Binding Site of the Rabbit Liver Lectin Specific for Galactose/*N*-Acetylgalactosamine†

Reiko T. Lee

ABSTRACT: Neoglycoproteins containing various monosaccharides and disaccharides were prepared by modifying bovine serum albumin (BSA) with thioglycosides using amidination or reductive alkylation [Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3963; Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 156–163]. The binding specificity of the rabbit liver galactose/*N*-acetylgalactosamine specific lectin was studied with these neoglycoproteins by assaying their ability to inhibit ^{125}I-labeled asialoorosomucoid binding to the isolated lectin or to the crude plasma membrane. The following observations were made: (1) All the galactose-containing neoglycoproteins (except two) had a similar inhibitory effect. (2) BSA derivatives containing glucose, with the sole exception of [Glc—S—CH_2C(=NH)—NH]$_n$-BSA, were poorer inhibitors than the galactose-containing BSA's, and the axial 4-OH of galactose appears to participate in the binding. (3) The binding site of the lectin for the C-6 region of galactose is apparently spacious, since a galactose substituted at the 6-OH with another monosaccharide can still be bound. (4) A negatively charged group at C-6 of galactose interfered with the binding to the lectin. (5) A bulky aglycon in an α-D-galactopyranoside considerably decreased the inhibitory power. For explanation of these results, it is proposed that the lectin possesses at least one negatively charged group at the binding site and the presence of this group is directly responsible for inability of the sialylated (negatively charged), native glycoproteins to bind to the mammalian hepatic lectin.

We have been studying the specificity of the galactose/*N*-acetylgalactosamine binding lectin of the rabbit liver (mammalian hepatic lectin) using neoglycoproteins derivatized with sugar imidates (Glyc-AI structure; see Chart I) as inhibitors of ^{125}I-labeled asialoorosomucoid (ASOR)[1] binding to the lectin (Krantz et al., 1976; Stowell et al., 1980). From these studies we reached the following conclusions on the binding requirements of the lectin: (i) 2-OH (equatorial) and 3-OH (equatorial) are needed for the binding, (ii) 4-OH can be axial (as in galactose) or equatorial (as in glucose), and (iii) $-CH_2OH$ at C-5 can be absent or can be modified (e.g., $-CH_3$ or $-CH_2OCH_3$).

We have now expanded these studies to include neoglycoproteins containing disaccharides and neoglycoproteins of different sugar-to-protein linking groups. The results obtained from these studies indicate that (i) the binding site of the lectin for the C-6 region of galactose is rather spacious and is capable of accommodating a C-6 linked group the size of *N*-acetylglucosamine, (ii) the specificity of the lectin with regard to the 4-OH group is not as lax as originally thought, and (iii) the negative charge of the sialic acid on intact serum glycoproteins rather than the fact that the galactose is penultimate to the nonreducing end is probably responsible for the failure of these glycoproteins to be bound by the hepatic lectin.

Experimental Procedures

Materials. Unless otherwise specified, sugars and sugar derivatives were obtained from Pfanstiehl Lab., Inc. (Waukegan, IL). BSA (fraction V, fatty acid free) was obtained from Miles Laboratories, Inc. α_1-Acid glycoprotein (orosomucoid) was kindly provided by the American Red Cross and desialylated by a mild acid hydrolysis to produce ASOR. Carrier-free sodium [^{125}I]iodide was obtained from Amersham Corp.

The following sugar derivatives were prepared by the methods previously reported: cyanomethyl 1-thioglycosides of galactose, glucose, maltose, melibiose, lactose, and cellobiose (Lee et al., 1976; Stowell & Lee, 1980); [[*N*-(2,2-dimethoxyethyl)amino]carbonyl]methyl 1-thio-β-D-galactopyranoside and [[*N*-(2,2-dimethoxyethyl)amino]carbonyl]pentyl 1-thio-β-D-galactopyranoside (Lee & Lee, 1979a); per-*O*-acetyl derivatives of *N*-acetyllactosamine (Lee & Lee, 1979b), Galβ(1→6)Glc, Galβ(1→6)Gal, Galα(1→6)Gal, and [[*N*-(2,2-dimethoxyethyl)amino]carbonyl]methyl 1-thioglycosides of the last two disaccharides (Lee & Lee, 1981a); 2-oxoethyl 1-thio-β-D-galactopyranoside and -glucopyranoside and 3-oxopropyl 1-thio-β-D-galactopyranoside and -glucopyranoside (Lee & Lee, 1981b). Cellobiose, lactose, melibiose, and *N*-acetyllactosamine were converted to the corresponding [[*N*-(2,2-dimethoxyethyl)amino]carbonyl]methyl 1-thioglycosides essentially in the same way as described (Lee & Lee, 1979a). The preparation of the galacturonic acid derivatives will be described elsewhere.

