in LBL is a ligand for Ca²⁺ or Mn²⁺ binding. Whereas this model is consistent with the pH dependence of sulfhydryl reactivity and the effects of EDTA on the thiol, it is difficult to rationalize with complete protection of these groups by carbohydrate binding. It seems more plausible that removal of metals induces a conformational change that both increases the accessibility of the sulfhydryl groups of LBL to Nbs₂ and disrupts the carbohydrate binding site, as was observed in crystallographic studies of demetallized concanavalin A (35).

Formation of a noncovalent complex between Nbs₂ and LBL is also notable. The Kₐ for this complex decreased with increasing ionic strength, suggesting that the cysteine may become less accessible under these conditions. Such a change could also account for the increase in affinity for Me-α-GalNAc at high ionic strength. Thus, it is not possible to distinguish between conformational effects and hydrophobic binding as the basis for the enhanced affinity of LBL for Me-α-GalNAc at high ionic strength.

In summary, the reactivity of the cysteine sulfhydryl groups present on each subunit of LBL has proved to be a very useful tool for understanding the interactions of this protein with both carbohydrate and metal ligands. We have presented preliminary characterization of both structural and thermodynamic parameters for carbohydrate interactions based on sulfhydryl modification. In addition, we have demonstrated correlation between the reactivity of the sulfhydryl groups and metal ion binding. It is hoped that these studies will provide a basis for further thermodynamic and kinetic characterization of the carbohydrate and divalent cation binding sites of LBL.

Acknowledgments—We would like to thank Linda Carriere for typing the manuscript, Dr. D. Baker for providing oligosaccharides, Dr. R. Rossman for atomic absorption analyses, and Drs. R. Blake and J. Shafer for numerous helpful discussions during the course of this work.

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