Site-directed mutagenesis studies on the lima bean lectin

Altered carbohydrate-binding specificities result from single amino acid substitutions

Elizabeth T. JORDAN and Irwin J. GOLDSTEIN

Department of Biological Chemistry, The University of Michigan, Ann Arbor, USA

The wild-type seed lima bean lectin (LBL), and recombinant LBL expressed in *Escherichia coli* show specificity for the human blood group A immunodominant trisaccharide GalNAcα1-3[Fucα1-2]Galβ1-R. We have generated four site-specific mutants of LBL, two of which show altered specificity for extended carbohydrate structures. Four mutants, [C127Y]LBL, [H128P]LBL, [H128R]LBL and [W132F]LBL were expressed in *E. coli*. Two mutants show altered specificity for the substituent at the C2 hydroxy group of the penultimate Gal in the wild-type ligand which is α-L-fucose in the A trisaccharide. The mutant [C127Y]LBL showed specificity for the A disaccharide (GalNAcα1-3Gal) and GalNAcα1-4Gal, with free hydroxyl groups at the C2 position of Gal. The mutant [H128P]LBL bound the Forssman disaccharide structure GalNAcα1-3GalNAc, in which the C2 hydroxyl group is substituted with an acetamido group. The third and fourth mutants, [H128R]LBL and [W132F]LBL, exhibited wild-type specificities, both recognizing the A trisaccharide. All of these mutant lectins bound the terminal GalNAc residues exposed on asialoovine submaxillary mucin, thus indicating that the monosaccharide-binding site had not been altered. We also determined that all but one mutant ([C127Y]LBL) retained the high-affinity binding site for N^6 derivatives of adenine, indicative of tetramer formation; each mutant also expressed the low-affinity binding site for 8-anilinonaphthalene 1-sulfonate (1/monomer). Thus, by targeting two residues in LBL, we have identified a region of the protein that is part of the extended carbohydrate-binding site and which is specifically involved in the binding/recognition of substituents at the C2 position of the penultimate Gal of the A disaccharide. We have determined, by site-directed mutagenesis, that an essential Cys residue is involved in the specificity of LBL for the A trisaccharide.

Keywords. Lima bean lectin; lima bean lectin mutants; mutant lectins.

The specificity of legume lectins for known oligosaccharide structures has made them useful tools in the study of cell surface glycoconjugates. Through the synthesis of substituted oligosaccharides, many requirements for lectin binding have been defined in an effort to elucidate their specificity. The crystal structures of *Canavalia ensiformis* agglutinin (ConA) in complex with methyl α-D-mannopyranoside [1], *Lathyrus ochrus* lectin in complex with an octasaccharide [2] and *Griffonia simplicifolia* isolectin IV in complex with the Lewis b tetrasaccharide [3] have clearly demonstrated that both hydrogen bonding and Van der Waal's forces are involved in carbohydrate binding. Additionally, many of the hydrogen bonds are mediated by water molecules, thus making a correlation between primary structure and specificity difficult. The structure of *Erythrina corallodendron* in complex with lactose displayed similar features, al-though interaction is directed primarily toward the terminal monosaccharide, a β-linked galactosyl unit [4].

Legume lectins appear to have similar secondary structures, composed predominantly of β sheets. However, sequence variability in the carbohydrate-binding regions could have an effect on the potential Van der Waal's surfaces of the binding pocket, in addition to the hydrogen-bonding possibilities with aminoacyl side chains. Thus, there are many factors that govern the carbohydrate specificity of each legume lectin, including the conformation of the carbohydrate ligand to which they bind. By modifying one amino acid residue at a time, site-directed mutagenesis affords an opportunity to study structure/function relationships directly.

Mutagenesis studies on legume lectins are still in their infancy. In an interesting study, a chimeric lectin with altered specificity was generated by replacing a heptapeptide of *Bauhinea purpurea* lectin with the corresponding region of the *Lens culinaris* lectin [5]. In a second study, substitution of Asn125 with Asp in the homologous region of the pea lectin resulted in an inactive pea lectin, thus implicating this residue in carbohydrate binding [6], whereas substitution of Ala126 with Val had no apparent effect on pea lectin activity. A further study on pea lectin focused on residues 103 and 104 (Val and Phe, respectively) in which each was separately replaced by Ala [7]. It was determined that the Val to Ala substitution yielded a mutant pea lectin which retained activity, but was temperature sensitive, whereas the Phe to Ala mutation had no such effect. Other site-

specific mutations have been reported in pea lectin, which appear to have little effect on its specificity, but did change the thermodynamic parameters of binding [8]. On the basis of sequence homology with peanut agglutinin, *E. corallodendron* lectin was mutated such that the Pro134-Trp dipeptide was replaced with Ser-Glu-Tyr-Asn, corresponding to the sequence in peanut agglutinin [9]. This mutant still retained its wild-type activity, but had a decreased affinity for GalNAc and its *N*-dansyl derivatives, indicating that this region of the monosaccharide-binding site recognizes the hydroxy group at the C2 position of the terminal Gal unit. These studies have helped to define the requirements for lectin-carbohydrate interaction from the 'lectin's point of view'. It is hoped that an accumulation of such data may assist in our understanding of peptide sequences which recognize specific sugars.

In this paper, we report on the mutagenesis of the lima bean lectin (LBL) in a region of the protein which was found to be important in carbohydrate binding. Protein modification studies with 5,5'-dithiobis(2-nitrobenzoic acid) demonstrated the involvement of a sulfhydryl residue in carbohydrate binding [10]. The essential Cys residue was ultimately identified by isolation of a peptide containing the modified residue [11]; the peptide is homologous to the portion of the carbohydrate-binding site investigated in *B. purpurea*, pea and *E. corallodendron* lectins [5–9]. Expression of LBL in *Escherichia coli* [12] allowed for site-directed mutagenesis studies, despite the lack of crystal-structure information available for this lectin. We describe four site-specific mutations of LBL in the region containing the essential Cys residue; two of these mutations had no effect on binding of oligosaccharides, whereas the other two mutants display altered specificities for extended carbohydrate structures.

