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Articles

Specificity of Chicken Liver Carbohydrate Binding Protein†

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ABSTRACT: Chicken hepatic lectin was isolated with affinity chromatography by using neoglycoproteins of bovine serum albumin (BSA) to which *n* moles of glycosides has been attached by amidination ($Glyc_n$-AI-BSA) [Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3963] attached to Sepharose 4B. The same protein could be isolated from Man-, GlcNAc-, and Glc-AI-BSA–Sepharose columns and was identical with the protein previously reported [Kawasaki, T., & Ashwell, G. (1977) *J. Biol. Chem. 252*, 6536–6543]. The sugar specificity for binding to the isolated chicken hepatic lectin examined with $Glyc_n$-AI-BSA showed the order of potency for binding $Glyc_n$-AI-BSA to be D-GlcNAc > D-Glc, D-Man, L-Fuc > D-Gal, and the estimated K_i's for binding $GlcNAc_{36}$-AI-BSA, Glc_{37}-AI-BSA, Man_{33}-AI-BSA, and L-Fuc_{28}-AI-BSA were (6–20) × 10^{-11}, (2–3) × 10^{-8}, (3–9) × 10^{-8}, and 5 × 10^{-8} M, respectively. The binding requirements of the binding protein were studied with a wide variety of $Glyc_n$-BSA's with different sugars and aglyconic linkages, as well as simple sugars and glycosides. It was concluded that (1) GlcNAc is the most potent sugar for binding, (2) the requirement for C-2 substituents is flexible, (3) an equatorial OH group at C-3 and C-4 must be present, (4) the 5-CH_2OH group is not required for binding, (5) the lectin cannot accommodate a negative charge at C-6, and (6) D-Man and L-Fuc bind equally well. Unlike the mammalian hepatic lectin, the chicken hepatic lectin has little preference for the type of sugar to protein linkage group.

The Gal/GalNAc specific receptor found in mammalian liver is absent in avian species and presumably reptilian species (Regoeczi & Hatton, 1976; Lunney & Ashwell, 1976). Furthermore, an analogous receptor in chicken liver which binds GlcNAc[1]-terminated glycoproteins has been isolated, purified, and characterized to have an absolute specificity for GlcNAc (Kawasaki & Ashwell, 1977). The conclusion about the sugar specificity were based on results obtained by using sequentially deglycosylated derivatives of orosomucoid or other serum glycoproteins and with various blood group substances (Sikdu et al., 1983).

Previous studies of the mammalian Gal/GalNAc receptor using neoglycoproteins with various sugars and different linkages to protein have proved to be valuable probes contributing to the understanding of the specificity for binding to this lectin (Stowell et al., 1980; Lee, 1982). Likewise, similar studies have been done with the mammalian macrophage Man-binding system (Shepherd et al., 1981; Hoppe & Lee, 1982).

The specificity of the chicken liver receptor was studied by using simple sugars and glycosides as well as neoglycoproteins with different sugar to protein linking groups to evaluate the structural requirement of various sugars. Our results indicate that the specificity of the isolated receptor is broad and in addition to GlcNAc, Man, Glc, and L-Fuc derivatives can be bound by the isolated protein.

Experimental Procedures

Materials. The following tissues or materials were obtained from the indicated sources: adult chicken livers from Dover Poultry, Baltimore, MD; carrier-free $Na^{125}I$ from Amersham Corp.; Sepharose, Sephadex, and Triton X-100 from Sigma Chemical Co.; bovine serum albumin (BSA)[2] (Pentex, fraction

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[1] All sugars are of the D configuration in pyranoside form, unless otherwise specified.

[2] Abbreviations: BSA, bovine serum albumin; $Glyc_n$-AI-BSA, neoglycoproteins of BSA to which *n* moles of glycosides has been attached by amidination (Lee et al., 1976); HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; OR, orosomucoid; AGOR, asialoagalactoorosomucoid; AHOR, asialoagalactoahexosaminoorosomucoid; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; [I_{50}], concentration of an inhibitor that causes 50% inhibition.

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Table I: Amino Acid Composition of the GlcNAc Lectin from Chicken Liver[a]

amino acid	affinant used for purification			
	Man-AI-BSA	Glc-AI-BSA	AGOR[b]	AGOR[c]
Asp	8.4	8.2	8.8	8.7
Thr	4.9	4.2	4.5	4.3
Ser	10.2	10.6	9.2	11.1
Glu	9.7	9.2	12.6	12.1
Pro	2.6	2.8	2.6	2.4
Gly	6.2	6.2	6.1	5.3
Ala	6.1	5.9	6.1	5.3
Val	4.5	4.7	4.6	4.3
$^1/_2$-Cys	1.6	0.8	3.0	3.3
Met	1.7	2.1	1.9	1.9
Ile	3.2	3.1	3.1	2.9
Leu	11.0	11.3	10.2	10.1
Tyr	4.7	7.1	4.0	4.8
Phe	6.2	6.6	5.9	5.8
Trp	ND[d]	ND	4.9	3.9
Lys	5.7	5.4	4.2	4.3
His	2.9	2.9	2.0	2.4
Arg	6.5	7.4	6.0	6.8

[a] Protein samples (ca. 30 μg) were hydrolyzed with 6 N HCl for 24 h at 105 °C in sealed tubes. The hydrolysate was analyzed with a Beckman amino acid analyzer. The amino acid compositions of proteins (in residues per 100 residues) isolated with different affinants are compared. [b] Kawasaki & Ashwell (1977). [c] Drickamer (1981). [d] Not determined.