Methods. Sugars were determined with a modified phenol–sulfuric acid method (McKelvy & Lee, 1969). Uronic acids and their esters were determined by a carbazole method (Bitter & Muir, 1962). The protein concentration of the modified BSA was determined by the microbiuret method (Zamenhof, 1957) using dried, unmodified BSA as standard. The radioactivity (^{125}I) was counted with a Packard PRIAS automatic γ counter, Model PGC.

Preparation of neoglycoproteins of BSA was by either amidination (Lee et al., 1976; Stowell & Lee, 1980) or reductive alkylation (Lee & Lee, 1980), using the appropriate thioglycosides described under Materials. The sugar content of

† From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218. *Received August 18, 1981.* Contribution No. 1132 from the McCollum-Pratt Institute, The Johns Hopkins University. Supported by U.S. Public Health Service, National Institutes of Health Research Grant AM 9970.

[1] Abbreviations: ASOR, asialoorosomucoid; BSA, bovine serum albumin; Mal, maltose; Cel, cellobiose; Mel, melibiose; Lac, lactose; LacNAc, *N*-acetyllactosamine; GalUA, galacturonic acid; Me-GalUA, methyl galacturonate; NeuAc, *N*-acetylneuraminic acid; RIP, relative inhibitory power; Glyc$_n$-BSA, bovine serum albumin which contains *n* residues of the specified mono- or disaccharide per molecule. See Chart I for abbreviations used for various neoglycoproteins.

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Chart I

BSA derivative	reagent (thioglycoside)	sugar–protein linkage
	NH ‖	NH ‖
Glyc-AI-BSA	Glyc–S–CH_2C–OMe	Glyc–S–CH_2C–NH–
Glyc-GD-BSA	Glyc–S–CH_2CHO	Glyc–S–$(CH_2)_2$–NH–
Glyc-PD-BSA	Glyc–S–$(CH_2)_2$CHO	Glyc–S–$(CH_2)_3$–NH–
Glyc-AD-BSA	Glyc–S–CH_2CONHCH$_2$CHO	Glyc–S–CH_2CONH$(CH_2)_2$–NH–
Glyc-HD-BSA	Glyc–S–$(CH_2)_5$CONHCH$_2$CHO	Glyc–S–$(CH_2)_5$CONH$(CH_2)_2$–NH–

neoglycoproteins was determined by hydrolyzing the thiosugar linkage with mercuric acetate and analyzing the freed sugar with an automated neutral sugar analyzer (Lee & Lee, 1980). The sugar content of neoglycoproteins containing various derivatives of galacturonic acid (except methyl 4-deoxygalacturonate) was determined by the carbazole method, using an appropriate thioglycoside as standard. The sugar content of BSA containing methyl 4-deoxygalacturonate was determined by the phenol–sulfuric acid method using [[*N*-(2,2-dimethoxyethyl)amino]carbonyl]methyl 1-thioglycoside of methyl 4-deoxygalacturonate as standard.

Plasma membranes were prepared from rabbit liver by a modification (Morell & Scheinberg, 1972) of the method of Ray (1970). The galactose/*N*-acetylgalactosamine binding lectin was isolated and purified by a modification (Stowell & Lee, 1978) of the method of Hudgin et al. (1974) using either Sepharose coupled with asialofetuin or Gal-AI-BSA as affinant. ASOR was iodinated by using Chloramine T (Greenwood et al., 1963) as described previously (Krantz et al., 1976).

Inhibition of [^{125}I]ASOR binding to the lectin by various neoglycoproteins was studied by using three assay methods.

Assay I. Sequential Inhibition Assay with Plasma Membranes. The method is essentially that described by Van Lenten & Ashwell (1972). Various amounts of a neoglycoprotein under study were incubated with the membrane preparation for 1 h at 37 °C in a buffer (pH 7.3). After addition of [^{125}I]ASOR, the assay mixtures were further incubated for 30 min before filtration through Whatman GF/C filter disks (diameter 2.4 cm). The radioactivity on the filter disks was then counted to determine membrane-bound [^{125}I]ASOR.

Assay II. Simultaneous Inhibition Assay with the Plasma Membrane. Various amounts of a neoglycoprotein under study and [^{125}I]ASOR ($\sim 3 \times 10^{-10}$ M) were incubated with the membrane preparation (120 μg/mL) at 37 °C for 80 min. The incubation buffer and procedures for filtration and counting were the same as in assay I.