MATERIALS AND METHODS

Materials. *Synsorb A.* Beads containing immobilized type-A trisaccharide GalNAcα1-3[Fucα1-2]Galβ1-O-(CH$_2$)$_8$CONH-, the type-A trisaccharide hapten, GalNAcα1-3[Fucα1-2]Galβ1-O-(CH$_2$)$_8$CONH$_2$, and the corresponding BSA conjugate were obtained from Alberta Research Council. Forssman disaccharide Synsorb beads containing immobilized Forssman disaccharide [GalNAcα1-3GalNAcβ1-O-(CH$_2$)$_8$CONH-], Forssman disaccharide hapten [GalNAcα1-3GalNAcβ1-O-(CH$_2$)$_8$CONH$_2$] and BSA conjugate were obtained from Alberta Research Council. Type-A disaccharide Synsorb beads containing immobilized type-A disaccharide [GalNAcα1-3Galβ1-O-(CH$_2$)$_8$CONH-] were obtained from Chembiomed. The type-A disaccharide hapten, Gal-NAcα1-3Galβ1-O-(CH$_2$)$_8$COCH$_3$, was obtained from Professor O. Hindsgaul (University of Alberta, USA). The type-A disaccharide hapten, without linker arm, GalNAcα1-3Galβ1-OH, was prepared in our laboratory by Dr Younus M. Meah. Other BSA conjugates, including GalNAcα1-4Gal and Galα1-R, were obtained from the Alberta Research Council. Melibionate-oval-bumin was prepared in our laboratory. Asialoovine submaxillary mucin was prepared in our laboratory by Dr Hanqing Mo, from ovine submaxillary mucin obtained from Dr A. Eckhardt (Duke University, USA).

Site-directed mutagenesis and DNA sequencing. Site-directed mutagenesis was carried out by the method of Kunkel [13] as previously described [12]. Further simplification and overall efficiency was achieved using PCR for mutagenesis. Mutagenic primers were synthesized (by the DNA Synthesis Core of the Biomedical Research Core Facilities at the University of Michigan, USA) which also incorporated a silent mutation, to create a unique restriction site 5' of the desired mutation. A second primer, which was complementary to the antisense strand,

was made in which only the silent mutations were incorporated. We were able to generate two halves of the LBL gene using the 5' and 3' primers previously described [12] along with the mutagenic primers. PCR conditions used for 30 cycles were: 94°C, 30 s; 50°C, 30 s; 72°C, 1 min. The 5' half of the gene was modified at its 3' end with the unique restriction sites incorporated by the silent mutations. Ligation of the two halves of the gene at the unique restriction site resulted in an LBL gene which had several unique restriction sites just 5' of a specific mutation. The 5' half, as well as each mutant 3' half, was sequenced by the standard procedures previously described [12]. The Sequenase version 2.0 Kit (United States Biochemical Corp.) was used in double-stranded dideoxy sequencing; both strands were sequenced.

Expression of wild-type and mutant recombinant LBL in *E. coli*. The mutated LBL genes were inserted into the *Nco*I and *Bam*HI restriction sites on the T7 expression vector pET3d (Novagen). The wild-type recombinant LBL expression vector was prepared as previously reported [12]. Induction of recombinant LBL expression in BL21(DE3)pLysE host strain was with 0.4 mM isopropyl β-D-thiogalactopyranoside (dioxane free). After 3 hours induction at 30°C, cells were harvested and washed with 0.1 M sodium phosphate, 0.15 M NaCl, 10 μM CaCl$_2$, 10 μM MnCl$_2$, pH 6.8 (NaCl/P$_i$), with 1 mM phenylmethyl-sulfonyl fluoride, 10 μM dithiothreitol (buffer A). The washed pellet was resuspended in lysis buffer, and passed through a French Pressure cell three times at 12000 Pa at 4°C. The crude lysate was centrifuged at > 120000×g for 70 min at 4°C in a Beckman ultracentrifuge to remove insoluble material (inclusion bodies) and cell membranes. The supernatant, containing soluble recombinant LBL, was removed for further analysis.

Analysis of mutant recombinant LBL carbohydrate binding. Hemagglutination assays were performed as described [12]. A modified ELISA assay was developed in which a glycoconjugate diluted to 10 μg/ml in 100 mM sodium carbonate, pH 9.6, was used to coat the wells of a 96-well microtiter plate (Corning). Specifically, the wells were coated with 50 μl solution for 3 hours at 37°C, and then washed twice (3 min each) with 100 μl 1% BSA (mass/vol.) in NaCl/P$_i$ (buffer B) at room temperature. Blocking was achieved by incubating coated, washed plates with 100 μl blocking buffer overnight at 37°C. Blocked wells were washed once, then incubated with lectin sample (in a final volume of 100 μl) for 30 min at 37°C. All dilutions were in buffer B. Wells were washed three times (3 min each) with buffer B between incubations. Primary antibody [rabbit IgG anti-(seed LBL) or anti-(recombinant LBL), as previously described [12]], was diluted 1:1500, and 100 μl was added to each well, then incubated for 30 min at 37°C. Following three washes, secondary antibody (goat anti-(rabbit IgG)-alkaline-phosphatase conjugate; Bethesda Research Laboratories) was diluted 1:3000; 100 μl was added to each well, and incubated for 30 min at 37°C. Following the final washes, 200 μl *p*-nitrophenyl phosphate substrate (Sigma or BRL), containing 1 mg/ml in 100 mM sodium carbonate, pH 9.6, was added, and incubated for 1 hour at room temperature in the dark. The absorbance at 405 nm was measured using a Titertek Multiskan MC plate reader. All samples were assayed in duplicate. Both crude extracts (of *E. coli* soluble proteins upon expression of recombinant LBL, and without the gene as a control), as well as purified protein were assayed by this method. Duplicates with values differing by less than 10% were accepted.