V) from Miles Laboratories, Inc.; HEPES, MES, and PIPES from Research Organics, Inc.; SDS, acrylamide, and protein standards for electrophoresis from Bio-Rad. All other chemicals used in this work were of the highest purity available commercially and were used without further purification. AGOR (4 Gal, 17 Man, and 28 GlcN) was provided by Dr. S. Roseman, The Johns Hopkins University. AHOR was kindly provided by Dr. G. Ashwell, National Institutes of Health, Bethesda, MD.

Preparation of Neoglycoproteins. BSA-derived neoglycoproteins of the amidino type ($Glyc_n$-AI-BSA), containing 2–44 mol of thioglycosides per mol of BSA, were prepared by established methods (Lee et al., 1976; Stowell & Lee, 1980). The neoglycoprotein preparations to be radioiodinated were purified by gel filtration on Sephadex G-150. Only the protein peak corresponding to the monomeric BSA derivative was used for experiments in which direct binding was measured. Neoglycoproteins prepared by reductive alkylation (Lee & Lee, 1980) (designated by AD-, PD-, GD-, and HD-) were made by Dr. Reiko Lee as previously described (Lee, 1982).

Protein Determination. The protein concentration of neoglycoprotein solutions was determined by the microbiuret method (Zamenof, 1957). Chicken liver binding protein in Triton X-100 was determined by a modification of the fluorescamine reaction using BSA as a standard (Stowell et al., 1978). This protein value so obtained was found to be 14% higher than the protein concentrations determined from the amino acid composition of the binding protein (Table I). The protein concentration of orosomucoid derivatives was determined by using $E^{1\%}_{278nm} = 8.9$ (Schmid, 1975).

Iodination of Protein. Neoglycoproteins or orosomucoid derivatives (10–50 μg) were iodinated with 1 mCi of $Na^{125}I$ by a modification of the procedure of Greenwood et al. (1963). The iodination mixture was chromatographed on a column (0.7 × 20 cm) of Sephadex G-25, and the radioactive material at the void volume was pooled. Recovery of protein after iodination and chromatography was estimated to be 60–70% as determined by the modified fluorescamine reaction (Stowell et al., 1978), and the specific radioactivity was 25–50 μCi/μg of protein. After a sample of the radioiodinated protein was taken for analysis, the solution was made to 0.1% (w/v) BSA and was stored at 4 °C and used within 2 weeks. For most experiments, the preparation was diluted with the appropriate unlabeled protein for the desired specific activity, but for those experiments using subnanomolar concentrations of labeled ligand, the iodinated protein was not diluted with unlabeled ligand. The iodination did not result in alteration of the ligand protein, shown as follows: when the protein was diluted with the corresponding unlabeled protein, binding remained constant on the basis of the combined quantity of labeled and unlabeled species.

Preparation of Affinity Materials. BSA–Sepharose was prepared according to the method of Stowell & Lee (1978). Typically, 5 mg of BSA was incorporated per mL of gel. Amidination was accomplished by adding BSA–Sepharose 4B in 0.25 M sodium borate (pH 8.5) to a 10-fold excess of sugar imidate to amino group of BSA (Lee et al., 1976). This mixture was shaken at room temperature for 3 h and then stored in the cold overnight. The beads were washed until the effluent was free of the sugar derivatives. Analysis of the sugar content in the prepared gel (Lee, 1972) showed that 20–30 mol of thioglycosides was coupled per mol of BSA under the described conditions. AGOR was coupled to CNBr-activated Sepharose 4B essentially as described (Kawasaki & Ashwell, 1977). The level of AGOR was approximately 1 mg/mL of gel.

Isolation of Binding Protein. The binding protein was isolated from fresh or frozen and thawed chicken liver by a modification of published procedures (Kawasaki & Ashwell, 1977; Tanabe et al., 1979). All operations were carried out at 4 °C. The buffers used in the isolation and purification were as follows: buffer A, 5 mM EDTA, 50 mM HEPES, and 0.1 mM PMSF, pH 7.8; buffer B, 0.1 M sodium acetate, 0.01 M EDTA, and 0.2 M NaCl, pH 5.4; buffer C, 0.01 M HEPES, 0.4 M KCl, and 2% (w/v) Triton X-100, pH 7.8; buffer D, 0.01 M HEPES, 1 M NaCl, 0.05 M $CaCl_2$, and 0.5% (w/v) Triton X-100, pH 7.8; buffer E, 0.04 M sodium acetate, 1.25 M NaCl, 0.01 M EDTA, and 0.5% (w/v) Triton X-100, pH 5.4. Chilled livers (200 g wet weight) were minced and homogenized in a 2.5-fold volume of buffer A, filtered through four layers of cheese cloth, and mixed with cold acetone (−20 °C) while being stirred to attain a final concentration of 90% (v/v). The precipitated material was collected on Whatman 1 filter paper under mild suction. The cake was dispersed in cold acetone and filtered, and this operation was repeated again. The final precipitate was air-dried and suspended in 1 L of buffer B. The suspension was gently stirred at 4 °C for 30 min and then centrifuged at 23000*g* for 15 min. The process was repeated twice in buffer B and then in distilled water. The final pellet was suspended in 1 L of buffer C, gently stirred in the cold for 30 min, and centrifuged, and the supernatant was made 50 mM in $CaCl_2$ and the pH adjusted to 7.8 with 0.1 M NaOH. A suspension of affinity gel (40 mL of settled gel per 300 mL of extract) in buffer D was shaken with the extract at 4 °C overnight. The gel was allowed to settle, and the supernatant was decanted. The settled beads were poured into a column and washed with buffer D until free of fluorescamine-positive materials (about 5–10 column volumes). The column was eluted with buffer E, and the effluent was assayed for binding activity (see below). The binding protein appeared between the first and second column bed volumes. The fractions containing binding protein were pooled, made to 0.02% with sodium azide and 0.1 mM PMSF, and stored at 4 °C. In some preparations, the pooled effluent was readjusted to pH 7.8, made to 50 mM

in $CaCl_2$, and chromatographed on a smaller affinity column containing 10 mL of gel. The yield of binding protein was 3–5 mg/100 g of liver.