Assay III. Inhibition Assay with the Solubilized Lectin (Kawaguchi et al., 1980). This assay is essentially assay A of Hudgin et al. (1974). Various amounts of a neoglycoprotein under study and [^{125}I]ASOR ($\sim 6 \times 10^{-10}$ M) were incubated with the solubilized and purified lectin (4×10^{-10} M) at pH 7.8 and 25 °C for 30 min in the presence of Triton X-100. The complex was precipitated in 50% saturated ammonium sulfate, filtered through Whatman GF/C filters, and counted as described above.

Results

The binding affinities of neoglycoproteins were estimated by their inhibitory action on [^{125}I]ASOR binding to the liver plasma membranes or to the isolated hepatic lectin. Most of the neoglycoproteins used as inhibitors had the sugar-to-BSA linkage of –$SCH_2CONH(CH_2)_2NH$– (Glyc-AD-BSA), and unless otherwise stated, this type of neoglycoprotein was used in the inhibition assays. However, for investigation of the effect of the linkage group on the inhibition potency, four other types

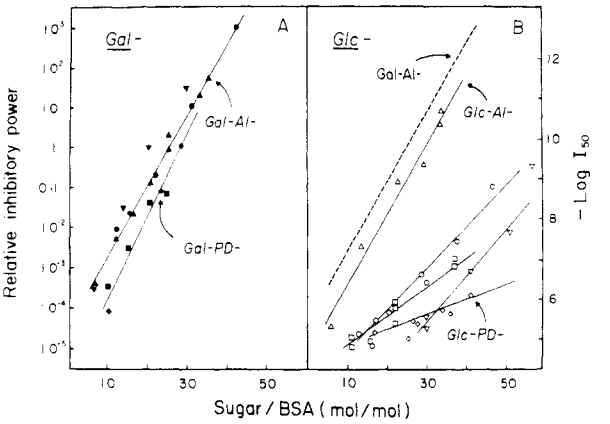


FIGURE 1: Plot of RIP and –log I_{50} (in M) vs. sugar content of various galactose- and glucose-containing BSA's (assay I). The right and left panels have the same ordinate. (A) Galactose derivatives: (●) Gal-AD-; (▲) Gal-AI-; (■) Gal-GD-; (♦) Gal-PD-; (▼) Gal-HD-. (B) Glucose derivatives: (○) Glc-AD-; (△) Glc-AI-; (□) Glc-GD-; (◇) Glc-PD-; (▽) Glc-HD-.

of neoglycoproteins were used. All five types of neoglycoproteins, their abbreviation symbols, their sugar-to-BSA linkages, and the reagent used in their preparation are presented in Chart I.

Assay I. This sequential membrane assay was used almost exclusively in our earlier studies of specificity (Stowell et al., 1980) and was also used most frequently in this study. In this assay, the inhibitory potency of neoglycoproteins is expressed in terms of the relative inhibitory power (RIP) as had been done in all our earlier studies (Krantz et al., 1976; Lee & Lee, 1980; Stowell et al., 1980). The RIP is defined as the ratio of the amounts in nanograms of ASOR and the neoglycoprotein inhibitor needed for the 50% inhibition of [^{125}I]ASOR binding. In Figures 1–3 the relationship between RIP and the number of sugar residues per molecule of BSA for various neoglycoproteins is shown. The figures also show I_{50} (molar protein concentration of an inhibitor that causes 50% inhibition) values on the right ordinate. The results are summarized below:

(1) Derivatized BSA's containing either mannose or *N*-acetylglucosamine were very poor inhibitors. The RIP values were too low to be shown in the figures. For Man_{35}-AD-BSA (containing 35 residues of thiomannoside per molecule of BSA), RIP was estimated to be $\sim 4 \times 10^{-6}$, while that of $GlcNAc_{46}$-AD-BSA was too low to be determined. These results parallel the earlier findings using another type of BSA-neoglycoprotein, Man-AI-BSA and GlcNAc-AI-BSA, as inhibitors (Krantz et al., 1976).

(2) With the exception of BSA derivatives containing galacturonic acid and methyl 4-deoxygalacturonate which showed barely measurable inhibitory power, the RIP values of all other neoglycoproteins increased exponentially with the increase in the number of sugar residues per molecule of BSA.

(3) All the BSA derivatives containing thiogalactoside (also those containing lactose and *N*-acetyllactosamine) had similar RIP values regardless of the linking group.