Hapten inhibition assays. Hapten inhibition assays were performed by serially diluting hapten in 1.5 ml tubes, and adding lectin and blocking buffer to the desired volume and concentration. Each tube contained a final volume sufficient for duplicates to be assayed on each type of glycoconjugate coating. Hapten

```
            117                   127           132
LBL     Q  T  V  A  V  E  F  D  T  C  H  N  L  D  W      D  P
ConA    T  I  V  A  V  E  L  D  T  Y  P  N  T  D  I  G   D  P
GS IV   Q  V  V  A  V  E  F  D  T  W  I  N  K  D  W  N   D  P
ECorL   Q  T  L  G  V  E  F  D  T  F  S  N        Q  W   D  P
DBA     Q  T  V  A  V  E  F  D  T  L  S  N  S  G  W      D  P
LTA     Q  F  V  A  V  E  F  D  S  Y  H  N  I     W      D  P

C127Y                              Y
H128R                                 R
H128P                                 P
W132F                                           F
```

Fig. 1. Sequence alignment of legume lectins in the region subjected to site-directed mutagenesis in LBL. Shown are Con A [15] *G. simplicifolia* isolectin IV (GS IV) [3], *E. corallodendron* lectin (ECorL) [25], *D. biflorus* agglutinin (DBA) [24] and *L. tetragonalobus* agglutinin (LTA) [26]. Individual mutations of LBL are listed in the lower part.

and lectin were incubated for 1 hour at room temperature and assayed as described above. Each plate contained, as references, duplicates of 100% binding (no hapten) and 0% binding (excess hapten) of the lectin being studied. The type-A disaccharides (both free and with linker arm) were assayed at 4°C for seed LBL, recombinant LBL, [H128R]LBL and [W132F]LBL.

Purification of mutant recombinant LBL. Based upon binding to the glycoconjugates tested, we were able to purify mutant [C127Y]LBL on Synsorb A-disaccharide resin (Chembiomed). [H128P]LBL was purified at room temperature on Synsorb beads conjugated with the Forssman disaccharide (Chembiomed). Wild-type recombinant LBL, [H128R]LBL and [W132F]LBL were purified on Synsorb A (trisaccharide form; Chembiomed). Elution from these columns was with 0.1 M acetic acid. Columns were washed with 2% ammonium hydroxide in 50 mM NaCl, followed by extensive washing in NaCl/P$_i$ to remove any remaining protein between samples.

Hydrophobic binding. Measurements to detect binding of the hydrophobic ligands 8-anilinonaphthalene 1-sulfonate (ANS) and 2-*p*-toluidinylnaphthalene-6-sulfonic acid were recorded using an Aminco Spectrofluorimeter. Excitation wavelengths were 350 nm and 420 nm and emission was at 430 nm and 480 nm (2-*p*-toluidinylnaphthalene-6-sulfonic acid and ANS, respectively). Stock solutions were prepared as follows: ANS was in NaCl/P$_i$; 2-*p*-toluidinylnaphthalene-6-sulfonic acid was in sodium phosphate buffer; N^6-benzylaminopurine and 4-aminopyrazolo[3,4,*d*]pyrimidine were in 75% ethanol.

RESULTS

The mutations of recombinant LBL (Fig. 1) were generated based on alignment of the recombinant LBL sequence with several other legume lectin sequences over the region of interest for this study. The mutations correspond to a region in the polypeptide chain of legume lectins which has been shown in the crystal structures of ConA, *Lathyrus ochrus* isolectin 1, *G. simplicifolia* isolectin IV and *E. corallodendron* to form part of the carbohydrate-binding site [1−4]. We chose the mutations shown (Fig. 1) based on two criteria; (a) conservation of the size and/or charge of the aminoacyl side chain or (b) preparation of a mutation to a homologous residue in Con A, arbitrarily considering this protein to be a proto-type lectin as it was one of the few crystal structures known at the time this work was initiated. A more appropriate model might have been *E. corallodendron*, since it represents a single-chain legume lectin with a monosaccharide-binding site for Gal/GalNAc. We generated the mutations in this region of the primary structure based upon the observation that modification of the free sulfhydryl group inactivated LBL [10];

this led us to consider that the Cys residue played an essential role in carbohydrate recognition, and/or metal binding. In an earlier study [14], it was reported that a decapeptide corresponding to the sequence around this Cys residue bound Ca^{2+}. This decapeptide is homologous to a region in lectins from the families Vicieae, Diocleae and others which have been shown to be involved in metal binding. Our rationale for replacing Cys127 with a non-conservative residue such as Tyr was that most other legume lectins have an aromatic residue at this position. Additionally, the Tyr residue would be expected to possess a hydrogen-bonding ability if this was an important feature of the Cys residue. We also prepared a mutant in which Cys was mutated to Ser; it did not appear to have any detectable carbohydrate-binding activity (Jordan, E. T. and Goldstein, I. J., unpublished results).