SDS–PAGE of Purified Binding Protein. Protein samples were heated at 100 °C for 5 min in 63 mM Tris (adjusted to pH 6.8 with HCl), 0.3% (w/v) SDS, 10% (w/v) glycerol, and 5% (v/v) β-mercaptoethanol. Bromophenol blue was added to the samples (0.1 mg/mL final concentration) and was electrophoresed in a slab of 10% polyacrylamide separating gel with a 5% stacking gel as described by Laemmli (1970). Gels were stained with Coomassie blue-G-250 (Fairbanks et al., 1971).

Standard Activity Assay for Soluble Binding Protein. The standard binding assay was a modification of the procedure of Hudgin et al. (1974). ^{125}I-Labeled ligand was incubated with purified binding protein or extract in a 0.5-mL mixture containing 0.2% (w/v) Triton X-100, 0.6% BSA, 50 mM HEPES, pH 7.8, 1 M NaCl, and 50 mM $CaCl_2$ (incubation buffer). After being shaken for 15 min at 25 °C, the mixture was chilled in an ice bath, and the bound ligand was precipitated by adjusting to 50% saturated ammonium sulfate. The mixture was stored for 10 min on ice, and the precipitate was collected on Whatman GF/C filter disks (2.4 cm in diameter) under suction and washed 3 times with an ice-cold solution of 40% saturated ammonium sulfate, pH 7.8, 10 mM in $CaCl_2$, containing 0.1% (w/v) BSA. The filter disks were air-dried, wrapped in a 2-in.^{2} piece of Parafilm, and counted for radioactivity in a Packard PRIAS PGD auto-γ counter.

Nonspecific precipitation was assessed by incubating the ^{125}I-labeled ligand in the absence of binding protein or was determined as the extrapolated value at zero protein concentration. Blank values so obtained were less than 1% of the total radioactivity added for ^{125}I-orosomucoid derivatives but were considerably higher (between 2 and 10%) for the ^{125}I-neoglycoproteins.

Measurement of [I_{50}] Values for Inhibition of the Chicken Liver GlcNAc-Binding Protein. The affinity of various carbohydrates and neoglycoproteins for the purified chicken liver GlcNAc-binding protein was determined by measuring the bound ligand (^{125}I-AGOR) at different concentration of inhibitors. A similar inhibition assay has been described (Connolly et al., 1982; Lee et al., 1982). Briefly, an inhibitor (in the concentration range of 10^{-12}–10^{-5} M for neoglycoproteins and 10^{-6}–10^{-1} M for sugars), ^{125}I-AGOR (approximately 0.1 nM), and purified liver GlcNAc-binding protein (about 0.1 nM, assuming M_r 210 000) were incubated at 25 °C for 45–60 min in the incubation buffer. Bound ^{125}I-AGOR was precipitated with 50% saturated ammonium sulfate and processed as described for the standard activity assay measurement. For each test compound, the precentage of inhibition was plotted vs. the logarithm of the inhibitor concentration to yield sigmoidal curves. The concentration of inhibitor causing 50% inhibition of ^{125}I-AGOR binding was determined from the curve. Since this assay was conducted with labeled ligand and binding protein at concentrations about one-tenth the K_D value, these [I_{50}] values should closely approximate the K_D, assuming a bimolecular, reversible binding reaction (Chang et al., 1975).

Results

Purity of Isolated Binding Proteins from Liver. The binding proteins isolated on AGOR–Sepharose and the neoglycoprotein affinity columns were assessed for purity by performing polyacrylamide gel electrophoresis of the proteins under reducing conditions in the presence of SDS (Figure 1). The results show that under these conditions all four preparations had a major protein band corresponding to a molecular weight of 27 000.

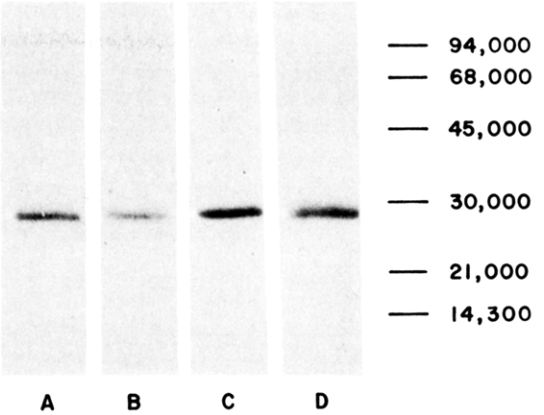

FIGURE 1: SDS–PAGE of liver binding proteins. Twenty micrograms of each binding protein isolated after chromatography on Man-AI-BSA–Sepharose (A), GlcNAc-AI-BSA–Sepharose (B), Glc-AI-BSA–Sepharose (C), and AGOR–Sepharose (D) in buffer E was denatured and subjected to SDS slab gel electrophoresis at 20 °C for 2 h at 20 mA per slab through the 5% stacking gel and for 5 h at 25 mA through the 10% separating gel (Laemmli, 1970). The gel was fixed and stained according to the procedure of Fairbanks (Fairbanks et al., 1971). A standard mixture containing 5 μg each of phosphorylase *b* (M_r 94 000), BSA (M_r 68 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 21 000), and lysozyme (M_r 14 300) was denatured and electrophoresed in the same slab gel as the binding protein.

