Binding of N-Linked Bovine Fetuin Glycopeptides to Isolated Rabbit Hepatocytes: Gal/GalNAc Hepatic Lectin Discrimination between Galβ(1,4)GlcNAc and Galβ(1,3)GlcNAc in a Triantennary Structure†

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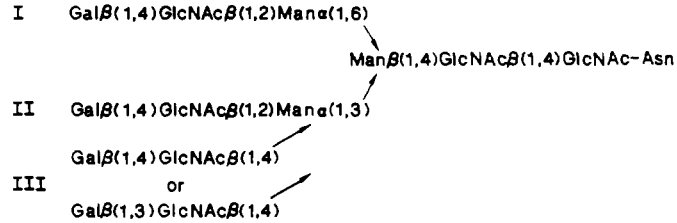
ABSTRACT: Glycopeptides were isolated from bovine fetuin after digestion with Pronase, aminopeptidase M, and carboxypeptidase Y. The glycopeptides were derivatized with *tert*-butyloxycarbonyltyrosine and separated on the basis of peptide by using reverse-phase high-performance liquid chromatography. Using 400-MHz ^{1}H NMR, the asialotriantennary oligosaccharides at each of the three N-linked glycosylation sites were found to be combinations of the following two structures in which the third branch is either Galβ(1,4)GlcNAc or Galβ(1,3)GlcNAc:



The asialotriantennary glycopeptides containing all β(1,4)-lactosamine as the branches were designated Galβ(1,4)GlcNAc-TRI while triantennary glycopeptides containing β(1,3)-lactosamine as branch III were termed Galβ(1,3)GlcNAc-TRI. The Galβ(1,3)GlcNAc unit was localized predominantly to the branch III arm on the basis of a downfield shift (−0.027 ppm) in the H-1 and upfield shift (0.01 ppm) in the NAc methyl signals from the branch III GlcNAc resulting from Galβ(1,3) instead of Galβ(1,4) substitution. Revised assignments are proposed for the H-1's of Gal residues 6 (δ 4.464) and 8 (δ 4.471) [Vliegenthart, J. F. G., Dorland, L., & van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–373] in a Galβ(1,4)GlcNAc-TRI. The proportion of Galβ(1,3)GlcNAc-TRI glycopeptides from the Asn-Asp, Asn-Gly, and Asn-Cys sites was found to be 40%, 60%, and 20%, respectively. Analysis of the binding of these glycopeptides, containing from 20% to 60% Galβ(1,3)GlcNAc as branch III, to rabbit hepatocytes revealed that the greater the proportion of Galβ(1,3)GlcNAc, the lower the affinity of the mixture. The K_d for Galβ(1,4)GlcNAc-TRI was found to be between 3.6 and 5.4 nM (P = 0.10) with a mean of 4.4 nM from binding data analyzed by using the LIGAND program [Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239] and computer simulations of the binding of two ligands as a mixture to one receptor site. The K_d of Galβ(1,3)GlcNAc-TRI oligosaccharide, prepared by hydrazinolysis, was found to be 305 nM from inhibition studies.

The hepatic Gal/GalNAc lectin binds *N*-acetyllactosamine-type oligosaccharides over a millionfold affinity range from K_d = 1 nM to K_d = 1 mM (Lee et al., 1983). In addition to the well-recognized specificity for the monosaccharides Gal and GalNAc (Sarkar et al., 1979; Lee et al., 1982), we have shown that the affinity can be affected by (a) the number of Gal residues per oligosaccharide, (b) the linkage positions of the branches to the trimannosyl core, and (c) the number of monosaccharides in each antenna (Lee et al., 1983). The inter-Gal distances (Bock et al., 1982), which are related to the above and other structural features, have been correlated with the binding affinity (Lee, Y. C., et al., 1984). NONA I, a synthetic ligand in which the branch III lactosamine (see Figure 3 for branch numbering) is β(1,4)-linked to the α-(1,3)-linked Man, has an affinity of K_d = 1 nM for the mammalian hepatic Gal/GalNAc lectin (Lee et al., 1983). The Gal residues of one of the minimal energy conformers of NONA I are arranged at the vertices of a triangle with sides of dimensions 15, 22, and 25 Å (Bock et al., 1982). We have designated the above branching pattern as a type 1 TRI[1] in contrast to a type 2 TRI carbohydrate (represented by NONA II) in which the branch III lactosamine is β(1,6)-linked to the

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[1] Abbreviations: ASOR, asialoorsomucoid; TRI, asialotriantennary glycopeptide with a type 1 lactosamine branching pattern (for structure, see Figure 3); GP, glycopeptide; BSA, bovine serum albumin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; *t*-BOC, *tert*-butyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid; K_d, equilibrium dissociation constant; R, concentration of ligand binding sites; N, nonspecific binding parameter used in the LIGAND program (Munson & Rodbard, 1980); HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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α(1,6)-linked Man. The importance of the arrangement of the lactosamine units around the internal mannoses is evident from the 50-fold lower affinity of the type 2 TRI for the Gal/GalNAc lectin on the surface of isolated hepatocytes compared to the type 1 TRI, which displays the highest affinity on a per Gal basis of ligands so far described (Baenziger & Fiete, 1980; Lee et al., 1983). To correlate further the effect of the above structural features of triantennary carbohydrates with binding and subsequent cellular events in hepatic lectin mediated endocytosis, we undertook to prepare substantial quantities of type 1 triantennary glycopeptides.

Bovine fetuin is reported to be a rich source of N-linked triantennary carbohydrates (Baenziger & Fiete, 1979; Nilsson et al., 1979). However, these two studies proposed different attachment sites for the branch III lactosamine approximating type 2 and type 1 triantennary structures, respectively. Subsequently, the presence of only a type 1 structure, as proposed by Nilsson et al. (1979) was supported by using Smith degradation coupled with methylation analysis (Kruis & Finne, 1981). During the course of the preparation of TRI from fetuin, we found it to be less tightly bound by rat or rabbit hepatocytes than type 1 TRI (NONA I). Analysis by 400-MHz ^{1}H NMR of fetuin glycopeptides, compared to previous spectral findings (Carver & Grey, 1981; Vliegenthart et al., 1983), supported the presence of only a type 1 TRI. Unexpectedly, the N-linked oligosaccharides of bovine fetuin were found to be a mixture of type 1 (Nilsson et al., 1979) TRI's in which the lactosamine unit linked β(1,4) to the α(1,3)-linked Man is either a Galβ(1,4)GlcNAc or a Galβ(1,3)GlcNAc unit. Further, we showed that the Galβ(1,3) positional isomer of type 1 TRI binds with a 70-fold lower affinity.

Materials and Methods

Materials. Acetonitrile and ammonium acetate were HPLC grade (J. T. Baker, Phillipsburg, NJ). Nylon filters (0.2 μm) for filtration of HPLC solvents were from Anspec Co. (Ann Arbor, MI). *N*-BOC-L-Tyr ester of 2,4,5-trichlorophenol was from Sigma Chemical Co. (St. Louis, MO). Deuterium oxide (99.996%) and anhydrous hydrazine were obtained from Aldrich Chemical Co. (Milwaukee, WI). The amino acids and vitamins used in the preparation of cell media were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-(2-Hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (Hepes) was from Research Organics (Cleveland, OH), and the medium salts were of reagent quality from J. T. Baker (Phillipsburg, NJ). Bovine serum albumin (BSA) was obtained from Armour Pharmaceuticals (Kankakee, IL). Crude collagenase (type 1, lot no. 4N6783) was from Cooper Biomedical (Malvern, PA), and carboxypeptidase Y and aminopeptidase M were from Boehringer-Mannheim (Indianapolis, IN). Bovine fetuin was from Calbiochem (San Diego, CA). Orosomucoid was a gift from Dr. M. Wickerhauser of the American Red Cross National Fractionation Center (Bethesda, MD). Neuraminidase from *Arthrobacter ureafaciens* was generously supplied by Dr. Y. Uchida of Kyoto Research Laboratories (Uji, Kyoto, Japan). A unit of neuraminidase is the amount of enzyme that will hydrolyze 1 μmol of sialic acid from colominic acid per minute under the reaction conditions described (Uchida et al., 1977).