We chose to mutate His to Arg in order to maintain the presence of an ionizable side chain (although, in a protein, Arg has a higher pKa than His). Additionally, inasmuch as the Arg side chain is somewhat extended, it might mimic His more closely than Lys. The substitution of His by Pro was generated on the basis of a Pro residue present in this position in ConA; this would allow us to determine whether the residues of the Cys-His dipeptide might act in concert, in which case the ionizable group of His would be important. The fourth mutant, [W132F]LBL, was prepared on the basis of what appears to be an evolutionarily conserved residue. Trp is present at this position in most legume lectins, with the exception of Con A [15], *Dioclea grandiflora* [16], and *Bowringia mildbraedii* [17], in which this amino acid is replaced by two residues with smaller side chains; Ile and Gly. This suggested a possible structural involvement of Trp in the legume lectin-binding region. The mutation of Trp to Phe was considered to be a conservative substitution which would maintain the overall size of the side chain. The site-specific mutations were incorporated by either the Kunkel method [13] of mutagenesis, or by PCR-mediated mutagenesis using synthetic oligodeoxynucleotide primers.

We assayed crude extracts of mutants for hemagglutinating activity against human type A, B or O cells, as well as against rabbit erythrocytes. Whereas both wild-type recombinant LBL and seed LBL showed hemagglutinating activity towards type-A cells, only two mutants, [H128R]LBL and [W132F]LBL, exhibited agglutinating activity towards these cells, and one, [C127Y]LBL, agglutinated fresh rabbit cells. More sensitive binding data were afforded using an enzyme-linked lectin assay (ELLA) [18, 19], in which microtiter plates were coated with glycoconjugates. We used this assay to screen mutants in crude extracts for carbohydrate-binding activity (data not shown) in parallel with crude extracts of *E. coli* harboring the original pET vector (no LBL gene insert). In these experiments, [H128P]LBL bound most significantly to GalNAcα1-3GalNAcβ1-R-BSA (the Forssman-disaccharide−BSA conjugate). [C127Y]LBL bound the type-A disaccharide (GalNAcα1-3Galβ1-R-BSA) and GalNAcα1-4Galβ1-R-BSA conjugates, whereas [H128R]LBL and [W132F]LBL were similar to the seed and recombinant LBL in binding the type-A trisaccharide best. We used these carbohydrate-binding properties to purify each mutant on the corresponding disaccharide-Synsorb or trisaccharide Synsorb columns.

Affinity-purified lectins were assayed for binding against a panel of glycoconjugates using the ELLA assay (Figs 2A and B). It should be noted that we compare the relative patterns of binding activity of recombinant LBL with mutant binding activities. The amount of binding relative to the best bound BSA conjugate is shown (Fig. 2). We have divided the data into two parts; (a) the binding of lectins which activity resembles that of the wild type, and (b) the binding of lectins which have activities

A
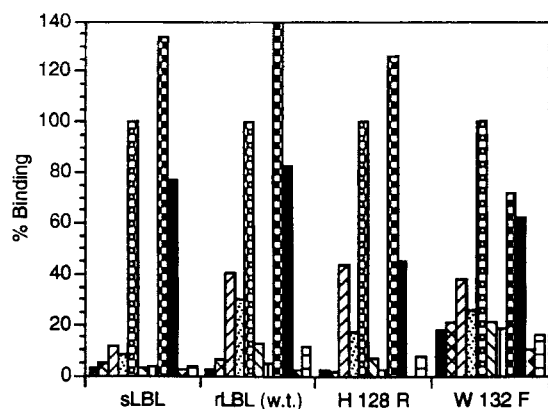
B
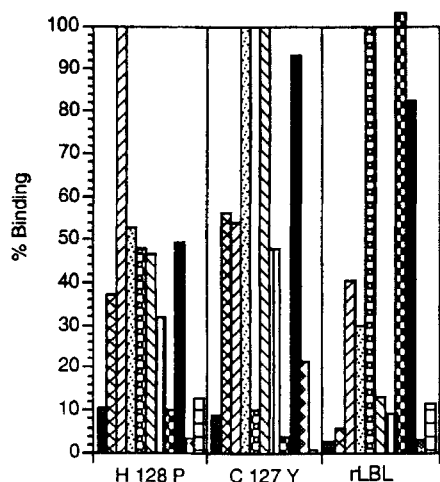
Fig. 2. Binding patterns of purified lectins to various glycoconjugates, determined using the ELLA assay. Symbols represent glycoconjugates used to coat wells. ▨, BSA; ▨, Gal α1-BSA; ▨, Forssman-disaccharide−BSA; ▨, type-A-disaccharide−BSA; ▨, type-A-trisaccharide−BSA; ▨, GalNac-α-1,4-Gal; ▥, melibionate-ovalbumin; ▨, human A substance; ■, asialo ovine submaxillary mucin; ▨, ovine submaxillary mucin; □, fetuin. (A) Lectins which bind the type-A trisaccharide−BSA structure best; seed LBL (sLBL), recombinant LBL (rLBL), [H128R]LBL (H128R) and [W132F]LBL (W132F). Binding is relative to 100% binding on the type-A trisaccharide−BSA conjugate. (B) lectins which display an altered specificity; [H128P]LBL (H128P) and [C127Y]LBL (C127Y) are shown with recombinant LBL (rLBL) from (A) for comparison. [H128P]LBL binding is relative to 100% binding on the Forssman-disaccharide−BSA conjugate. [C127Y]LBL binding is relative to 100% binding on the type-A-disaccharide−BSA conjugate.

Table 1. Relative inhibitory potencies of haptens used to inhibit binding of wild-type and mutant recombinant LBLs to the type-A-trisaccharide−BSA conjugate. Values of relative inhibitory potencies are relative to those of GalNAc, as determined by the ELLA assay. The actual observed concentration of GalNAc required to achieve 50% inhibition of binding is shown as a reference. n.i., not inhibitory. The Forssman disaccharide and the type-A disaccharide-OH were assayed at 4°C.