Amino Acid Composition of Isolated Chicken Hepatic Lectin. The amino acid composition of the binding proteins isolated from Man-AI-BSA–Sepharose and Glc-AI-BSA–Sepharose and published data for AGOR–Sepharose-isolated proteins are shown in Table I. There are few differences among the preparations. With the exception of tryptophan which was not determined for the "Man-binding protein" and the "Glc-binding protein", the largest deviations from the data published for the liver "AGOR-binding protein" were that the tyrosine content for the Glc-binding protein is 1.8 times the published value and the number of half-cystine residues in the Glc-binding protein is one-fourth of the published value. All of the amino acid composition data are generally in good agreement with the theoretical amounts as determined from the complete amino acid sequence analysis (Drickamer, 1981) of the chicken GlcNAc receptor.

Binding of ^{125}I-Orosomucoid Derivatives and ^{125}I-Neoglycoproteins to the Isolated Chicken Hepatic Lectin. Figure 2 shows that ^{125}I-AHOR binds to the isolated chicken liver protein, contrary to an earlier report indicating that AHOR was inactive in this system (Kawasaki & Ashwell, 1977). These results implied that the chicken liver binding protein recognized Man-terminated glycoproteins (AHOR) in addition to GlcNAc-terminated glycoproteins (AGOR).

The ability of the chicken liver GlcNAc-binding protein to recognize Man was tested in a more direct manner by using neoglycoproteins prepared by attaching GlcNAc, Man, Glc derivatives to BSA. When increasing amounts of GlcNAc-binding protein were added to a large excess of neoglycoproteins, increasing amounts of neoglycoproteins were bound to the binding protein (Figure 3). Equal amounts of ^{125}I-$GlcNAc_{36}$-, ^{125}I-Man_{33}-, or ^{125}I-Glc_{37}-AI-BSA were bound to the binding protein, despite their difference in binding affinity (see below). Binding affinities of these and other potential ligands were assessed with an inhibition assay. Because the binding affinity of ^{125}I-AGOR to the purified binding protein has been previously characterized and because ^{125}I-AGOR has

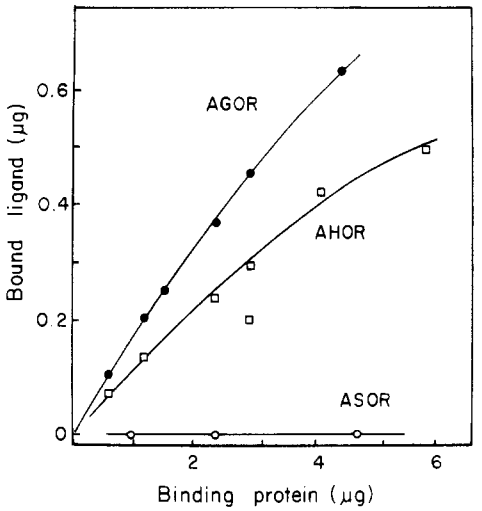

FIGURE 2: Binding of orosomucoid derivatives to the chicken hepatic lectin. Purified chicken liver binding protein (0.6–6.2 μg) and 1 μg of ^{125}I-ASOR (○), ^{125}I-AHOR (□), or ^{125}I-AGOR (●) were incubated for 15 min at 25 °C in the standard assay as described under Experimental Procedures. Specifically bound ligand was determined after precipitation with cold 50% saturated ammonium sulfate. Nonspecific precipitation was determined as the extrapolated value at zero protein concentration.

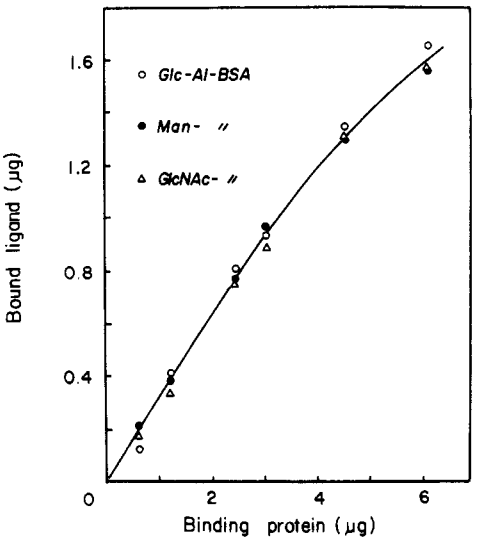

FIGURE 3: Binding of ^{125}I-neoglycoproteins to chicken hepatic lectin. Purified chicken liver binding protein (0.3–6 μg) and 1 μg of ^{125}I-GlcNAc$_{36}$-AI-BSA (△), 20 μg of ^{125}I-Man$_{33}$-AI-BSA (●), or 5 μg of ^{125}I-Glc$_{37}$-AI-BSA (○) were incubated for 15 min 25 °C in the standard binding assay as described under Experimental Procedures. Specifically bound ligand was determined after precipitation with cold 50% saturated ammonium sulfate. Nonspecific precipitation was determined as the extrapolated value at zero protein concentration.

relatively low nonspecific precipitation in the ammonium sulfate assay, it was chosen as the reference labeled ligand with which to measure inhibition.