Glycopeptide Preparation. Five grams of bovine fetuin was digested for 3 days under sterile conditions as previously described (Townsend et al., 1982). The lyophilized digest was dissolved in 20 mL of 0.5 M pyridine–acetate, pH 4.7, and applied to a Bio-Gel P-2 column (5 × 125 cm) equilibrated in the same buffer. The fractions containing carbohydrate, detected by a phenol–sulfuric acid method (McKelvy & Lee, 1969), were pooled and lyophilized. The above Pronase digestion was repeated, followed by chromatography on a Sephadex G-50 column (2.5 × 200 cm) equilibrated in the above buffer. The neutral sugar containing fractions were pooled, lyophilized, and desialylated as follows. Glycopeptides (approximately 800 mg) were dissolved in 20 mL of acetate buffer, pH 5.0, and incubated with 2 units of neuraminidase for 48 h at 37 °C. Greater than 99% of the sialic acid was removed as determined by assaying glycopeptides, treated for 1 h at 80 °C with 0.1 M HCl, by use of a modified thiobarbituric acid assay (Uchida et al., 1977). The neuraminidase digest was adjusted to pH 6.0 with 1 M Hepes at pH 8.0 and made 2 mM in $MnCl_2$. Aminopeptidase M (5 units) was added each of 3 days through a Nalgene 100-mL filter unit (0.2 μm). The digest was fractionated on a Sephadex G-50 column (conditions described above), and the glycopeptides were incubated in 20 mL of acetate buffer, pH 5.0, with carboxypeptidase Y for 3 days with additions of 5 units of enzyme every 24 h. The digest was reisolated on a Sephadex G-50 column as described above and lyophilized. The approximate overall yield of asialoglycopeptides from fetuin was 50%.

Tyrosylation and Reverse-Phase HPLC of Glycopeptides. Tyrosine was coupled to the dried glycopeptides by dissolving 50 μmol of glycopeptides in 2 mL of dry Me_2SO to which was added a 3-fold excess of redistilled triethylamine. *N*-BOC-L-Tyr ester of 2,4,5-trichlorophenol in 3-fold molar excess was added as a solid, and the reaction was allowed to proceed for 1 h at room temperature. The Me_2SO was evaporated, and the dried glycopeptides were dissolved in 1 mL of 0.1 M acetic acid. The resulting precipitate was removed by centrifugation at 10000*g* for 15 min, and the precipitate was extracted twice with 0.5 mL of 0.1 M acetic acid. The combined supernatants were applied to a Bio-Gel P-6 column (1.5 × 90 cm) equilibrated in 0.1 M acetic acid. The resulting single carbohydrate-containing peak was pooled, lyophilized, and dissolved in 2 mL of water. The *t*-BOC-Tyr-glycopeptides were separated by reverse-phase HPLC on a column (4.6 mm × 25 cm) of Supelcosil 5-μm spherical octadecylsilica (Supelco). Recovery of glycopeptides from the reverse-phase column was >80%. The chromatograph consisted of two Gilson Model 302 pumps controlled by Gilson Gradient Manager software executed on an Apple II Plus computer, Gilson Model 802 manometric module, and Gilson Model 811 dynamic mixer. Injection was by a Rheodyne 7125 valve equipped with a 100-μL sample loop. The column effluent was monitored at 280 nm with an ISCO Model V-4 variable-wavelength detector with built-in peak separator, interfaced to an ISCO Fraction Optimizing X-Y fraction collector. After HPLC purification and lyophilization, *t*-BOC was removed by dissolving the glycopeptides in 75% trifluoroacetic acid at room temperature. After 6 h, the trifluoroacetic acid was lyophilized, and the glycopeptide was isolated by using a G-10 column (0.8 × 40 cm) equilibrated in water with effluent monitoring at 280 nm. The concentration of glycopeptide was based on the Gal content (3 mol of Gal/mol of glycopeptide) determined by a modified version of the neutral sugar analyzer (automated liquid chromatography; Lee, 1972).

Iodination of Tyr-Glycopeptides. The glycopeptides were labeled with ^{125}I by using a modified chloramine-T method (Greenwood et al., 1963) as follows. Stock solutions (0.1 mM) of the compounds to be iodinated were prepared in water. Radioiodine (as Na^{125}I) was obtained from Amersham (Arlington Heights, IL) in vials containing approximately 0.5 nmol of $^{125}I^-$ (1 mCi). After the addition of 10 μL of 0.5 M sodium

phosphate buffer, pH 7.5, to the V-vial container, 10 μL of glycopeptide stock solution was added. Chloramine-T (121 nmol) was added in 10 μL of the phosphate buffer, and the solution was gently mixed with a Pipetteman tip. After 1 min, sodium metabisulfite (1300 nmol) in 100 μL of the phosphate buffer was added, and the reaction mixture was desalted on a G-10 column (0.5 × 20 cm) equilibrated in 50 mM Hepes buffer, pH 7.5, containing 0.15 M NaCl. A single radioactive peak (the $^{125}I^-$ was much more retarded under these conditions) was pooled to a final volume of approximately 2 mL, and BSA (50 μg) was added. The ratio of incorporation of ^{125}I was routinely 0.25–0.36 mol/mol and was independent of the glycopeptide mixture used.

Amino Acid Analysis. The amino acid composition of the glycopeptides was determined by reverse-phase HPLC analysis of the (phenylthio)carbamyl derivatives (Bidlingmeyer et al., 1984). Doubling the concentration of triethylamine in gradient buffer A was necessary to effect resolution of galactosamine, glucosamine, and serine.

Preparation of Oligosaccharides. Sialylated fetuin glycopeptides prepared as described above were subjected to hydrazinolysis and re-N-acetylation (Bendiak & Cumming, 1986). A tetrasialylated triantennary oligosaccharide was obtained by DEAE-Sephacel (Pharmacia, Piscataway, NJ) chromatography as follows. The sialylated oligosaccharides (20 μmol) were applied to a 1.5 × 25 cm column equilibrated in 10 mM Tris-HCl buffer, pH 7.6. The oligosaccharides were eluted with a linear gradient up to 50 mM NaCl over 6 column volumes at a flow rate of 0.33 mL/min. After isolation, the tetrasialylated oligosaccharide was treated at 80 °C with 0.1 M HCl to remove sialic acid, and the concentration of oligosaccharide based on Gal content was determined by automated liquid chromatography (Lee, 1972).

^1H NMR Spectroscopy at 400 MHz. Glycopeptide and oligosaccharide samples were exchanged twice with deuterium oxide (99.996 atom % ^2H) by lyophilization and stored in a dry nitrogen atmosphere. Immediately prior to spectroscopy, the samples were dissolved in deuterium oxide and transferred to a thin-walled 5-mm NMR tube (Wilmad, Buena, NJ). Spectra were obtained on a Varian XL-400 spectrometer by pulsed Fourier transform with quadrature detection. Data were accumulated in 20K memory (double precision) with a spectral width of 5 kHz to give a digital resolution of 0.306 Hz. For resolution enhancement, a combination of an exponential multiplier and a Gaussian apodization function (Varian software) was used in processing the free induction decay data. The data were also zero-filled to a higher Fourier number to obtain better peak definitions. The spectra were obtained at 27 °C, and chemical shifts were referenced to an internal acetone signal at δ 2.225 relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

Preparation of Isolated Rabbit Hepatocytes. Isolated rabbit hepatocytes were prepared from male New Zealand white rabbits (800–1000 g) by a modified collagenase technique (Seglen, 1976) with the following modifications. The animals were given 1000 units of sodium heparin from Upjohn (Kalamazoo, MI), followed by a lethal dose of pentobarbital (both through the marginal ear vein). Buffer I, consisting of 5.96 g/L NaCl, 0.4 g/L KCl, and 50 mM Hepes buffer, pH 7.4 at 37 °C, was perfused through the portal vein at 80 mL/min via a polystyrene cannula (4.5-mm o.d. and 3.0-mm i.d.). After 15 min the liver was perfused with buffer I containing 5 mM $CaCl_2$ and 2 mg/mL collagenase. The flow as stopped after 45 s, and the liver was quickly excised and placed into 100 mL of medium 4C at 0–2 °C. Medium 4C is a modified Eagle's medium containing 10 mM Hepes buffer, pH 7.4 at 4 °C, with amino acid supplementation as described (Schnaar et al., 1978). The gallbladder was excised, and the capsule was removed to allow the cells to flow out of the liver. The cell suspension was filtered, sequentially, through a 100-μm and then a 35-μm nylon mesh filter (Tetko Co., Elmsford, NY), followed by purification with three centrifugation (50*g*)–resuspension cycles at 4 °C using 50-mL polycarbonate flasks. The cells were suspended to a concentration of 25–30 million/mL. A minimum of 90% of the hepatocytes excluded trypan blue and were without blebs both prior to and following incubations with ligand.