Hapten	Relative inhibitory potency for			
	seed LBL	recombinant LBL	[H128R]-LBL	[W132F]-LBL
GalNAc	1	1	1	1
	(5 mM)	(5 mM)	(10 mM)	(3.5 mM)
Me-α-GalNAc	4.1	2.5	3.1	2.7
Me-β-GalNAc	2.4	1.2	1	1.8
Me-α-Gal	0.2	0.2	0.4	0.6
Type-A disaccharide-OH	1.8	2.1	n.i.	n.i.
GalNAcα1-6 Gal	3.1	2.5	5.1	2.6
Forssman disaccharide	7.5	3.7	n.i.	4.8
Type-A trisaccharide	49	50	83	177

[W132F]LBL binds the type-A-trisaccharide conjugate better than the other glycoconjugates tested, it is notably different from seed LBL, recombinant LBL and [H128R]LBL in terms of binding to human A substance.

The binding of [H128P]LBL and [C127Y]LBL to the same panel of glycoconjugates was compared (Fig. 2B) to the binding of recombinant LBL (from Fig. 2A). The results indicate that [H128P]LBL binds the Forssman-disaccharide−BSA conjugate better than any other glycoconjugate tested; this mutant also bound rather weakly to the type-A-disaccharide−BSA conjugate. The [C127Y] LBL mutant bound the type-A-disaccharide−BSA (GalNAcα1-3Gal), GalNAcα1-4 Gal−BSA conjugates, and asialo ovine submaxillary mucin almost equally well. Additionally, binding to αGal-BSA, the Forssman-disaccharide−BSA conjugate and melibionate ovalbumin appeared to be enhanced over that of recombinant LBL (relative to the A disaccharide and A trisaccharide, respectively). Although [C127Y]LBL binds several monosaccharide and disaccharide conjugates, it did not bind the A-trisaccharide−BSA conjugate or human A substance.

In order to confirm this apparent change in the carbohydrate-binding specificity of several of the mutants, we performed hapten inhibition assays in which the purified lectins were incubated with hapten sugars and assayed by ELLA for binding to glycoconjugate-coated plates. In Table 1, we present data obtained in the type-A-trisaccharide−BSA system, in which inhibitory potencies (for 50% inhibition) are given relative to those for GalNAc. In this system, the purified lectins (Fig. 2A) are compared by inhibition assay on type-A-trisaccharide−BSA-coated plates. The values obtained are all within the same range, except for the type-A trisaccharide. The type-A trisaccharide inhibited seed LBL and recombinant LBL approximately 32−50-fold better than GalNAc. However, the potency of this haptenic trisaccharide appears to be increased for mutant [H128R]LBL, and was about threefold higher than that observed for [W132F]LBL. These data confirm the specificity of these lectins for the type-A trisaccharide. Neither the free type-A disaccharide nor the disaccharide with the linker arm were inhibitory at 37°C, but they were slightly inhibitory at 4°C.

Table 2 summarizes the inhibitory potencies of several haptens in the [H128P]LBL−Forssman-disaccharide−BSA binding system and the [C127Y]LBL−A-disaccharide−BSA system, relative to that for GalNAc. The best inhibitor of [H128P]LBL

differing from that of the wild type. The purified lectins which were isolated on the type-A-trisaccharide−Synsorb column (seed LBL, recombinant LBL, [H128R]LBL and [W132F]LBL) all bind the type-A-trisaccharide−BSA conjugate better than any other BSA conjugate assayed (Fig. 2A). The binding patterns of these lectins are very similar for the glycoconjugates tested, the main differences being that recombinant LBL and [H128R]LBL appear to bind the Forssman disaccharide and possibly the type-A disaccharide conjugates somewhat better than seed LBL. Also, [H128R]LBL binds asialo-ovine submaxillary mucin less well than seed and recombinant LBL. While

Table 2. Relative inhibitory potencies of haptens in the inhibition of [H128P]LBL binding to Forssman-disaccharide–BSA conjugate and [C127Y]LBL binding to the type-A-disaccharide–BSA conjugate. Values of relative inhibitory potencies are relative to those of GalNAc, as determined by the ELLA assay. The observed concentration of GalNAc required to achieve 50% inhibition of binding is shown as a reference. n.i., not inhibitory.

Hapten	Relative inhibitory potency for	
	[H128P]LBL	[C127Y]LBL
Gal	n.i.	0.02
GalNAc	1 (2.5 mM)	1 (0.65 mM)
Me-α-GalNAc	1.8	4.3
Me-β-GalNAc	0.4	0.9
Type-A disaccharide-free	n.i.	1.8
Type-A disaccharide–linker-arm	n.i.	3
GalNAc-α-1,6-Gal	1.4	2.7
Forssman disaccharide	2.8	n.i.
Type-A trisaccharide	2.1	n.i.

Table 3. Binding of hydrophobic probes ANS and 2-*p*-toluidinyl-naphthalene-6-sulfonic acid to wild type and mutant LBLs. Pyrazolo, 4-aminopyrazolo[3,4,*d*]pyrimidine.

Lectin sample	ANS K_d	Pyrazolo K_{act}	TNS site 1 K_d	TNS site 2 K_d
	μM			
Seed LBL	56.6	16.3	51.2	57.7
Recombinant LBL	23.1	75.3	48.0	24.7
[W132F]LBL	25.4	68.2	46.1	27.3
[H128R]LBL	23.0	10.0	10.6	26.1
[H128P]LBL	30.8	84.0	10.3	29.0
[C127Y]LBL	26.8	–	–	–

was determined to be the Forssman disaccharide, although it was only threefold better than GalNAc. At room temperature, however, this hapten inhibited binding 6.4-times better than Gal-NAc. Other potential inhibitors of [H128P]LBL were the type-A trisaccharide and Me-α-GalNAc. The type-A trisaccharide hapten was almost twice as potent an inhibitor as GalNAc.