Using the conditions described in Figure 4, we obtained the following concentrations of inhibitor which produced 50% inhibition ([I_{50}]): 2.2×10^{-10} M for GlcNAc$_{36}$-AI-BSA, 2.7×10^{-8} M for Man$_{28}$-AI-BSA, 1.8×10^{-8} M for Glc$_{37}$-AI-BSA, and 4.7×10^{-8} M for L-Fuc$_{28}$-AI-BSA. The [I_{50}] value for AGOR inhibition of ^{125}I-AGOR was found to be 4×10^{-10} M (Table II) which is close to the K_D value of 7.1×10^{-10} M determined by the direct binding method (see below).

When the binding proteins purified with different affinants (i.e., GlcNAc-, Man-, or Glc-AI-BSA) were compared with respect to inhibition of AGOR binding by AGOR, GlcNAc$_{36}$-AI-BSA, Glc$_{37}$-AI-BSA, Man$_{33}$-AI-BSA, and L-Fuc$_{28}$-AI-BSA, the [I_{50}] values were very close to each other (Table II).

Similarly, the inhibitory power of free sugars and simple glycosides was tested, and the [I_{50}] values are shown in Table

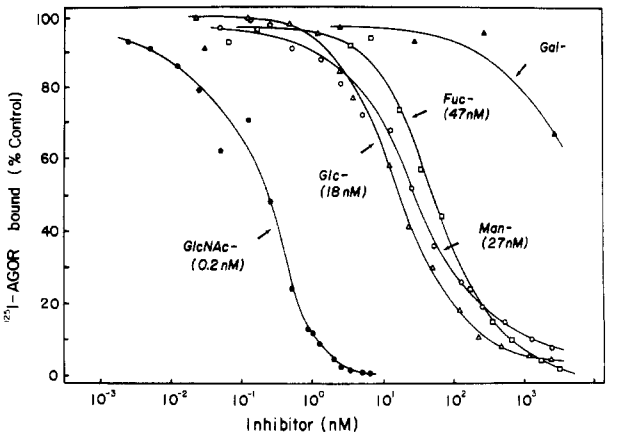

FIGURE 4: Inhibition of ^{125}I-AGOR binding to isolated chicken hepatic lectin. ^{125}I-AGOR (1.5 ng), GlcNAc-binding protein (7 ng), and inhibitor (up to 850 nM) were incubated in the standard assay mixture for 1 h at 25 °C. Bound ^{125}I-AGOR was measured by precipitation in 50% saturated ammonium sulfate as described under Experimental Procedures. The binding of ^{125}I-AGOR in the absence of added inhibitor is referred to as 100%.

Table II: Inhibition of ^{125}I-AGOR Binding to the Chicken Liver Lectin Prepared with Different Affinants[a]

	affinant used for purification		
inhibitor	AGOR	GlcNAc-AI-BSA	Man-AI-BSA
AGOR	4×10^{-10}	4×10^{-10}	4×10^{-10}
GlcNAc$_{36}$-AI-BSA	7×10^{-11}	2×10^{-10}	6×10^{-11}
Man$_{33}$-AI-BSA	7×10^{-8}	3×10^{-8}	9×10^{-8}
Glc$_{37}$-AI-BSA	3×10^{-8}	2×10^{-8}	3×10^{-8}
Fuc$_{28}$-AI-BSA	7×10^{-8}	5×10^{-8}	5×10^{-8}

[a] The binding assays were performed as described in Figure 4, and the concentration of inhibitor required to produce 50% inhibition of ^{125}I-AGOR binding, [I_{50}] (in molar units), was obtained from the inhibition curves.

Table III: Inhibition of ^{125}I-AGOR Binding to Chicken Liver Lectin by Sugars and Simple Glycosides[a]

	affinant used for purification	
inhibitor	AGOR	Man-AI-BSA
Me α-D-GlcNAc	2×10^{-5}	
Me β-D-GlcNAc	6×10^{-5}	8×10^{-5}
D-GlcNAc	6×10^{-5}	8×10^{-5}
Me α-D-Man	1×10^{-2}	1×10^{-2}
Phe α-D-Man	7×10^{-3}	
Me β-D-Man	8×10^{-3}	
D-Man	6×10^{-3}	1×10^{-2}
Me α-D-Glc	6×10^{-3}	
Me β-D-Glc	1×10^{-2}	2×10^{-2}
Me β-6-deoxy-D-Glc	1×10^{-2}	
D-Glc	7×10^{-3}	1×10^{-2}
Me α-D-Gal	3×10^{-1} (35%)	
Me β-D-Gal	3×10^{-1} (35%)	3×10^{-1} (17%)
D-Gal	7×10^{-2}	7×10^{-2}
D-GalNAc	2×10^{-1} (27%)	
D-ManNAc		6×10^{-3}
Me α-L-Fuc	1×10^{-2}	9×10^{-3}
L-Fuc	8×10^{-3}	2×10^{-2}

[a] Two binding protein preparations obtained with two different affinity columns (AGOR–Sepharose and Man-AI-BSA–Sepharose) are compared. The binding assays were performed as described in Figure 4, and the concentration of inhibitor required to produce 50% inhibition of ^{125}I-AGOR ([I_{50}], in molar units), except where noted, is tabulated.