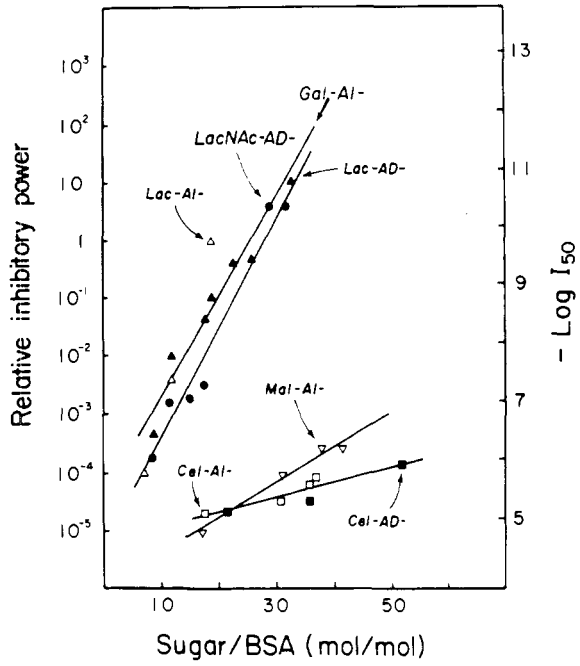


FIGURE 2: Plot of RIP and $-\log I_{50}$ (in M) vs. sugar content of BSA neoglycoproteins containing 1,4-linked disaccharides (assay I). (●) *N*-Acetyllactosamine-AD-; (▲) lactose-AD-; (△) lactose-AI-; (■) cellobiose-AD-; (□) cellobiose-AI-; (▽) maltose-AI-. For comparison, the line for Gal-AD-BSA from Figure 1 is indicated.

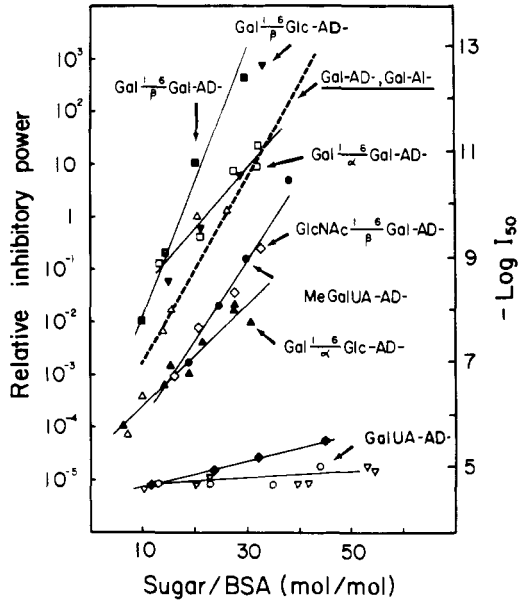


FIGURE 3: Plot of RIP and $-\log I_{50}$ (in M) vs. sugar content of various BSA neoglycoproteins containing either a 1,6-linked disaccharide or a galacturonic acid derivative (assay I). (▲) Melibiose-AD-; (△) melibiose-AI-; (▼) Galβ(1→6)Glc-AD-; (■) Galβ(1→6)Gal-AD-; (□) Galα(1→6)Gal-AD-; (◆) GlcNAcβ(1→6)Gal-AD-; (●) methyl galacturonate-AD-; (○) galacturonic acid-AD-; (♦) methyl 4,5-anhydrogalacturonate-AD-; (▽) methyl 4-deoxygalacturonate-AD-. The line for Gal-AD-BSA from Figure 1 is included for comparison.

(4) All the BSA derivatives containing glucose were inhibitors. However, with the sole exception of Glc-AI-BSA, the inhibitory power of glucose-containing neoglycoproteins was much weaker than that of the corresponding galactose derivatives.

(5) Since BSA derivatives containing GlcNAcβ(1→6)Gal, a disaccharide consisting of one binding (galactose) and one nonbinding (*N*-acetylglucosamine) sugar residue, were good inhibitors, the lectin apparently can accommodate a bulky substituent (*N*-acetylglucosamine) at 6-OH of galactose. Also

Table I: Comparison of I_{50} Values of Various Galactose- and Glucose-Containing BSA's (30 mol of Sugar per mol) Obtained by the Three Different Assay Methods

linking arm[a]	I_{50} (nM) Gal-BSA I[b]	Gal-BSA II[b]	Gal-BSA III[b]	Glc-BSA I	Glc-BSA II	Glc-BSA III
AI	0.03	0.03	5	0.11	0.06	9
GD	0.11	ND[c]	25	600	ND	2000
PD	0.11	ND	47	2500	ND	3500
AD	0.03	0.25	26	200	280	2500
HD	0.008	ND	9	4000	ND	5000

[a] See Chart I for chemical structure of various linking arms. [b] See Experimental Procedures for description of assays I, II, and III. [c] Not determined.