The binding of mutant [C127Y]LBL to the type-A disaccharide conjugate was inhibited best by Me-α-GalNAc. The type-A disaccharide hapten (with linker arm) was the next best inhibitor (three-times better than GalNAc). Interestingly, the type-A disaccharide with a free anomeric hydroxy group was only about two-times more active than GalNAc as an inhibitor. This might suggest that the sugar specificity of this mutant is mainly for the α anomer of GalNAc. Both of the type-A disaccharide haptens (free and with linker arm) had inhibitory activities at 37°C, in contrast to the other systems discussed in which inhibition was observed only at 4°C. Interestingly, whereas [C127Y]LBL bound the type-A disaccharide–BSA conjugate, and was purified on the type-A disaccharide–Synsorb column, the type-A disaccharide hapten was not the best inhibitor of this system. The best inhibitor of [C127Y]LBL was Me-α-GalNAc. The disaccharide GalNAcα1-6Gal had an inhibitory potency similar to that of the type-A disaccharide (with linker arm), and was slightly better than that of the free type-A disaccharide.

As a test of the overall molecular structure of the LBL mutants, we made use of the fact that LBL has one low-affinity binding site/monomer for ANS and 2-*p* toluidinylnaphthalene-6-sulfonic acid, and one high affinity site/tetramer for N^6 derivatives of adenine [20, 21]. We were interested in ascertaining whether there were any gross conformational changes in the mutants we generated which would disrupt ANS binding to each monomer. From titrations of the lectins with these hydrophobic fluorescent probes, we demonstrated that each mutant, as well as recombinant LBL and seed LBL, was able to bind ANS; the calculated K_d values are shown (Table 3). The results indicate that each protein has a similar K_d value, in the range 20–50 μM. We investigated the occurrence of LBL mutants as tetrameric structures using two methods; (a) enhancement of ANS fluorescence with the addition of 4-aminopyrazolo [3,4-*d*]pyrimidine, a compound previously shown to have such an effect on seed LBL [22], and (b) observation of both high-affinity and low-affinity sites for 2-*p*-toluidinylnaphthalene-6-sulfonic acid. The observed K_{act} of the lectins for 4-aminopyrazolo[3,4-*d*]pyrimidine ranged over 6.8–16.3 μM (Table 3). However, upon titra-

tion of [C127Y]LBL with this compound, we observed no enhancement of ANS fluorescence. We also found that 2-*p*-toluidinylnaphthalene-6-sulfonic acid did not bind to [C127Y]LBL. However, the other lectins showed both high-affinity and low-affinity binding, with the observed K_d values ranging over 4.6–10 μM (high affinity) and 24.7–57 μM (low affinity). These results indicate that seed LBL, recombinant LBL and mutants [H128R]LBL, [H128P]LBL and [W132F]LBL appear to be similar in terms of their quaternary structures. However, mutant [C127Y]LBL differs in that adenine did not cause any enhancement in the binding of ANS. This could indicate that the binding sites have been disrupted, or that mutant LBL does not form tetramers. Nevertheless, this lectin agglutinated rabbit cells, which implies the presence of at least dimers.

DISCUSSION

Using site-directed mutagenesis, we have demonstrated that three amino acids of LBL are apparently involved in the extended carbohydrate combining site. Only two of the generated mutants had detectable hemagglutination activity for human type-A cells, and one mutant agglutinated fresh rabbit cells. Making use of a very sensitive ELLA assay for mutant binding to a panel of glycoconjugates, we showed that all four of the mutants had carbohydrate-binding activities (Fig. 2A and B). We were able to purify each mutant on the basis of its carbohydrate-binding specificity, as determined by ELLA in crude extracts, rather than by immunoaffinity chromatography which could result in the purification of a mixture of active and inactive proteins.

The specificity of seed LBL and recombinant LBL and all of the mutants for terminal GalNAc residues is demonstrated (Fig. 2A and B). Seed LBL, recombinant LBL, [H128R]LBL and [W132F]LBL bind best to the type-A trisaccharide structure GalNAcα1-3[Fucα1-2-]Gal (Fig. 2A), in which the penultimate Gal has an α-L-Fuc substituent at the C2 position. The slight difference in binding between seed LBL and recombinant LBL could be due to the fact that recombinant LBL is a non-glycosylated homotetramer whereas seed LBL is a glycosylated heterotetramer [12]. Replacement of L-Fuc with -NAc or -OH, as in the case of the Forssman disaccharide (GalNAcα1-3GalNAc) and type-A disaccharide (GalNAcα1-3Gal), respectively, diminished binding by seed LBL, but allowed binding by [H128P]LBL and [C127Y]LBL (Fig. 2B). Thus, two mutants ([H128R]LBL and [W132F]LBL) have wild-type activities, and two mutants ([C127Y]LBL and [H128P]LBL) have altered specificities. Confirmation of the changes in the carbohydrate-binding specificity is presented (Table 2), in which the best inhibitors of

[H128P]LBL and [C127Y]LBL were the Forssman disaccharide, and Me-α-GalNAc, respectively.

Our results indicate that two of the positions in which we made amino acid substitutions are directly involved in determining the carbohydrate specificity of LBL. The Cys residue which was previously proposed to be involved in carbohydrate and/or metal binding [10, 11, 14], has been shown in this study to be directly involved in the carbohydrate specificity. The mutant lectin resulting from replacement of the Cys residue with a bulky aromatic Tyr side chain, [C127Y]LBL, failed to bind the human type-A trisaccharide, although the less bulky *N*-acetyl substitution at the C2 position of Gal (Forssman disaccharide) is tolerated. This mutant bound best to structures in which the penultimate Gal was not substituted at the C2 position (type-A disaccharide and GalNAcα1-4Gal). Additionally, this substitution caused a loss of specificity for α1-3 compared to α1-4 linkages. Our observation that substitution of the Cys residue with Ser resulted in a loss of carbohydrate-binding activity suggests that the Cys residue is also involved in metal binding, and that Ser cannot act in this capacity, thus leading to an inactive mutant. We also found that agglutination of rabbit cells by [C127Y]LBL is both metal and carbohydrate dependent. Additionally, the [C127Y]LBL mutant was the only mutant LBL that was inhibited by high concentrations of galactose, lactose, raffinose, melibiose and stachyose.