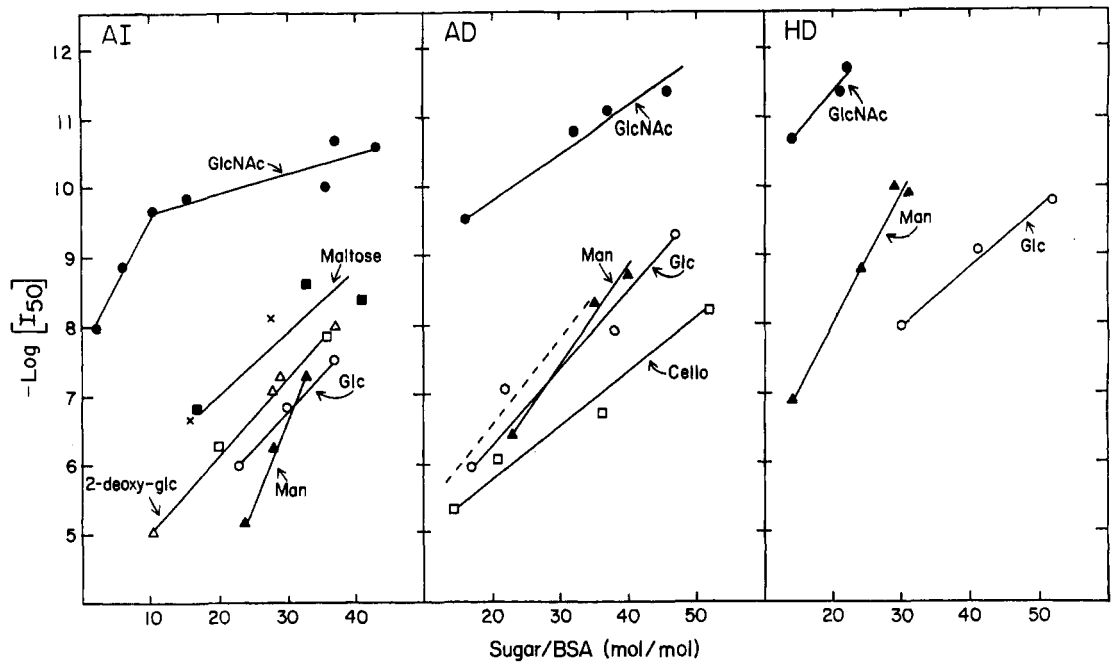

FIGURE 5: Effect of sugar density on the $[I_{50}]$ value. The inhibition assay was performed as described in Figure 4. The concentrations of neoglycoprotein which produced 50% inhibition of ^{125}I-AGOR binding were designated by the $[I_{50}]$ value. The negative logarithm of the $[I_{50}]$ value in molar vs. the moles of sugar per mole of BSA is plotted. (AI) Neoglycoproteins of the Glyc-AI-BSA series: (●) GlcNAc; (○) Glc; (▲) Man; (△) 2-deoxy-Glc; (■) maltose; (×) cellobiose. (AD) Neoglycoproteins of the Glyc-AD-BSA series (symbols as in AI). (HD) Neoglycoproteins of the Glyc-HD-BSA series (symbols as in AI).

III. The methyl glycosides were not significantly better inhibitors than the free sugars. Although the methyl α-glycoside of GlcNAc was a 3-fold better inhibitor than its β anomer, discrimination of anomers by the binding protein is practically negligible for glycosides of other sugars. A recent study conducted with immobilized chicken hepatic lectin (Sikdu et al., 1983) also indicated a preference for the α-glycoside of GlcNAc.

Effect of Sugar Density and Linking Arm Structure on Binding Affinity. The relative binding affinity of the appropriate neoglycoprotein derivatives increases dramatically as the number of sugar residues increases (Figure 5). These results are similar to those obtained with the purified rabbit liver binding protein (Stowell et al., 1980; Lee, 1982). In contrast to the Gal/GalNAc lectin of rabbit liver, which showed a preference for the amidino linkage (Kawaguchi et al., 1981), the avian hepatic binding protein showed no such effect (Figure 5). Figure 5 also shows the effect of increasing length of arm. The $[I_{50}]$ value decreases slightly with increasing length of arm. For both GlcNAc and Glc derivatives, the HD derivatives (the longest linkage arm) were better than the AD derivatives, which in turn are better than the AI derivatives (the shortest linkage arm).

Stoichiometry of Binding. The stoichiometry of binding of ^{125}I-AGOR and ^{125}I-neoglycoproteins to various preparations of isolated chicken liver binding protein is summarized in Table IV. Assuming that the molecular weight of the binding protein is 210 000 (Kawasaki & Ashwell, 1977), the stoichiometry of binding is 0.7–1.0 mol of ^{125}I-AGOR bound per mol of binding protein for the various preparations and 1.1–2.2 mol of ^{125}I-neoglycoprotein per mol of binding protein. However, within any one preparation, there are no significant differences between the binding of $GlcNAc_{36}$-AI-BSA, Man_{33}-AI-BSA, and Glc_{37}-AI-BSA.

Table IV: Stoichiometry of Binding of ^{125}I-AGOR and ^{125}I-Neoglycoproteins to Purified Chicken Liver Binding Protein[a]

affinant[c] for affinity chromatography	^{125}I-ligand[b]			
	AGOR	$GlcNAc_{36}$-AI-BSA	Man_{33}-AI-BSA	Glc_{37}-AI-BSA
AGOR	0.7 ± 0.08[d]	1.6 ± 0.09	1.4 ± 0.04	1.1 ± 0.07
GlcNAc-BSA	0.8 ± 0.06	2.0 ± 0.09	1.7 ± 0.06	1.8 ± 0.21
Man-BSA	1.02 ± 0.02	2.1 ± 0.12	2.2 ± 0.11	1.7 ± 0.14
Glc-BSA	0.9 ± 0.06	2.0 ± 0.10	1.4 ± 0.25	1.8 ● 0.08

[a] Binding protein (in three different concentrations ranging from 0.1 to 1.7 μg) was incubated in the standard assay with 1 μg of ^{125}I-AGOR or 7 μg of each ^{125}I-neoglycoprotein. [b] Assumed molecular weights are the following: AGOR, 36 000; $GlcNAc_{36}$-AI-BSA, 69 000; binding protein, 210 000. [c] Compounds attached to Sepharose 4B. [d] Data are expressed as mean ± standard deviation.