Table II: I_{50} of ASOR and Some Neoglycoprotein Inhibitors ($Glyc_{30}$-AD-BSA): Comparison of Assays I, II, and III

thioglycoside	I_{50} (nM) I[a]	II[a,b]	III[a]
ASOR	0.2	0.2	4
lactose	0.03	0.45	50
melibiose	6.5	10	1500
GlcNAcβ(1→6)Gal	1	9	400
Galβ(1→6)Gal	$\sim 10^{-4}$	0.15	13
galacturonic acid	10000	ND[c]	100000
methyl galacturonate	1	ND	250
methyl 4,5-anhydrogalacturonate	6000	ND	10000
methyl 4-deoxygalacturonate	10000	ND	60000

[a] For description of assays I, II, and III, see Experimental Procedures. [b] I_{50} in this assay was obtained by using one BSA derivative for each sugar, which contained 28-31 residues of sugar per molecule of BSA. [c] Not determined.

BSA derivatives containing Galβ(1→6)Gal had RIP values much higher than the BSA derivatives containing thiogalactosides when compared at the same level of glycoside coupling (mole per mole of BSA), suggesting that both galactose residues in Galβ(1→6)Gal could be bound by the lectin.

(6) A negatively charged group at C-6 of galactose interferes with binding, since BSA containing galacturonic acid was only weakly inhibitory while esterification of this carboxylic acid (BSA containing methyl galacturonate) essentially restored the inhibitory potency to the level of galactose-containing BSA's.

(7) The axial 4-OH of galactose contributes to strong binding, since the BSA derivatives containing methyl 4,5-anhydrogalacturonate and methyl 4-deoxygalacturonate were very poor inhibitors compared to BSA derivatives containing methyl galacturonate.

(8) A bulky aglycon (such as a glycopyranosyl group) which is α-anomerically linked to galactose might interfere with the binding of the galactosyl moiety, as the BSA derivatives containing Galα(1→6)Glc (melibiose) and Galα(1→6)Gal were weaker inhibitors than Galβ(1→6)Glc and Galβ(1→6)Gal derivatives, respectively.

Assay II. ASOR and a few selected neoglycoproteins were used as inhibitors in this simultaneous membrane assay. The results are presented in Figure 4 and in Tables I and II. In general the results from the two membrane assays were comparable within an order of magnitude. One interesting observation is that in assay II Gal-AI-BSA and Glc-AI-BSA were better inhibitors than Gal-AD-BSA at a comparable level of sugar substitution (mole per mole of BSA), while in assay I they possessed a comparable inhibitory potency.

Assay III. The inhibition assay with the purified, Triton-solubilized lectin was carried out by using neoglycoproteins

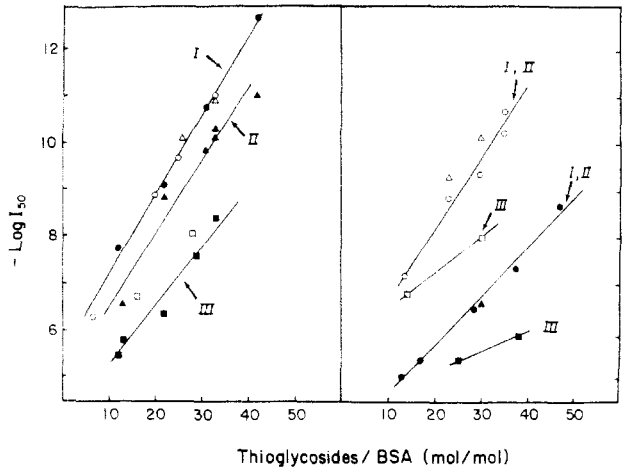


FIGURE 4: Plot of I_{50} (in M) vs. sugar content of galactose- and glucose-containing BSA's using the three assay methods. Solid symbols indicated AD derivatives and open symbols indicate AI derivatives. Circles, assay I; triangles, assay II; squares, assay III. (Left panel) Galactose derivatives: (●) Gal-AD- and (○) Gal-AI-, assay I; (▲) Gal-AD- and (△) Gal-AI-, assay II; (■) Gal-AD- and (□) Gal-AI-, assay III. (Right panel) Glucose derivatives: (●) Glc-AD- and (○) Glc-AI-, assay I; (▲) Glc-AD- and (△) Glc-AI-, assay II; (■) Glc-AD- and (□) Glc-AI-, assay III.