Binding Assays and Parameter Estimations. Before binding assays at 0–2 °C, the hepatocytes (2–3 million/mL) were gyrated (100 rpm) in a polycarbonate flask for 30 min at 37 °C in medium 37C with 1 mg/mL of BSA in a polycarbonate flask. Medium 37C contains an additional 40 mM Hepes and is pH 7.4 at 37 °C compared to medium 4C. Both media contain 130 mM sodium ion. The cells were recovered by centrifugation (50*g*) at 4 °C and resuspended into medium 4C containing 1 mg/mL BSA. The binding assays were performed in 1.5-mL polypropylene tubes. Using a tube that is almost filled with the incubation volume reduces the cell damage during the end-over-end rotation at 1 rpm for 2 h. The hepatocytes (0.25 mL) were added to 0.75 mL of medium 4C containing the desired concentration of ligand. The ratio of labeled to total ligand was never greater than 1:10. After 2 h of incubation, the total cell-associated radioactivity was determined by centrifuging 200 μL of the cell suspension over a silicone–mineral oil mixture (4:1) at 10000*g* for 30 s. The snipped pellet was counted in a Prias Autogamma Counter to a counting error of ≤2%. Nonspecific binding was experimentally determined by pipetting 200 μL of cell suspension into a polystyrene tube containing 4 μL of 0.5 M EDTA. After at least 10 min, 100 μL was removed and the total cell-associated radioactivity was measured. In the case of inhibition assays, the labeled ligand was ^{125}I-ASOR at a dose of 0.125 nM. Nonspecific binding was determined as the amount of labeled ligand bound in the presence of 2 mg/mL $[(\beta\text{-D-galactopyranosylthio})\text{acetamidino}]_{44}$-bovine serum albumin (Lee et al., 1983). The data were analyzed by using the LIGAND program (Munson & Rodbard, 1980). All parameters were generated by using the LIGAND program with data in which nonspecific binding was not subtracted and by employing the *N* parameter to estimate nonspecific binding as recommended (Munson & Rodbard, 1980). Similar parameter estimates were obtained by using data with the experimentally determined nonspecific binding subtracted from each bound value and by fitting the data with the nonspecific parameter constrained to 0.

Simulation of Simultaneous Binding of Two Ligands. The binding of two different ligands (in a mixture) to a single class of sites was simulated by an approach analogous to that described by Mendel et al. (1985). The relevant equations are detailed in the Appendix. The apparent bound and the apparent free were calculated and plotted according to Scatchard (1949) and compared to data calculated by using the parameters from the LIGAND program for binding data of different glycopeptide mixtures.

RESULTS

Purification and Characterization of N-Linked Fetuin Glycopeptides. Figure 1 shows separations using a calibrated Sephadex G-50 column of sialylated fetuin (peak I) and asialofetuin (peak II) glycopeptides after two cycles of Pronase digestions. Peak I eluted just prior to the position for sialylated

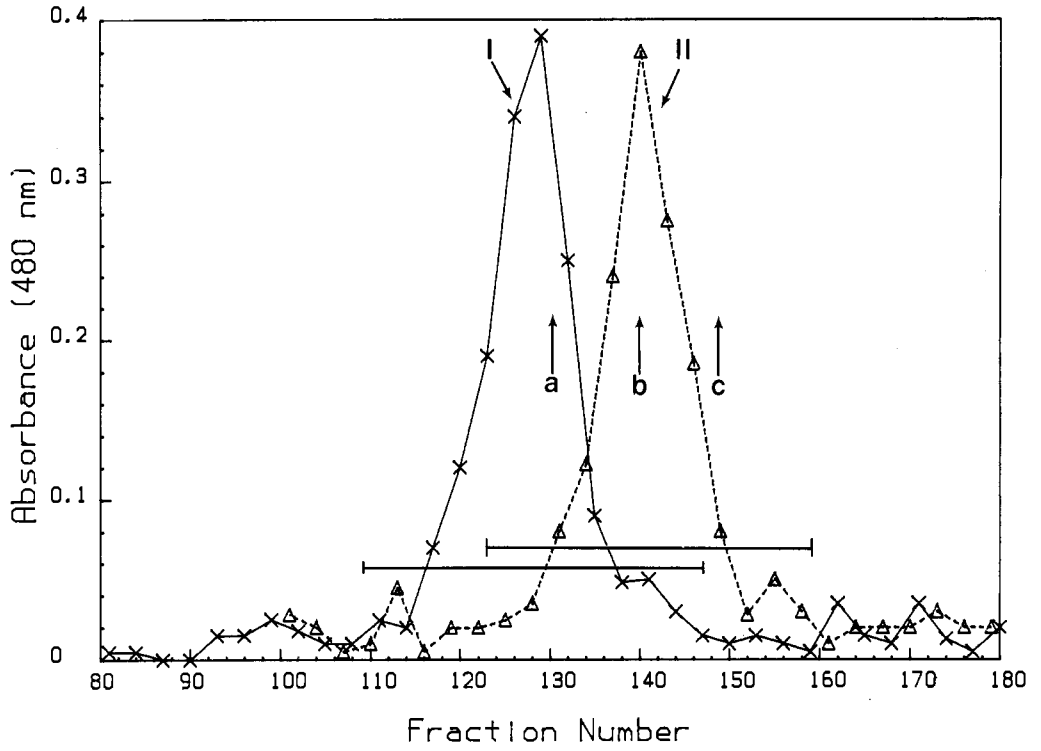

FIGURE 1: Sephadex G-50 chromatography of fetuin glycopeptides. Sialylated glycopeptides from bovine fetuin (800 mg), prepared as described under Materials and Methods, were dissolved in 10 mL of 0.5 M pyridine–acetate buffer, pH 4.7. Fractions (5.7 mL) were collected at a flow rate of 15 mL/h. The column was standardized with sialylated biantennary (a), asialobiantennary (b), and agalactobiantennary (c) glycopeptides prepared from human fibrinogen as previously described (Townsend et al., 1982). The figure is a composite of two separate runs. Neutral sugars were assessed by using a phenol–sulfuric acid method (McKelvy & Lee, 1969). Peak I (×) contains the sialylated glycopeptides. Peak II (△) was obtained after treatment of the pooled fractions of peak I (indicated by the bar) with neuraminidase as described under Materials and Methods.

biantennary glycopeptides obtained from human fibrinogen. The fractions indicated by the bar under the first peak were pooled, and the sialic acid was removed as described under Materials and Methods. The resulting asialotriantennary glycopeptides (peak II) eluted at the position of asialobiantennary glycopeptides on the G-50 column. These fetuin asialoglycopeptides were pooled, lyophilized, and further fractionated by using reverse-phase HPLC on an octadecylsilica column. The underivatized glycopeptides were poorly resolved with four or five major, overlapping broad peaks observed by using a 0–5% acetonitrile gradient (data not shown). Tyrosine, as its *N*-*t*-BOC derivative, was coupled to the glycopeptides to enhance hydrophobicity for improved reverse-phase HPLC, to permit specific column monitoring, and to allow radioiodination for cell binding studies. This chemical modification resulted in superior resolution of the glycopeptides as shown in Figure 2. The single major peak by G-50 chromatography was resolved into approximately 30 peaks by using these HPLC conditions. Eight major peak fractions were selected for further analysis, and they were assigned the numbers shown in Figure 2.