Discussion

The finding (Kuhlenschmidt & Lee, 1980) that the purified GlcNAc-binding protein from chicken liver recognizes Man- and Glc-modified BSA's was surprising on two accounts. First, chicken hepatocytes failed to bind to Man- or Glc-derivatized polyacrylamide gels (Schnaar et al., 1978). Second, Kawasaki & Ashwell (1977) reported that the solubilized receptor from whole liver showed absolute specificity for glycoprotein derivatives with exposed GlcNAc residues rather than Man residues. Examination of the sugar specificity for binding to the purified receptor using sequentially deglycosylated derivatives of orosomucoid revealed that contrary to the earlier reports (Kawasaki & Ashwell, 1977) both the asialoagalacto derivative (AGOR) and the asialoagalactoahexosamino derivative (AHOR) were bound (Figure 2). This latter finding has since been confirmed (Harford & Ashwell, 1982). In view of the heterogeneity of deglycosylated glycoproteins, it was decided to use neoglycoproteins of defined sugar composition and structure (Lee & Lee, 1982) to answer this question. The isolated protein could bind a synthetic glycoprotein bearing "*S*-glycosides", as anticipated from the previous reports of chicken hepatocyte adhesion to gels containing *O* or *S*-glycosides of GlcNAc derivatives (Schnaar et al., 1978).

A convenient method for assessing the relative binding affinities of various ligands is to use the ligands to inhibit binding of a labeled reference ligand. Under conditions pre-

Table V: Summary of Sugar Inhibition of ^{125}I-AGOR Binding to Chicken Liver GlcNAc-Binding Protein

sugar or sugar derivative[c]	$[I_{50}]^a$ (M)	sugar or sugar derivative[c]	$[I_{50}]^a$ (M)
$GlcNAc_{36}$-AI-BSA	1×10^{-10}	$GalNAc_{37}$-AI-BSA	$>2 \times 10^{-6}$ [b]
Glc_{37}-AI-BSA	2×10^{-8}	L-Ara_{36}-AI-BSA	$>2 \times 10^{-6}$ [b]
Man_{33}-AI-BSA	5×10^{-8}	Lac_{34}-AI-BSA	$>1 \times 10^{-6}$ [b]
L-Fuc_{28}-AI-BSA	6×10^{-8}	Mel_{29}-AI-BSA	$>1 \times 10^{-6}$ [b]
2-deoxy-Glc_{37}-AI-BSA	1×10^{-8}	GlcNAc	6×10^{-5}
		Glc	7×10^{-3}
Xyl_{37}-AI-BSA	2×10^{-7}	Man	6×10^{-3}
Mal_{41}-AI-BSA	4×10^{-9}	L-Fuc	8×10^{-3}
Cel_{36}-AI-BSA	1×10^{-8}	ManNAc	6×10^{-3}
All_{25}-AI-BSA	$>1 \times 10^{-6}$ [b]	GalNAc	$>1 \times 10^{-1}$ [b]
3-*O*-Me-Glc_{30}-AI-BSA	$>1 \times 10^{-5}$ [b]	Gal	$>1 \times 10^{-3}$ [b]
		Galβ(1→3)GlcNAc	$>1 \times 10^{-3}$ [b]
$(Man6P)_{47}$-AD-BSA	$>2 \times 10^{-6}$	Galβ(1→6)GlcNAc	4×10^{-4}
		Galβ(1→4)GlcNAc	$>1 \times 10^{-3}$ [b]
D-Fuc_{29}-AI-BSA	$>6 \times 10^{-6}$ [b]		

[a] Measurements as described in Figure 4. [b] Indicates that <50% inhibition was seen at this concentration. [c] Abbreviations: Mal, maltose; Cel, cellobiose; All, allose; Lac, lactose; Mel, melibiose.

scribed by Jacobs et al. (1975) and Chang et al. (1975) in which the concentrations of binding sites and ligand are 10% or less of the dissociation constant of the binding protein–ligand complex, the concentration of inhibitor which decreases binding by 50% is approximately equal to the K_I of the inhibitor. The validity of this approach was tested by fitting some sets of inhibition data with a nonlinear regression program, SCAFIT (Munson & Rodbard, 1980). Inhibition of ^{125}I-AGOR by unlabeled AGOR yielded a K_D of 3.2×10^{-10} M with the SCAFIT program, while the $[I_{50}]$ value for AGOR by the aforementioned graphic method was 4.0×10^{-10} M. Dissociation constants of some other neoglycoprotein inhibitors obtained by SCAFIT analysis also lie within the experimental range of $[I_{50}]$ values determined graphically. These data also support the validity of the one-site model used in the calculation.