The charge of the His residue appears to be important in binding to the type-A trisaccharide, in that replacement of His by Arg has no net effect on the carbohydrate-binding capacity of this mutant. Substitution with Pro at this position yielded a mutant which bound the Forssman disaccharide, but not the type-A trisaccharide. Since Pro is often involved in disrupting protein structures, it is possible that this is the cause of the failure to bind the type-A trisaccharide. It is interesting to note that previous studies indicated that a hydrophobic interaction between Fuc and protein may enhance the binding of the type-A trisaccharide [10]. The Trp to Phe mutation did not affect the carbohydrate-binding specificity of the resulting LBL mutant; a similar finding was reported for the pea lectin [8] in which an identical substitution did not change the carbohydrate-binding specificity of the lectin. Thus, the Trp may be a conserved structural feature of the monosaccharide-binding sites of legume lectins.

We also demonstrated, by hydrophobic binding studies, that three of the mutants appear to have an overall structure similar to that of seed LBL and recombinant LBL. It was shown previously that ANS fluorescence is blue-shifted upon binding to the hydrophobic site of seed LBL monomers [20], and that addition of N^6 derivatives of adenine and similar compounds resulted in the enhancement of ANS fluorescence [22]. (There is one adenine binding site/tetramer.) We found that each mutant has a binding site for ANS, indicating that this site had been preserved, and thus confirming the overall structure of the monomer. However, upon titration with 4-aminopyrazolo [3,4,*d*]pyrimidine, we observed ANS fluorescence enhancement for only five of the six proteins studied (all but [C127Y]LBL). This suggests that a change has occurred in which binding to the high-affinity adenine site on the tetramer did not enhance ANS fluorescence, or that [C127Y]LBL does not form tetramers. However, [C127Y]LBL must exist, at least as a bivalent aggregate, as indicated by its ability to agglutinate rabbit cells. This is confirmed by the fact that Cys260, involved in linking two monovalent monomers by disulfide-bond formation, is intact, as determined by electrophoresis in the presence and absence of 2-mercaptoethanol.

In summary, we have generated four site-specific mutations of recombinant LBL, in a region implicated in carbohydrate

binding based on biochemical studies [10, 11] and sequence homology with other legume lectins. The use of an ELLA-based assay has allowed us to determine the carbohydrate-binding activities of the mutant lectins on human erythrocytes using a wider variety of glycoconjugates than is normally available. We have presented evidence which clearly demonstrates that two of these mutants, [C127Y]LBL and [H128P]LBL, have carbohydrate-binding specificities for extended structures which are distinctly different from that of seed LBL and wild-type recombinant LBL. We have also found that the two mutants, [H128R]LBL and [W132F]LBL, have retained the carbohydrate-binding specificity of the wild-type lectin. Our study indicates that a single amino acid substitution in the carbohydrate-binding region of LBL is sufficient to cause a change in the specificity of the lectin for extended carbohydrate structures. We also confirmed our previous observation that Cys127 [11] is involved in the carbohydrate binding and possibly in the metal binding of LBL.

Bourne et al. [23] have determined that a Fuc moiety on *N*-acetyllactosamine-type glycans enhances the affinity of *Vicieae* lectins for carbohydrate ligands by binding in a small crevice on the surface of the lectin. Additionally, comparison of the structure of the isolectin II-lactotransferrin N2 fragment complex from *L. ochrus* with that of ConA, shows that ConA lacks this crevice; this correlates with the finding that fucosylated glycans show no enhanced reactivity towards ConA. A similar hypothesis may be proposed for the role of the fucosyl moiety in the interaction of the type-A trisaccharide with LBL, in which Cys127 may be part of such a crevice on LBL. Our results indicate that Cys127, located two amino acids before the essential Asp (Asp129), plays a role in the binding of oligosaccharides to LBL. A similar function for the Phe residue, located two residues before Asp129, was elegantly described in the crystallographic analysis of LOL [2]. Thus, despite the clear difference in the structures of these oligosaccharides (the A trisaccharide compared to a biantennary oligosaccharide), one might speculate that the residue at this position (Cys127) also plays a role in the specificity of binding, showing a preference for a ligand of a particular structure. When Cys127 is replaced by Tyr, the crevice may be filled, and thus prevent binding of the fucosyl moiety of the A trisaccharide. This work corroborates the crystallographic data of Bourne and colleagues [23] which described the importance of the Phe residue (which aligns with the Cys of LBL) in the binding of oligosaccharide structures in *Vicieae* lectins. Comparing the fine specificities and sequence homologies of other Gal/GalNAc-binding lectins, site-directed mutagenesis studies may be employed to gather more insightful information on the correlation of the amino acid sequence with carbohydrate recognition.

REFERENCES

1. Derewenda, Z., Yariv, J., Helliwell, J. R., Kalb, A. J., Dodson, E. J., Papiz, M. Z., Wan, T. & Campbell, J. (1989) The structure of the saccharide-binding site of concanavalin A, *EMBO J. 8*, 2189–2193.

2. Bourne, Y., Rouge, P. & Cambillau, C. (1992) X-ray structure of a biantennary octasaccharide-lectin complex refined at 2.3-A resolution, *J. Biol. Chem. 267*, 197–203.

3. Delbaere, L. T. J., Vandonselear, M., Prasad, L., Quail, J. W., Wilson, K. S. & Dauter, Z. (1993) Structures of the lectin IV of *Griffonia simplicifolia* and its complex with the lewis b human blood group determinant at 2.0 A, *J. Mol. Biol. 230*, 950−965.