Sufficient quantities of purified glycopeptides for further analysis and cell binding studies were obtained by multiple injections of up to 200 nmol of total glycopeptides without loss of the resolution shown in Figure 2. After multiple chromatographic runs were pooled, the samples were lyophilized, and stock solutions were analyzed for neutral and amino sugars. All the selected fractions contained carbohydrate except peak 5, which contained only peptide. Galactosamine was detected in GP-6 and GP-7, while the remainder of the selected peaks contained only glucosamine. GP-6 and GP-7 consist largely of the O-linked carbohydrates of fetuin (Spiro & Bhoyroo, 1974) and were not analyzed further. By analytical HPLC,

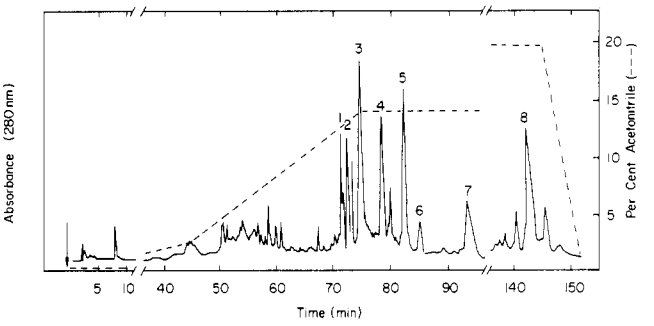

FIGURE 2: Reverse-phase HPLC of *t*-BOC-Tyr-fetuin glycopeptides. Derivatized glycopeptides (50 nmol) in water were chromatographed on an octadecylsilica column (Supelco) equilibrated in 50 mM ammonium acetate, pH 6.9. The arrow indicates the point of injection. A gradient of acetonitrile shown by the dashed line was developed as described under Materials and Methods. The system gradient delay time was 6 min for a flow rate of 0.5 mL/min. The numbers indicate the peak fractions that were pooled for further analysis.

the purified GP fractions were greater than 90% pure with the exception of GP-2, which contained approximately 30% of an earlier eluting material (data not shown).

The results of amino acid analysis are shown in Table I. All the fractions examined contained Tyr and Asp. The ratio of Asp to Tyr was 1:1 except in GP-1 and GP-2 in which the ratio was 2:1. Every purified GP apparently contained between 0.17 and 0.24 mol of proline relative to Asp. It is surmised that the peak eluting in the position of proline in the purified fractions is ammonia since the proline peak is difficult to resolve routinely from ammonia. This is supported by the reported absence of proline around the N-linked glycosylation sites (Begbie, 1974). However, some of the material in the proline position found in the unfractionated glycopeptides is

Table I: Amino Acid Composition of HPLC-Purified Fetuin Glycopeptides

amino acid	fractions					
	total[a]	GP-1	GP-2	GP-3	GP-4	GP-8
Asp	0.94	1.83	1.81	1.15	1.23	0.82
Glu	0.16	0.15	–[b]	–	–	–
Ser	0.32	0.16	0.75	–	–	–
Gly	0.41	0.16	0.09	0.16	0.76	–
Thr	0.15	–	–	–	–	–
Ala	0.13	–	–	–	–	–
Pro	0.38	0.17	0.17	0.24	0.14	0.19
Tyr	0.82	1.02	1.14	1.01	0.81	1.12
Cys[c]	0.31	–	–	–	–	1.01

[a] Amino acid composition given as mol/3.0 mol of Gal. [b] Amino acids present to less than 5% indicated by dash. [c] Cysteine was determined as cysteic acid and is reported relative to Asp in the performic acid hydrolyzate. Recovery of Asp was 37%.

probably from the O-linked glycopeptides that contain Pro (Begbie, 1974). The peptide sequence of the isolated N-linked GP's, based on the compositional data and the reported sequence information (Begbie, 1974; Plummer et al., 1984), is as follows: GP-1, Tyr-Asn-Asp; GP-2, Tyr-Asn-Asp-(Ser); GP-3, Tyr-Asn; GP-4, Tyr-Asn-Gly; and GP-8, Tyr-Asn-Cys, representing the three N-linked glycosylation sites of fetuin (Spiro, 1962).

Approximately 1 mg (500 nmol) of the purified N-linked GP's was exchanged with deuterium oxide and analyzed by 400-mHz ^{1}H NMR spectroscopy as described under Materials and Methods. Table II gives the chemical shifts and signal integrations from the 400-MHz ^{1}H NMR spectra of GP-1, GP-2, GP-3, GP-4, and GP-8, as well as the unfractionated *t*-BOC-Tyr-GP's. The chemical shifts not enclosed in the braced *areas I and II* of Table II agreed (within 0.003 ppm) with reported spectral data (Vliegenthart et al., 1983) for a type 1 triantennary structure (for structure, see Figure 3) except for the presence of an NAc methyl signal at δ 2.034 in the spectrum of the total glycopeptides and a quartet at δ 4.193 in the spectrum of GP-2. A consideration was that the NAc resonance is from the methyl group of α(2,3)NeuAc (Vliegenthart et al., 1983). This possibility was ruled out since the total glycopeptides were more than 99% desialylated and this signal integrated to approximately 0.3 methyl group. The signal (δ 2.034) was tentatively assigned to the GalNAc residue of the O-linked sugars and was not present in the selected fractions containing only N-linked carbohydrates. The signal at δ 4.193 was found only in GP-2 which by analytical HPLC contained 30% of an earlier eluting peak. This reporter group and a doublet at δ 5.048 (H-1 of GlcNAc), which integrated to 0.2 proton (Table II), are likely to be from asialobiantennary glycopeptides. The other signals from a biantennary structure would overlap those from a triantennary GP (Vliegenthart et al., 1983).

Major spectral differences among the HPLC-purified fractions were apparent from signals that have been assigned to the branched lactosamine residues (Vliegenthart et al., 1983). These chemical shifts and signal integrations are indicated by the braced areas (areas I and II) of Table II. Figure

Table II: Chemical Shifts and Signal Integrations from 400-MHz ^{1}H NMR Spectra of Asialoglycopeptides from Bovine Fetuin

protons	residues[a]	chemical shifts and signal integrations[b]											
		total		GP-1		GP-2		GP-3		GP-4		GP-8	
H-1	1	5.079	0.6	5.051	0.9	5.038	0.7	5.043	0.9	5.024	0.8	5.041	0.9
		5.039				5.048	0.2						
	2	4.613	0.9	4.616	0.9	4.616	1.0	4.612	1.0	4.613	1.0	4.604	0.8
	4	5.116	1.0	5.115	1.0	5.115	1.0	5.116	1.0	5.114	1.0	5.116	1.0
	4′	4.924	1.0	4.924	0.9	4.924	1.0	4.924	1.0	4.924	1.0	4.924	1.0
						Area I							
	5	4.568	1.2	4.572	1.2	4.570	0.9	4.569	1.2	4.569	0.7	4.571	1.2
	5′	4.581	1.2	4.582	1.1	4.581	1.3	4.581	1.2	4.581	1.1	4.581	1.0
	7	4.546	0.6	4.547	0.6	4.547	0.6	4.548	0.7	4.546	0.5	4.547	0.8
	7*									4.573	0.6		
	total		3.0		3.0		2.9		3.3		3.1		2.9
	6	4.463	0.9	4.468	0.9	4.465	0.9	4.465	1.2	4.464	1.0	4.465	1.5
	6′	4.474	1.2	4.475	1.0	4.474	1.0	4.475	1.1	4.475	1.0	4.475	1.3
	8	4.469	0.7	4.471	0.8	4.470	0.9	4.471	0.9	4.471	0.4	4.471	0.7
	8*	4.442	0.4	4.444	0.4	4.444	0.3	4.444	0.5	4.444[c]	0.6	4.444	0.2
	total		3.2		3.0		3.0		3.7		3.1		3.7
H-2	3	4.214	1.9	4.216	1.7	4.215	1.6	4.213	2.3	4.214	1.9	4.214	1.7
	4	4.217		4.220		4.218		4.217		4.217		4.217	
	4 (BI)					4.193	0.3						
	4′	4.112	1.1	4.114	0.9	4.113	1.1	4.111	1.2	4.112	1.1	4.114	0.8
NAc of	1	1.995		2.008	1.0	1.997		2.009	0.9	1.997	1.2	2.004	0.9
		2.006				2.006							
		2.002	0.8			2.008	0.9						
	2	2.076	0.9	2.075	0.9	2.076	1.0	2.072	0.9	2.074	1.0	2.071	0.9[d]
	5	2.049	1.0	2.051	1.0	2.051	1.0	2.051	1.0	2.050	1.0	2.051	1.0
	5′	2.045	0.9	2.046	1.2	2.047	1.1	2.047	1.0	2.046	1.1	2.046	1.3
						Area II							
	7	2.081	0.5	2.078	0.6	2.079	0.6	2.078	0.7	2.078	0.3	2.080	0.8
	7*	2.067		2.069	0.4	2.070	0.3	2.069	0.4	2.068	0.7	2.064	0.3
GalNAc		2.034											

[a] The numbering of residues is as described (Vliegenthart et al., 1983) and is shown with the structure in Figure 3. [b] The integration of the anomeric and Man H-2 protons is based on the proton signal of residue 4 set to 1.0. The NAc methyl integrations are given as one methyl group (three protons) on the basis of the NAc methyl protons of residue 5. [c] The integration of the doublet at δ 4.444 is given as 2 times the area of the higher upfield resolved signal of the partially resolved doublet. [d] The chemical shifts and values for the NAc methyl protons for GP-8 were obtained at 70 °C to allow resolution of the 7* NAc methyl protons.