bearing various sugars as inhibitors at two different sugar levels (usually one at <30 and the other at >30 mol of sugar per mol of BSA). The results were plotted as exemplified in Figure 4 (most of the plots are not shown), and from the plots the I_{50} values were estimated at 30 mol of sugar per mol of BSA for the derivatized BSA's (Tables I and II). Qualitatively one arrives at the same conclusions from this assay and assay I with regard to the relative inhibitory potency of various sugars. However, some quantitative differences do exist between the results obtained from assays I and III: (1) The I_{50} values for neoglycoproteins were in general higher in assay III than in assays I and II (Tables I and II). (2) The difference in I_{50} for a good and weak inhibitor is more pronounced in assay I than in assay III (Table II). (3) An increase in the level of sugar substitution (moles per mole of BSA) causes a more pronounced increase in the I_{50} in assay I than in assay III (slope of the lines in Figure 4).

Discussion

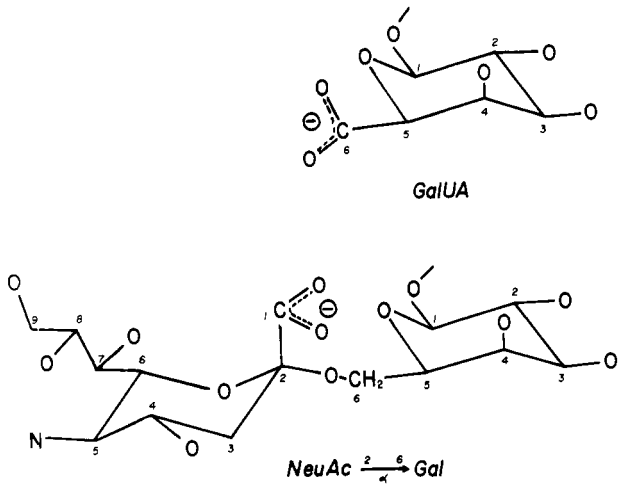
We have previously studied the sugar specificity of the galactose/*N*-acetylgalactosamine-binding lectin of the rabbit liver using exclusively BSA derivatized with sugar imidates (Glyc-AI-BSA) as inhibitors of [^{125}I]ASOR binding to the lectin (Stowell & Lee, 1978; Stowell et al., 1980). It was found that the glucose-containing BSA (Glc-AI-BSA) was bound by this lectin as well as that containing galactose (Gal-AI-BSA). This phenomenon was difficult to explain, since their 2-acetamido analogues behaved totally differently; i.e., BSA derivatized with *N*-acetylgalactosamine was bound even stronger than the galactose derivative, while binding of BSA containing *N*-acetylglucosamine was negligible.

In our present study, we prepared several other types of neoglycoproteins (shown in Chart I) and tested them as inhibitors of [^{125}I]ASOR binding to the rabbit liver lectin. All the galactose-containing BSA's [with exceptions of those containing melibiose and Gal(1→6)Gal] had remarkably similar inhibitory power. This includes BSA containing *N*-acetyllactosamine, which is the disaccharide unit of the nonreducing end in the desialylated, serum-type glycoproteins. These results are in agreement with the idea that the binding site of this lectin is rather small, involving mainly the binding of the terminal galactose (or *N*-acetylgalactosamine) moiety of various glycosides and oligosaccharides (Sarkar et al., 1979). On the contrary, glucose-containing BSA's had variable inhibitory power depending on the sugar-to-protein linking group. The most inhibitory glucose-containing BSA was Glc-AI-BSA, and as reported earlier (Stowell & Lee, 1978), it had inhibitory power comparable to that of the galactose-containing BSA's. All other types of glucose-containing BSA's including cellulose-AI-BSA and maltose-AI-BSA were poorer inhibitors than the galactose-BSA's. These facts, together with the finding that the BSA derivatives containing methyl 4-deoxygalacturonate were much poorer inhibitors than BSA derivatized with methyl galacturonate (Figure 3), suggest that the axial 4-OH contributes significantly to the binding. Since the lectin binds *N*-acetylgalactosamine more strongly than galactose (Sarkar et al., 1979; Stowell et al., 1980), the equatorial 2-acetamido group must contribute more to the binding than the equatorial 2-hydroxyl group. On the other hand, the BSA derivatives of *N*-acetylglucosamine, the 4 epimer of *N*-acetylgalactosamine, do not bind at all, indicating that the equatorial 4-hydroxyl group is prohibitory to the binding when the 2-acetamido group is present. The same equatorial 4-hydroxyl group on a glucose moiety, however, apparently does not offer a formidable obstruction to the binding, since various BSA derivatives containing glucose were bound by the lectin to different degrees. It may be that the strong binding of the equatorial 2-acetamido group requires the pyranose moiety to be bound rigidly, and only in this rigidly bound state the equatorial 4-hydroxyl group (in *N*-acetylglucosamine) interferes significantly with the binding.