4. Shaanan, B., Lis, H. & Sharon, N. (1991) Structure of a legume lectin with an ordered n-linked carbohydrate in complex with lactose, *Science 254*, 862−866.

5. Yamamoto, K., Konami, Y., Osawa, T. & Irimura, T. (1992) Alteration of the carbohydrate-binding specificity of the *Bauhinia purpurea* lectin through the preparation of a chimeric lectin, *J. Biochem. 111*, 87−90.

6. van Eijsden, R. R., Hoedemaeker, F. J., Diaz, C. L., Lugtenberg, B. J. J., de Pater, B. S. & Kijne, J. W. (1992) Mutational analysis of pea lectin. Substitution of Asn125 for Asp in the monosaccharide-binding site eliminates mannose/glucose-binding activity, *Plant Mol. Biol. 20*, 1049−1058.

7. Hoedemaeker, F. J., van Eijsden, R. R., Diaz, C. L., de Pater, B. S. & Kijne, J. W. (1993) Destabilization of pea lectin by substitution of a single amino acid in a surface loop, *Plant Mol. Biol. 22*, 1039−1046.

8. Stubbs, M. E., Pang, H., Dunn, R. J. & Carver, J. P. (1991) The effect of amino acid substitution at position 128 of recombinant pea lectin, *Glycoconjugate J. 8*, 235−236.

9. Arango, R., Rodriguez-Arango, E., Adar, R., Belenky, D., Loontiens, F. G., Rozenblatt, S. & Sharon, N. (1993) Modification by site-directed mutagenesis of the specificity of *Erythrina corallodendron* lectin for galactose derivatives with bulky substituents at C-2, *FEBS Lett. 330*, 133−136.

10. Roberts, D. D. & Goldstein, I. J. (1984) Effect of carbohydrate and metal ion binding on the reactivity of the essential thiol groups of the lima bean lectin, *J. Biol. Chem. 259*, 903−908.

11. Roberts, D. D. & Goldstein, I. J. (1984) Isolation from the lima bean lectin of a peptide containing a cysteine residue essential for carbohydrate binding activity, *J. Biol. Chem. 259*, 909−914.

12. Jordan, E. T. & Goldstein, I. J. (1994) The sequence of a second member of the lima bean lectin gene family and the expression and characterization of recombinant lectin in *Escherichia coli*, *J. Biol. Chem. 269*, 7674−7681.

13. Kunkel, T. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection, *Proc. Natl Acad. Sci. USA 82*, 488−492.

14. Maliarik, M. J., Roberts, D. D. & Goldstein, I. J. (1990) Anitigenic and calcium binding properties of a peptide containing the essential cystein in lima bean lectin, *Plant Physiol. 95*, 286−290.

15. Carrington, D. M., Auffret, A. & Hanke, D. E. (1985) Polypeptide ligation occurs during post-translational modificatio of concanavalin A, *Nature 313*, 64−67.

16. Richardson, M., Campos, F. D. A. P., Moreira, R. A., Ainouz, I. L., Begbie, R., Watt, W. B. & Pusztai, A. (1984) The complete amino acid sequence of the major alpha subunit of the lectin from the seeds of *Diolea grandiflora* (Mart.), *Eur. J. Biochem. 144*, 101−111.

17. Chawla, D., Animashaun, T., Hughes, R. C., Harris, A. & Aitken, A. (1993) *Bowringia mildbraedii* agglutinin: polypeptide composition, primary structure and homologies with other legume lectins, *Biochem. Biophys. Acta 1202*, 38−46.

18. McCoy, J. P., Varani, J. & Goldstein, I. J. (1983) Enzyme-linked lectin assay (ELLA). II. Detection of carbohydrate groups on the surface of unfixed cells, *Anal. Biochem. 130*, 437−444.

19. van der Schaal, I. A., Logman, T. J., Diaz, C. L. & Kijne, J. W. (1984) An enzyme-linked lectin binding assay for quantitative determination of lectin receptors, *Anal. Biochem. 140*, 48−55.

20. Roberts, D. D. & Goldstein, I. J. (1982) Hydrophobic binding properties of the lectin from lima beans (*Phaseolus lunatus*), *J. Biol. Chem. 257*, 11274−11277.

21. Roberts, D. D. & Goldstein, I. J. (1983) Adenine binding sites of the lectin from lima beans (*Phaseolus lunatus*), *J. Biol. Chem. 258*, 13820−13824.

22. Roberts, D. D., Arjunan, P., Townsend, L. B. & Goldstein, I. J. (1986) Specificity of adenine binding to lima bean lectin, *Phytochemistry 25*, 589−593.

23. Bourne, Y., Mazurier, J., Legrand, D., Rouge, P., Montreuil, J., Spik, G. & Cambillau, C. (1994) Structures of a legume lectin complexed with the human lactotransferrin N2 fragment, and with an isolated biantennary glycopeptide: role of the fucose moiety, *Structure 2*, 209−219.

24. Harada, J. J., Spadoro-Tank, J., Maxwell, J. C., Schnell, D. J. & Etzler, M. E. (1990) Two lectin genes differentially expressed in *Dolichos biflorus* differ primarily by a 116-base pair sequence in their 5′ flanking regions, *J. Biol. Chem. 265*, 4997−5001.

25. Arango, R., Adar, R., Rozenblatt, S. & Sharon, N. (1992) Cloning and sequence analysis of the *Erythrina corallodendron* lectin cDNA, *Eur. J. Biochem. 205*, 575−581.

26. Konami, Y., Yamamoto, K. & Osawa, T. (1990) The primary structure of the *Lotus tetragonolobus* seed lectin, *FEBS Lett. 268*, 281−286.