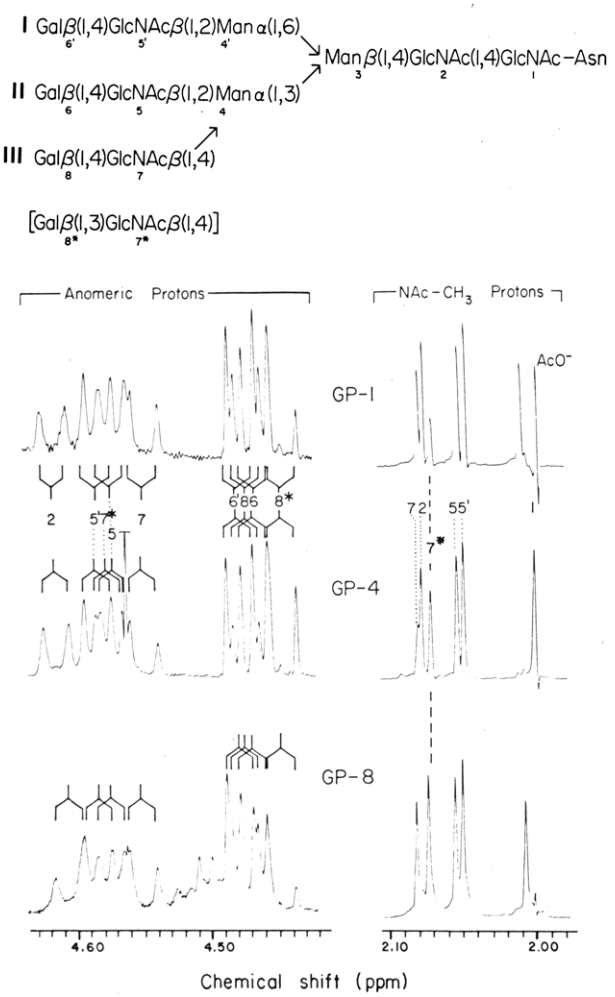

FIGURE 3: 400-MHz ^{1}H NMR spectra of HPLC-purified fetuin glycopeptides. Spectra from each glycosylation site (GP-1, GP-4, and GP-8) were obtained and resolution was enhanced as described under Materials and Methods. The numbering and assignments were based on reported reference spectra (Vliegenthart, 1983) and data presented herein (Table II). The Roman numerals indicate the branch number of the triantennary structure. The bracketed β(1,3)-lactosamine unit is substituted as the third branch, to form Galβ-(1,3)GlcNAc-TRI in proportions measured by the signal area of the 8* doublet (Table II). The unassigned resonances in the spectrum of GP-8 are suspected to be from amino acids.

3 shows partial spectra of the anomeric and the NAc methyl proton signals from residues comprising the branches of GP-1, GP-4, and GP-8. Residues and branch numbering for the two triantennary structures to be discussed are shown in Figure 3. The chemical shifts cited below were those obtained for GP-4 (Table II) since the highest spectral resolution was achieved with this fraction and a long accumulation time was used to minimize noise to assist more accurate signal integrations.

The Gal H-1 region contained four sets of doublets (J = 7.9 Hz) instead of the expected three for an asialotriantennary structure. The two doublets at δ 4.471 and 4.444 in the Gal H-1 region integrated to 0.4 and 0.6 proton, respectively, whereas the other two doublets (δ 4.464 and 4.475) each represented 1 proton (Table II). A doublet at δ 4.444 has been assigned to the H-1 of Gal linked β(1,3) to GlcNAc in a variety of carbohydrate structures including the α(1,3)-linked β(1,3)-lactosamine branch of a biantennary structure (Bernard et al, 1984). Therefore, these NMR data suggest that about one in six triantennary branches consists of a Galβ(1,3)-GlcNAc unit. Since the chemical shift of the H-1 of unsubstituted Gal linked β(1,3) to GlcNAc changes by only about

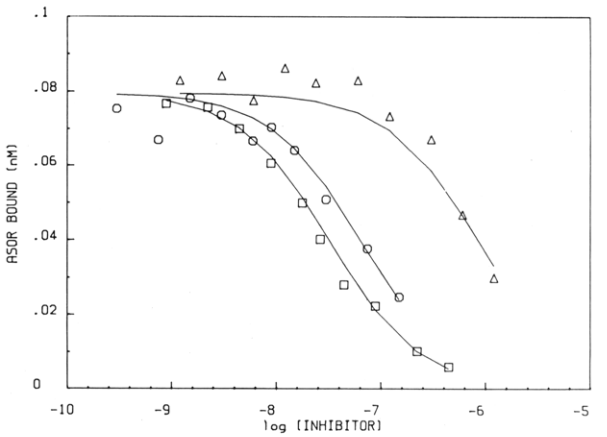

FIGURE 4: Inhibition of binding of ^{125}I-ASOR to rabbit hepatocytes by fetuin glycopeptides and synthetic oligosaccharides. Rabbit hepatocytes (0.25 mL) at 10 million/mL were added to 0.75 mL of medium containing the indicated concentration of either NONA I (□), GP-4 (○), or NONA II (△) and 1.25×10^{-10} M ^{125}I-ASOR at 2 °C. After 2 h of incubation at 0–2 °C, 200 μL of cell suspension was removed, and the total cell associated radioactivity was determined as described under Materials and Methods. The curves represent the "best-fit" parameters for a one receptor–two ligand model using the LIGAND program (Munson & Rodbard, 1980).

0.007 ppm in the various compounds examined so far (Bernard et al., 1984), it was not possible to determine branch location from the chemical shift of the H-1 of Gal alone. However, Bernard et al. (1984) found a corresponding downfield shift in the H-1 of Glc NAc-5 from δ 4.582 in a Galβ(1,4)GlcNAc unit to 4.600 for the Galβ(1,3)GlcNAc in a biantennary structure. Also a slight shift in the NAc methyl proton of GlcNAc-5 resulted from a Galβ(1,3) substitution of the GlcNAc residue. These above changes in the resonances of the H-1 and NAc of GlcNAc-5 after substitution with Galβ(1,3) (Bernard et al., 1984) were not present in the spectra we obtained from fetuin triantennary glycopeptides. However, we did observe six NAc methyl signals instead of the expected five from the GlcNAc residues of a triantennary structure. An NAc methyl signal not found in a type 1 triantennary glycopeptide from α-1-acid glycoprotein (Vliegenthart et al., 1983) was seen at δ 2.068 and was related directly in signal area to the doublet at δ 4.444. The signal area of the resonance at δ 2.078, which had been previously assigned to the NAc of GlcNAc-2, varied indirectly (area II) with the NAc signal at δ 2.068. A downfield shift (–0.027 ppm) was noted in the H-1 of GlcNAc-7 to δ 4.573 which we attributed to β(1,3) substitution of GlcNAc-7 with Gal. From these apparent changes in GlcNAc-7 resonances, we conclude that most of the Galβ(1,3)GlcNAc is located on branch III. For purposes of discussion, we have designated these two triantennary structures as Galβ(1,4)GlcNAc-TRI and Galβ(1,3)GlcNAc-TRI.

Binding of N-Linked Glycopeptides to Isolated Rabbit Hepatocytes. The signal area of the doublet at δ 4.444 ranged from 0.2 proton in GP-8 to 0.6 in GP-4. We next determined whether these mixtures of TRI's with either Galβ(1,3)GlcNAc or Galβ(1,4)GlcNAc as branch III could bind differently to the hepatic Gal/GalNac lectin. Figure 4 shows inhibition curves for GP-4 and related synthetic oligosaccharides; the binding constants are given in Table III. The GP-4 mixture was a 2-fold worse inhibitor (K_d = 22 nM) than NONA I (K_d = 11 nM) but a 10-fold better inhibitor than NONA II (K_d = 295 nM), which is a type 2 triantennary structure.