The variability in the inhibitory power of glucose-containing BSA's implies that the sugar-to-protein linkage somehow influences the binding of glucose. The linking group, —$CH_2C(=NH)NH$—, in Glc-AI-BSA apparently strengthens the binding of this neoglycoprotein to a level comparable to the galactose-containing BSA's. A distinctive structural feature in this linking group is the presence of a positively charged amidino group in the proximity of anomeric carbon (see Chart I). Therefore, it is thought that the binding site of the lectin may have a strategically positioned group (possibly a negatively charged group) which could interact with the amidino group of Glc-AI-BSA, making it possible for this derivative to be bound as tightly as galactose-containing BSA's. If indeed there is on the lectin a negatively charged group responsible for the binding of the amidino group, it is intriguing that another glucose derivative, Glc-GD-BSA, having a positively charged secondary amino group located similarly to that in Glc-AI-BSA, failed to show substantial binding. The small difference in the location of the positive charge in the two linking groups or the difference in basicity of the amidino group and the secondary amino group may explain the large difference in binding. The binding area of the lectin may be sufficiently hydrophobic as to cause significant deprotonation of the weaker base (the secondary amine in Glc-GD-BSA) at pH 7.5. On the other hand, it may be that the unsaturated structure C=NH rather than the positive charge is more important in promoting the binding. Glc-AD-BSA which has an oxygen analogue (C=O) of this structure similarly positioned as the amidino group of Glc-AI-BSA was a better inhibitor than either Glc-GD-BSA or Glc-PD-BSA which lack this structure (see Chart I).

Earlier studies from this laboratory revealed that the 5-CH_2OH group of galactose is not needed for the binding and that there is enough room in the binding site of the lectin to accommodate an extra methyl group (-CH_2-O-CH_3). In the

Scheme I



present study we have extended this further and substituted the 6-OH of galactose with a nonbinding monosaccharide, *N*-acetylglucosamine. The BSA derivatives containing GlcNAcβ(1→6)Gal had good inhibitory power, indicating that the internal galactose residue substituted at C-6 must still be effectively bound by the lectin. It is well-known that the lectin does not bind native serum glycoproteins. Much of the galactose moieties in these glycoproteins are penultimate to a nonreducing end, *N*-acetylneuraminic acid, and a significant portion of *N*-acetylneuraminic acid is linked through the 6-OH of galactose to yield the NeuAcα(2→6)Gal structure. The three-dimensional relationship of the two sugar rings in NeuAcα(2→6)Gal and GlcNAcβ(1→6)Gal is quite similar, since the C_5^2 (abnormal) conformation of *N*-acetylneuraminic acid results in its α-glycosidic linkage to be equatorial to the sugar ring (Scheme I). It was puzzling therefore that the NeuAcα(2→6)Gal structure in native serum glycoproteins is apparently not bound by the lectin in spite of its resemblance to GlcNAcβ(1→6)Gal. Though *N*-acetylneuraminic acid is larger than *N*-acetylglucosamine, it seems unlikely that the presence of the exocyclic 3-carbon chain of *N*-acetylneuraminic acid would cause almost total loss of binding. I suspected that the negatively charged carboxylic acid on the *N*-acetylneuraminic acid was responsible for the drastic reduction in the binding. As shown in Scheme I, the location of the negative charge on galacturonic acid approximates that of NeuAcα-(2→6)Gal remarkably well. The fact that BSA derivatives containing galacturonic acid were very poor inhibitors, while BSA derivatives containing the methyl ester of galacturonic acid were nearly as inhibitory as BSA containing galactose, strongly suggests that the negative charge of *N*-acetylneuraminic acid does not allow the NeuAcα(2→6)Gal structure to be bound by the lectin. It is proposed that the negatively charged *N*-acetylneuraminic acid is repulsed by a certain negatively charged group in the binding site of the lectin. It remains to be seen whether the lectin repels the negative charge near C-6 of galactose or if it repels all the negatively charged molecules regardless of its location. Such a knowledge may allow us to understand whether one negatively charged group is responsible for both the repulsion of the negative charge and the aforementioned attraction of the amidino-linked BSA derivatives.

It is known that the lectin binds an α-galactosyl moiety and a β-galactosyl moiety equally well if the aglycon is of simple, linear structure (Connolly et al., 1982; Connolly, 1981). However, results using melibiose-AD-BSA's as inhibitors suggest that a bulky α substituent does interfere with the binding (Figure 3). The result is in agreement with the observation made by Sarkar et al. (1979) that melibiose was a considerably poorer inhibitor than lactose when inhibition of [^{125}I]ASOR binding to rabbit hepatic lectin immobilized on Sepharose was assayed. Another supportive piece of evidence is that BSA containing Galα(1→6)Gal was less inhibitory than BSA containing Galβ(1→6)Gal (Figure 3).