From the results summarized in Table V, a number of generalizations can be made. (1) The most potent neoglycoproteins were GlcNAc-BSA derivatives, the best of which was nearly 10-fold more potent than AGOR. (2) Because Glc and Man can inhibit (albeit with lower affinity), it can be concluded that the requirement for C-2 substituents is flexible. The 2-OH can be equatorial (glucose), axial (mannose), or absent (2-deoxyglucose). The presence of the equatorial acetamido group at C-2, on the other hand, enhances binding tremendously (GlcNAc). However, the axial 2-acetamido group (ManNAc) does not have the enhancing effect. (3) No significant binding of allose AI-BSA (3-*O*-MeGlc-AI-BSA) or a disaccharide, Galβ(1→3)GlcNAc, was observed, indicating the importance of the 3-OH group. (4) Equatorial C-4 is extremely critical. No binding occurs if the group is axial (Gal) even if there is an equatorial acetamido group (GalNAc) at C-2. Substitution at this position is not tolerated as evidenced by the nonbinding of the nonbinding or Galβ(1→4)-GlcNAc, lactose and ASOR (Kawasaki & Ashwell, 1977). (5) The presence of the 5-CH_2OH group is not critical for binding, because Xyl_{37}-AI-BSA binds nearly as well as Glc_{37}-AI-BSA. (6) Some modification of C-6 of GlcNAc is tolerated [Galβ(1→6)GlcNAc], but the binding is reduced. However, modification at C-6 of mannose with a phosphate group is incompatible with binding. (7) L-Fuc and D-Man bind equally well. The hydroxyl groups at C-2(equatorial), C-3-(equatorial), C-4(axial), and the ring oxygen of L-Fuc can be superimposed on C-4(equatorial), C-3(equatorial), and C-2-(axial) or D-Man. If the binding protein does not have specific requirements for substituents at C-1 and C-5 of Man (or C-5 and C-1 of L-Fuc), then Man and L-Fuc would appear similar to the binding protein (Lee & Lee, 1982). In fact, simple glycosides of the various sugars were no more effective than the reducing sugars, and the anomeric configuration appears to be relatively unimportant (Table III). The recognition of L-Fuc and D-Man by the same receptor is not unprecedented. In addition to mammalian macrophages and purified liver Man-binding proteins which also bind L-Fuc (Shepherd et al., 1981), there is also a lectin which binds both L-Fuc and D-Man (Kameyama et al., 1979).

The specificity of the chicken hepatic lectin is closely parallel to the sugar binding specificities for the alveolar macrophage (Hoppe & Lee, 1982) or the purified mannan-binding protein in mammalian liver (Kawasaki et al., 1978), but unlike the mammalian species which do not distinguish between the Man and GlcNAc derivatives, the chicken liver protein clearly preferred the GlcNAc derivatives.

As in the mammalian hepatocytes or alveolar macrophages, the preference of the receptor for a multivalent ligand is quite pronounced. While the $[I_{50}]$ value for the most potent monosaccharide (GlcNAc) was 10^{-5}–10^{-4} M, the $[I_{50}]$ value for $GlcNAc_{44}$-AI-BSA was 10^{-10} M. This effect was seen (Figure 5) for other binding sugars and was also independent of the structure of the linkage group.

There are many other similarities between the mammalian Man/GlcNAc lectins and the chicken hepatic lectin. First, they both require calcium for binding. Second, only the terminal sugars, not the internal sugars, are directly participating in the binding, since neoglycoproteins with only monosaccharides attached to peptide side chains are excellent ligands. Another similarity is the effect of C-5 substituents. These lectins require no C-5 substituents, and both can accommodate bulky substituents at C-5, but neither can tolerate negative charges at this position. There are, however, some differences between the mammalian Man/GlcNAc lectin and the chicken hepatic lectin. The binding of Glc derivatives by the chicken hepatic lectin is not dependent on the presence of an amidino group in the aglycon as in the case of mammalian hepatic lectin (Lee, 1982). The effect of the length of the aglycon is much more pronounced for the chicken hepatic lectin. This can be due to either greater flexibility of the "long-armed" glycosides or the hydrophobic effect suggested by Sikdu et al. (1983).

The possibility existed that the isolated chicken liver binding protein preparations contained separate protein(s) which preferred Man- and/or Glc-terminated glycoconjugates. This seems unlikely because the chicken liver receptor purified with affinity chromatography on GlcNAc-, Man-, or Glc-AI-BSA–Sepharose resulted in the same protein as judged by the following criteria: (1) SDS–PAGE (Figure 1) showed that each of the proteins purified with different affinity columns was comprised of a single band (Coomassie blue staining), corresponding to M_r 27 000, in close agreement with the molecular weight of 26 000 reported previously (Kawasaki & Ashwell, 1977). (2) Amino acid compositions (Table I) of the affinity-purified proteins were comparable to those previously reported for AGOR–Sepharose-isolated protein (Kawasaki & Ashwell, 1977), as well as with the composition computed from the complete amino acid sequence of the chicken liver protein (Drickmer, 1981). (3) With high doses of ligand, the stoichiometry of binding AGOR or BSA derivatives was the same for the different binding protein preparations (Table IV). (4) GlcNAc-BSA could inhibit the

binding of either Man- or Glc-BSA's. (5) Antisera to the AGOR-purified protein reacted with the Man, Glc, and GlcNAc affinity-isolated proteins (Kuhlenschmidt, 1983).

Because the binding protein binds most tightly to GlcNAc derivatives, it may be expected that the major physiological function of the receptor would involve the GlcNAc moiety as the recognition signal. The finding that other sugars (i.e., Man, Glc, and L-Fuc) can also be effective ligands for this lectin suggests that, given the right conditions, naturally occurring Man-, L-Fuc, or Glc-containing ligands could interact with the receptor and thus be important biologically. An unresolved question is that this apparent counterpart of the mammalian Gal/GalNAc binding protein has a dramatically different sugar specificity. Whatever functions are speculated for the mammalian hepatic sugar receptor [such as translocation of IgA (Stockert et al., 1982) or intracellular translocation of glycosylation intermediates], they are also plausible in the chicken, which in turn suggest that the chicken glycoproteins may have a different terminal structure in the oligosaccharide chains. The recent finding that ovomucoid contains many GlcNAc-terminated oligosaccharides (Yamashita et al., 1983) suggests that such a possibility may exist.

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