We then compared the binding parameters of GP-8, GP-1, and GP-4 which varied in the relative amounts of Galβ-(1,3)GlcNAc-TRI and Galβ(1,4)GlcNAc-TRI (from 0.2 in

Table III: Comparison of Binding Affinity for GP-4 and Synthetic Oligosaccharides

compd[a]	K_d[b] (nM)
NONA I	15/ 11/ 8.0
NONA II	510/290/170
GP-4	36/ 22/ 14

[a] For structures of synthesis oligosaccharides, see Lee et al. (1983). [b] The constants were obtained by using the LIGAND program (Munson & Rodbard, 1980) and a one site–two ligand model. The data from the three inhibition curves were fit together with the C parameter constrained to 1 and the receptor concentration constrained to 1.1×10^{-9} M, which was obtained from direct binding data for ^{125}I-ASOR to rabbit hepatocytes (Stults et al., unpublished data). The mean parameter value is bracketed with the lower and upper 90% confidence limits on either side of the slashes. The 90% confidence intervals were calculated by using the t distribution and the logarithm of the approximate standard error given by the LIGAND program.

GP-8 to 0.6 in GP-4). Table IV gives parameters for the binding of GP-8, GP-1, and GP-4, and Figure 5 shows the Scatchard (1949) transformation of binding data. Although the data for GP-8 and GP-1 were best described with two independent classes of sites (as judged by the F test), the resulting parameters were indeterminant as is evident from the wide 90% confidence intervals (Table IV). This large error in parameter estimations is related to the number of degrees of freedom (15) and the number of parameters (5) sought. The significant variation in binding parameters that occurs among cell preparations (Hardy et al., 1985) limits advantages gained in parameter estimation by combining results (Munson & Rodbard, 1980) from different animals. The one-site model yielded narrow confidence intervals, and we have found that the rank of binding affinities among both synthetic oligosaccharides (Lee et al., 1983) and the glycopeptides described herein is maintained among different cell preparations of rabbit hepatocytes. Thus, three parameters (R, K_d, and N) were used to describe the binding data to compare the TRI-GP's using the same preparation of hepatocytes. The single-site K_d for GP-8, GP-1, and GP-4 was 5.0 nM, 8.5 nM, and 16 nM, respectively, with 420–500 kilosites/cell for all three glycopeptide mixtures (Table IV). It was noted that as the proportion of Galβ(1,3)GlcNAc increased, the overall affinity of the mixture decreased. This finding suggests that triantennary oligosaccharide containing Galβ(1,3)GlcNAc binds with a significantly lower affinity than triantennary with Galβ-(1,4)GlcNAc as all three lactosamine branches as is the case for NONA I.

We estimated the affinity of Galβ(1,3)GlcNAc-TRI present in the mixtures by computer simulation of the binding of two ligands of different affinities, an implementation of the approach described by Mendel et al. (1985) (see Appendix for equations). A theoretical mixture containing 60% Galβ-(1,3)GlcNAc-TRI and 40% Galβ(1,4)GlcNAc-TRI was used to simulate the binding of the GP-4 mixture. The K_d for Galβ(1,4)GlcNAc-TRI was approximated to be 5 nM, and the total site concentration was 2 nM. These values were

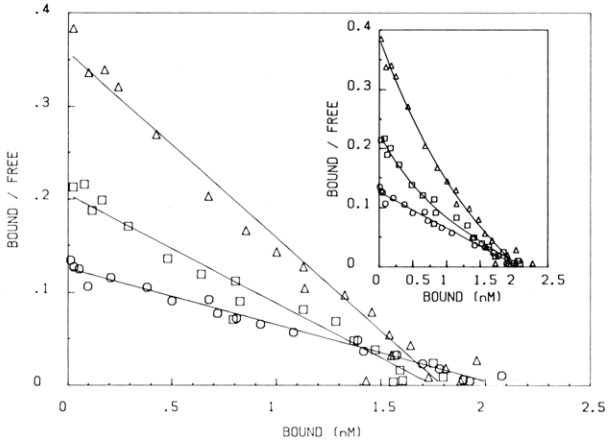

FIGURE 5: Binding of ^{125}I-GP-1, ^{125}I-GP-4, and ^{125}I-GP-8 to rabbit hepatocytes. Rabbit hepatocytes (0.25 mL) at 10 million/mL were added to 0.75 mL of medium containing the desired final concentration of either GP-8 (△), GP-1 (□), or GP-4 (○) at 0–2 °C. After 2 h the total cell-associated radioactivity was determined. The lines represent the best determined parameters for the one class of binding sites model. The *inset* shows the Scatchard transforms of the two-site fits for GP-1 and GP-8, which were significantly better than the one-site fits, based on the F test, for these ligands. GP-4, which was better described by the one-site model, is replotted for comparison.

obtained from the one-site fit of GP-8 [which contains 80% of Galβ(1,4)GlcNAc-TRI] binding. Binding simulations were performed, keeping the above values constant and varying the K_d of the component present as 60% of the total, Galβ(1,3)-GlcNAc-TRI. Scatchard transforms of the calculated binding data at selected K_d's for Galβ(1,3)GlcNAc-TRI are shown in Figure 6. It was found that the affinity of the 60% component [Galβ(1,3)GlcNAc-TRI] had to be between 200 and 1600 nM to approximate the affinity observed for GP-4 (open circles) in the presence of a 40% component, Galβ(1,4)GlcNAc-TRI, which possesses a K_d of 5 nM. The theoretical result that the K_d of Galβ(1,3)GlcNAc-TRI is between 200 and 1600 nM predicts that even at the highest dose employed (500 nM) in the binding experiments its occupancy would amount to <3% of the total bound ligand. In other words, free Galβ(1,3)-GlcNAc-TRI in the presence of Galβ(1,4)GlcNAc-TRI is an inactive ligand, and the observed differences in affinities of the three mixtures, GP-1, GP-4, and GP-8, are a result of overestimation of the active ligand concentration. We tested this hypothesis by subtracting the noncontributing unbound ligand concentration [Galβ(1,3)GlcNAc-TRI] (for equation, see legend to Figure 6) and refitting the data. This dose correction was extended to the analysis of binding data for all three mixtures and resulted in the collapse of the binding isotherms to approximate a single set of parameters after analysis using the LIGAND program (Figure 6, inset). These resulting parameters for the binding of Galβ(1,4)GlcNAc-TRI were found to be K_d = 4.4 nM, with 90% confidence limits of 3.6 and 5.4 nM, and a range of 390–500 kilosites per hepatocyte, with a mean value of 440.

Table IV: Comparison of Binding Constants for ^{125}I-GP-1, ^{125}I-GP-4, and ^{125}I-GP-8

	binding parameters[a]			
compd	K_{d1} (nM)	K_{d2} (nM)	R_1[b]	R_2
GP-8	6.2/ 5.0/ 4.0		380 /430/ 490	
	34.0/ 2.5/ 0.2	330/12/0.5	2 /170/14 000	44/320/2400
GP-1	11.4/ 8.5/ 6.3		350 /420/ 520	
	110 /3.0/ 0.08	300/19/1.2	0.3/ 99/30 000	160/420/1100
GP-4	20 /16/13		430 /500/ 580	

[a] The parameters for both a one-site and two-site fit were calculated by using the LIGAND program. The mean parameter value is bracketed with the lower and upper 90% confidence limits on either side of the slashes. The 90% confidence intervals were calculated by using the t distribution and the logarithm of the approximate standard error given by the LIGAND program. [b] Number of binding sites in kilosites/million hepatocytes.