In the simultaneous inhibition assays (assays II and III), concentrations of [^{125}I]ASOR and the lectin were kept near or below the dissociation constant, so that I_{50} should approximate K_I (Cheng & Prusoff, 1973; Chang et al., 1975). The I_{50} value for ASOR was (3–5) × 10^{-9} M when the Triton-solubilized, purified lectin was used (assay III) (Connolly et al., 1982), while a 10-fold lower value [(2–5) × 10^{-10} M] was obtained when the membrane-bound lectin was used (assay II)[2] (Table II). Assay I, in which the membrane is first incubated alone with the inhibitor and then with [^{125}I]ASOR at near saturation concentration, depends on the fact that the binding of ASOR (and presumably other multivalent glycoproteins) to membrane-bound lectin is operationally irreversible as originally reported by Van Lenten & Ashwell (1972) and that the second incubation with [^{125}I]ASOR is to tag that portion of the lectin that had not been occupied by the inhibitor. If the latter assumption is correct, the I_{50} values obtained in this assay should be equal to K_D. The I_{50} value obtained by assay I for ASOR was (1.5–3.5) × 10^{-10} M, which is very similar to the I_{50} values of ASOR determined by assay II, suggesting that the sequential inhibition assay is an operationally valid method to determine K_D.

Qualitatively, the inhibition assays with membranes (assay I) and with purified, Triton-solubilized lectin (assay III) depicted similar sugar specificities of binding. Quantitatively, however, there are significant differences. For assessment of whether these differences are due to the way assays are conducted (sequential vs. simultaneous) or due to the state of the lectin (in the membrane vs. in the Triton-solubilized, purified form), the simultaneous inhibition assay (assay II) was conducted with the membranes using several neoglycoproteins as inhibitors. Figure 4 and Tables I and II indicate that the I_{50} values obtained in the two membrane assays were, in general, quite similar and much lower than the value obtained in the soluble lectin assay. The state of the lectin such as its quaternary organization in the membrane may be influencing the inhibitory potency of the neoglycoprotein inhibitors.

One common feature seen in the simultaneous assays (assays II and III) but not in the sequential assay (assay I) is the superiority of Gal-AI-BSA (and Glc-AI-BSA) over Gal-AD-BSA as an inhibitor. Equilibrium and kinetic studies of ASOR binding to the purified, Triton-solubilized lectin indicated that the dissociation of the lectin–ASOR complex by multivalent inhibitors (polyglycosyl derivatives) behaved anomalously, in that only a small portion of the complex dissociated with the expected rate while the remainder dissociated with a very slow rate (Connolly et al., 1981). Thus, it is reasonable that in the simultaneous assays the rate of the forward reaction (the lectin–ligand complex formation) of an inhibitor relative to that of [^{125}I]ASOR weighs heavily in determining the relative inhibitory potency of that inhibitor. The aforementioned phenomenon of attraction between the amidino group in the Glyc-AI-BSA and the lectin binding site may increase the rate of complex formation, so that at a comparable level of sugar substitution (mole per mole of BSA) Gal- and Glc-AI-BSA

[2] Lowering of the [^{125}I]ASOR concentration in assay II from ~3 × 10^{-10} M to ~3 × 10^{-11} M did not lower the I_{50} value significantly (I_{50} = 2 × 10^{-10} M).

behaved as better inhibitors than Gal-AD-BSA (which lacks this amidino linkage) in the simultaneous inhibition assays (assays II and III).

The same reasoning can also explain the large difference in the inhibitory potency exhibited by BSA derivatives containing Galβ(1→6)Gal depending on the assay methods (sequential vs. simultaneous). As shown in Figure 3 and Tables I and II, in the sequential assay (assay I) the I_{50} of the BSA derivative containing 30 residues of Galβ(1→6)Gal was ~100-fold lower than that of the BSA derivatives containing 30 residues of galactose, while in the simultaneous inhibition assays (assays II and III), the BSA derivative containing 30 residues of Galβ(1→6)Gal was only a slightly better inhibitor than the BSA derivatives containing 30 residues of galactose. It appears that given sufficient time both galactose residues in Galβ(1→6)Gal can bind to the lectin (assay I). However, the binding of the second galactose residue to the lectin may be so much slower than the first that the binding of the second galactose residue does not contribute much to the inhibition in the simultaneous inhibition assays.

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