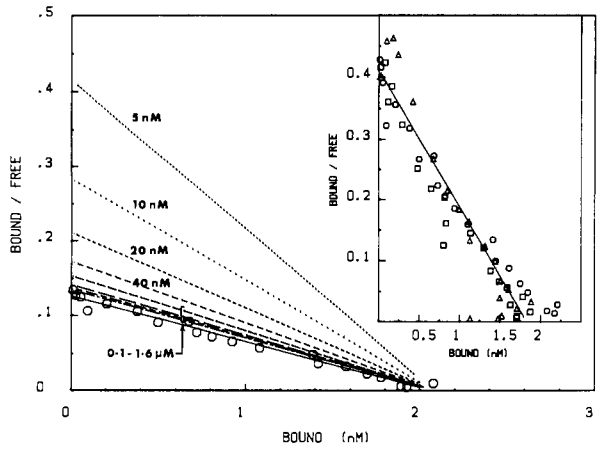

FIGURE 6: Comparsion of binding of ^{125}I-GP-1, ^{125}I-GP-4, and ^{125}I-GP-8 after dose corrections. Rabbit hepatocytes (0.25 mL) at 10 million/mL were added to 0.75 mL of medium containing the desired final concentration of GP-4 (○) at 0–2 °C. After 2 h the total cell-associated radioactivity was determined. Shown are theoretical data (dashed lines) generated by using the approach of Mendel et al. (1985) as described under Materials and Methods in which the affinity of one ligand is kept constant (K_d = 5 nM) and the second ligand K_d is varied from 5 to 1600 nM. The proportion of the first ligand to the total was 0.4, the relative amount of Galβ(1,4)-GlcNAc-TRI in GP-4. The open circles and solid line are the binding data and calculated one-site fit using the LIGAND program for ^{125}I-GP-4. The *inset* shows the experimental and calculated result when the doses for GP-8 (△), GP-1 (□), and GP-4 (○) were corrected [fraction of Galβ(1,4)GlcNAc-TRI × dose = effective dose]. The solid line represents the best-fit parameters describing the adjusted data for all three ligands, analyzed together with the LIGAND program.

Table V: Binding Parameters for Synthetic Oligosaccharides and Galβ(1,3)GlcNAc-TRI Oligosaccharide

compd	binding parameters[a] K_d (nM)
ASOR	14/ 0.7/ 0.03
NONA I	17/ 4.0/ 1.0
NONA II	270/ 57.0/12
Galβ(1,3)GlcNAc-TRI	1800/310 /54

[a] The parameters were calculated by using the LIGAND program. The mean parameter value is bracketed with the lower and upper 90% confidence limits on either side of the slashes. The 90% confidence intervals were calculated by using the *t* distribution and the logarithm of the approximate standard error given by the LIGAND program.

To substantiate our theoretical calculations and to exclude any potential contributions of the ^{125}I-peptide to the differential binding of the glycopeptide mixtures, we compared the binding of purified Galβ(1,3)GlcNAc-TRI oligosaccharide with a Gal(1,4)GlcNAc-TRI oligosaccharide (NONA I). Galβ-(1,3)GlcNAc-TRI oligosaccharide was obtained as the tetrasialylated triantennary oligosaccharide (von Halbeek et al., 1985) after DEAE-Sepahcel chromatography of hydrazinolyzed N-reacetylated fetuin glycopeptides. High-resolution ^{1}H NMR of this fraction revealed the presence of >90% Galβ(1,3)GlcNAc-TRI (data not shown). Figure 7 shows that Galβ(1,3)GlcNAc-TRI was the worst inhibitor among NONA II, NONA I, and ASOR. Table V shows that the affinity of Galβ(1,3)GlcNAc-TRI was 75-fold lower (K_d = 305 nM) than that for NONA I (K_d = 4 nM) and even slightly lower than the K_d for NONA II (K_d = 57 nM). It is noteworthy that changing the Gal linkage on branch III from β(1,4) to β(1,3) resulted in a greater affinity decrease than altering the attachment point of the third branch (type 1 vs. type 2 TRI).

Discussion

The hepatic Gal/GalNAc lectin binds carbohydrates of type 1 (NONA I) and type 2 (NONA II) triantennary structures

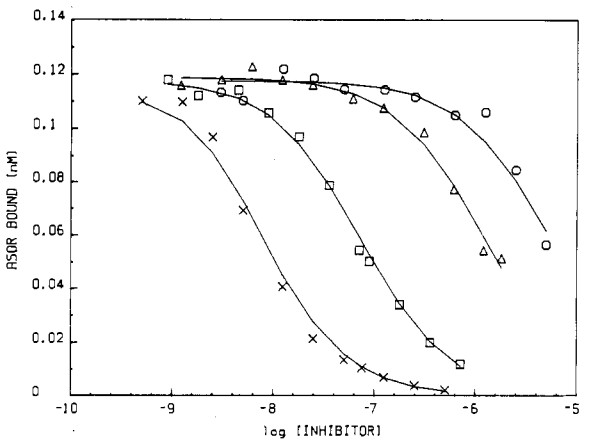

FIGURE 7: Inhibition of binding of ^{125}I-ASOR to rabbit hepatocytes by Galβ(1,3)GlcNAc-TRI and synthetic oligosaccharides. Rabbit hepatocytes (0.25 mL) at 10 million/mL were added to 0.75 mL of medium containing the indicated concentration of either ASOR (×), NONA I (□), NONA II (△), or Galβ(1,3)GlcNAc-TRI oligosaccharide (○) and 1.25 × 10^{-10} M of ^{125}I-ASOR at 0–2 °C. After 2 h of incubation, 200 μL of cell suspension was removed, and the total cell-associated radioactivity was determined as described under Materials and Methods. The curves represent the composite of best-fit parameters for a one receptor–two ligand model using the LIGAND program (Munson & Rodbard, 1980).

with a 30–50-fold difference in affinity. It became necessary to purify and distinguish these two structures for more detailed studies on the Gal-combining sites of this lectin. We chose bovine fetuin as a source for triantennary structures and undertook to evaluate the branching pattern of glycopeptides with homogeneous amino acid composition. Two reports on the structure of the N-linked oligosaccharides of fetuin proposed two different branching patterns (Baenziger & Fiete, 1979; Nilsson et al., 1979) corresponding to the synthetic oligosaccharides NONA I and NONA II (Lee et al., 1983). All subsequent investigations (Kruis & Finne, 1981; Carver & Gray, 1981; van Halbeek et al., 1985; Berman, 1986) have proposed that the NONA I or type 1 branching pattern is correct. In the fetuin GP's examined from each glycosylation site, the Man H-2 resonances agreed well (within 0.003 ppm) with previous NMR data indicating a type 1 TRI branching pattern (Carver & Grey, 1981; Vliegenthart et al., 1983). We found that the chemical shift of the H-2 of Man-4 was decremented by 0.027 ppm compared to the H-2 from Man-4 of a biantennary structure. This change in chemical shift indicates substitution of the α(1,3)-linked Man and is diagnostic of the type 1 branching pattern (Vliegenthart et al., 1983). The chemical shift of the H-2 of Man-4′ (δ 4.112) was unchanged in the GP's examined and agreed within 0.003 ppm with literature resonances (Vliegenthart et al., 1983). Therefore, from the ^{1}H NMR data we conclude that bovine fetuin TRI consists predominantly of the type 1 branching pattern or NONA I type structure at each of the three glycosylation sites.

Glycopeptides from each of the three glycosylation sites of fetuin (Begbie, 1974) were found to be mixtures of type 1 TRI isomers which differed in the relative amounts of Galβ(1,3)-GlcNAc and Galβ(1,4)GlcNAc units. We inferred that the β(1,3)-lactosamine was present primarily as branch III from differences in chemical shifts of the NAc methyl and H-1 protons of GlcNAc-7 caused by β(1,3) vs. β(1,4) substitution (Bernard et al., 1984). From ^{1}H NMR spectroscopy of a tetrasialylated triantennary oligosaccharide isolated from fetuin, van Halbeek et al. (1985) recently concluded that β-(1,3)-lactosamine constituted branch III. Using ^{13}C NMR spectroscopy and the resistance of Galβ(1,3)GlcNAc units to

cleavage by β-galactosidase from *Aspergillus niger*, Berman (1986) also found Galβ(1,3) linkages in bovine fetuin.

The identification of Galβ(1,3)GlcNAc as branch III of a type 1 TRI structure raised questions concerning the assignment of the H-1 Gal protons and the NAc methyl protons of GlcNAc residues 2 and 7 (Vliegenthart et al., 1983). We found that the signal from the H-1 of Gal linked β(1,3) to GlcNAc (δ 4.444), residue 8*, when summed with the signal that has been previously assigned to Gal-6 (δ 4.468) (Vliegenthart et al., 1983) equaled approximately 1 proton. The relatedness of these two doublets was evident in all the GP's examined (Figure 3 and Table II). The other two Gal H-1 doublets (δ 4.475 and 4.464) were present each in intensity equal to 1 proton. Thus, the H-1 doublet that had originally been assigned to Gal-6 (second branch) we now specify to the H-1 of Gal-8 (branch III). The chemical shift of Gal-6′ (first branch) at δ 4.475 is in agreement with previous intepretation (Vliegenthart et al., 1983) at δ 4.473. Apparently, β(1,4) substitution of Man-4 with either a β(1,4)- or β(1,3)-lactosamine unit causes an upfield shift (0.005 ppm) in the H-1 of Gal-6, and the H-1 of Gal-8 now resonates at δ 4.471. Subtraction of our chemical shifts (Table II) by 0.002 ppm results in agreement with previously reported data (Vliegenthart et al., 1983) to within 0.001 ppm. Examination of the spectra of glycopeptides with different amounts of Galβ-(1,4)GlcNAc-TRI and Galβ(1,3)GlcNAc-TRI also revealed that the NAc methyl signal originally assigned to GlcNAc-2 was related by signal intensity to a new resonance at δ 2.068. Since we could assign this newly described signal to the GlcNAc to which is linked β(1,3)-Gal, we then attributed the lowest field signal of the NAc methyl protons to GlcNAc-7 (δ 2.080) and the next upfield resonance to GlcNAc-2 (δ 2.071) instead of to GlcNAc-7, as originally proposed (Vliegenthart et al., 1983).

The importance of the branching pattern to the binding of TRI to the Gal/GalNAc lectin has been shown (Lee et al., 1983) and correlated with the inter-Gal distances of preferred conformers (Lee, Y. C., et al., 1984). The distances between branch II and III Gal's were found to be 15 and 23 Å in NONA I and NONA II structures, respectively, whereas the other inter-Gal distances were between 22 and 31 Å (Bock, 1983). On the basis of these and other findings (Lee, R. T., et al., 1984), we concluded that the shorter inter-Gal distance (15 Å) explained the higher affinity of NONA I. The comparable affinities of NONA II and Galβ(1,3)GlcNAc-TRI suggest that the branch II and III inter-Gal distances are different than 15 Å. Determination of the three-dimensional structure of Galβ(1,3)GlcNAc-TRI should provide further insight into the structural basis of these large affinity differences.

Although the minor peak fractions from the HPLC chromatogram were not examined (Figure 1), we did obtain spectra of triantennary structures from each N-linked glycosylation site. GP-1 and GP-2 were from the same site (Asn-Asp and Asn-Asp-Ser), and 30–40% of those TRI structures contained β(1,3)-lactosamine as branch III. The Asn-Gly site (GP-4) and the Asn-Cys (GP-8) site contained 60% and 20% Galβ-(1,3)GlcNAc-TRI, respectively. We explored the significance of this microheterogeneity by determining the binding parameters for these mixtures of Galβ(1,4)GlcNAc-TRI and Galβ(1,3)GlcNAc-TRI (GP-1, GP-4, and GP-8) for the hepatic Gal/GalNAc lectin on the surface of rabbit hepatocytes. We found that as the amount of Galβ(1,3)GlcNAc-TRI increased in the mixture, a drop in affinity was observed (Figure 5). The correlation between the amount of Galβ(1,3)-GlcNAc-TRI present and the affinity of the mixture agreed well with the theoretical binding data (Figure 6) if the affinity of Galβ(1,3)GlcNAc-TRI was much lower (K_d > 100-fold higher) than the affinity of Galβ(1,4)GlcNAc-TRI, which was estimated to have K_d = 4 nM. The model (see Appendix) predicts that in the above naturally occurring mixtures of ligands, at the receptor concentrations used ($K_d \cong [R]$), the lower affinity ligand is virtually not bound. We envision that the hepatic Gal/GalNAc lectin is able to prioritize ligands for endocytosis from the initial event (binding). In other words, the clearance of an asialoglycoprotein may not only be affected by its K_d but also by the presence of other asialoglycoproteins with higher affinity. Further, the larger affinity difference between Galβ(1,3)GlcNAc-TRI and Galβ(1,4)GlcNAc-TRI and the intersite heterogeneity not only point to an intriguing oligosaccharide biosynthetic problem but also underscore the resulting effects that apparently small changes in carbohydrate structure can have on glycoprotein binding by the hepatic Gal/GalNAc lectin.

While our work was in progress, Takasaki and Kobata (1986), using hydrazinolysis, glycosidase digestion, Smith degradation, and methylation techniques, independently discovered the presence of Galβ(1,3)GlcNAc from bovine fetuin samples. They also noted that this Galβ(1,3)GlcNAc segment was linked β(1,4) to the α-linked Man. Their studies also showed that biantennary oligosaccharides are present in bovine fetuin as minor components.

Acknowledgments

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Appendix

Simulation of the Binding of a Mixture of Two Ligands to a Single Class of Binding Sites. The binding of a mixture of two different ligands in a known proportion to a single class of binding sites can be simulated by using the mass action equations describing the binding of the individual ligands, L_1 and L_2, to the receptor:

$$K_{d1} = [R][L_1]/[RL_1] \quad \text{or} \quad [L_1] = K_{d1}[RL_1]/[R] \tag{1}$$

$$K_{d2} = [R][L_2]/[RL_2] \quad \text{or} \quad [L_2] = K_{d2}[RL_2]/[R] \tag{2}$$

where R, RL_1, and RL_2 represent unoccupied receptor sites, sites containing bound L_1, and sites containing bound L_2, respectively. The brackets denote concentration.

The relationships between the various species can be further stated by the mass conservation equations:

$$[R]_t = [R] + [RL_1] + [RL_2] \quad \text{(total receptor)} \tag{3}$$

$$[L_1]_t = [L_1] + [RL_1] \quad \text{(total ligand 1)} \tag{4}$$

$$[L_2]_t = [L_2] + [RL_2] \quad \text{(total ligand 2)} \tag{5}$$

$$[RL]_t = [RL_1] + [RL_2] \quad \text{(bound ligand)} \tag{6}$$

Note that bound in eq 6 would be the experimentally determined dependent variable. The total ligand dose (experimental independent variable), L_t, is given by

$$[L]_t = [L_1]_t + [L_2]_t \tag{7}$$

The ratio of $[L_1]$ to $[L_1]_t$ is defined thus:

$$f_1 = [L_1]_t/[L]_t \quad (8)$$

For constant values of K_{d1}, K_{d2}, $[R]_t$ and f_1, and a given value of $[L]_t$, the unknowns ($[RL_1]$, $[RL_2]$, $[RL]_t$, $[L_1]$, and $[L_2]$) may be calculated as follows:

(A) The dose for each individual ligand was calculated. From eq 8:

$$[L_1]_t = f_1[L]_t$$

and

$$[L_2]_t = [L]_t - [L_1]_t \quad (8a)$$

(B) An initial estimate is assigned to $[R]$, and $[RL_1]$ and $[RL_2]$ are calculated. From eq 1:

$$[L_1] = \frac{(K_{d1}/[R])[L_1]_t}{1 + (K_{d1}/[R])}$$

or, from eq 4:

$$[RL_1] = [L_1]_t - \frac{(K_{d1}/[R])[L_1]_t}{1 + (K_{d1}/[R])}$$

Similarly, from eq 2 and 5:

$$[RL_2] = [L_2]_t - \frac{(K_{d2}/[R])[L_2]_t}{1 + (K_{d2}/[R])}$$

(C) The value of $[R]$ is adjusted and the calculations in (A) and (B) are repeated until the approximate equalities below are within a present tolerance:

$$[R]_t(\text{calcd}) = [RL_1] + [RL_2] + [R] \quad (9)$$

Define

$$A \equiv [R]_t(\text{calcd})/[R]_t(\text{given}) \quad (10)$$

and

$$[R]_t(\text{new}) \equiv [R]/A \quad (11)$$

(D) The calculations in (B) and (C) are repeated until $A = 1$ within a preset tolerance (e.g., ± 0.001).

(E) The apparent bound ($[RL] = [RL_1] + [RL_2]$) and the apparent free ($[L] = [L_1] + [L_2]$) were calculated and plotted according to Scatchard (1949) and compared to data calculated by using the parameters from the LIGAND program of binding data of different glycopeptide mixtures.

Registry No. NONA I, 67299-25-6; NONA II, 103776-01-8; Galβ(1,3)GlcNAcβ(1,4), 103776-00-7.

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