Control of Glycoprotein Synthesis. Bovine Milk UDPgalactose:*N*-Acetylglucosamine β-4-Galactosyltransferase Catalyzes the Preferential Transfer of Galactose to the GlcNAcβ1,2Manα1,3- Branch of both Bisected and Nonbisected Complex Biantennary Asparagine-Linked Oligosaccharides†

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ABSTRACT: Bovine milk UDPgalactose:*N*-acetylglucosamine β-4-galactosyltransferase has been used to investigate the effect of a bisecting GlcNAc residue (linked β1,4 to the β-linked mannose of the trimannosyl core of asparagine-linked complex oligosaccharides) on galactosylation of biantennary complex oligosaccharides. Columns of immobilized lectins (concanavalin A, erythroagglutinating phytohemagglutinin, and *Ricinus communis* agglutinin 120) were used to separate the various products of the reactions. Preferential galactosylation of the GlcNAcβ1,2Manα1,3 arm occurred both in the absence and in the presence of a bisecting GlcNAc residue; the ratio of the rates of galactosylation of the Manα1,3 arm to the Manα1,6 arm was 6.5 in the absence of a bisecting GlcNAc and 2.8 in its presence. The bisecting GlcNAc residue reduced galactosylation of the Manα1,3 arm by about 78% probably due to steric hindrance of the GlcNAcβ1,2Manα1,3Manβ1,4 region of the substrate by the bisecting GlcNAc. This steric hindrance prevents the action of four other enzymes involved in assembly of complex asparagine-linked oligosaccharides and indicates the importance of the bisecting GlcNAc residue in the control of glycoprotein biosynthesis. The Manα1,3 arm of biantennary oligosaccharides is believed to be freely accessible to enzyme action whereas the Manα1,6 arm is believed to be folded back toward the core. This may explain the preferential action of Gal-transferase on the Manα1,3 arm of both bisected and nonbisected oligosaccharides.

Oligosaccharides attached to the polypeptide backbone by a *N*-acetyl-D-glucosamine–asparagine (GlcNAc–Asn)[1] linkage occur in a wide variety of glycoproteins. Complex Asn-linked oligosaccharides consist of a $(Man)_3(GlcNAc)_2Asn$ core to which are attached two or more antennae (Figure 1). The assembly of antennae occurs in a highly ordered manner (Schachter et al., 1983). An important factor in the process is the prevention of at least four enzyme-catalyzed steps by the insertion of a bisecting GlcNAc residue (linked β1,4 to the β-linked Man of the trimannosyl core). The enzyme that inserts the bisecting GlcNAc residue has been called UDPGlcNAc:GnGn (GlcNAc to Manβ1,4) β-4-GlcNAc-transferase III (Narasimhan, 1982) and the enzymes that cannot act on bisected substrates are (i) GlcNAc-transferase II which initiates the antenna-attached β1,2 to the Manα1,6 arm of the core (Harpaz & Schachter, 1980a), (ii) GlcNAc-transferase IV which initiates the antenna-attached β1,4 to the Manα1,3 arm (Gleeson & Schachter, 1983), (iii) the α-6-Fuc-transferase which inserts a Fuc into the Asn-linked GlcNAc of the core (Longmore & Schachter, 1982), and (iv) mannosidase II which removes the last two Man residues during the processing of high mannose to complex oligosaccharides (Harpaz & Schachter, 1980b).

The inhibitory action of the bisecting GlcNAc residue is believed to be due to steric obstruction of the GlcNAcβ1,2Manα1,3Manβ1,4 region of the substrate, and it has been suggested that this region is a binding site for all of the above four enzymes (Brisson & Carver, 1983). Indeed, addition of a Gal residue in β1,4 linkage to the terminal GlcNAc of GlcNAcβ1,2Manα1,3Manβ1,4 will prevent the actions of the above four enzymes even in the absence of a bisecting GlcNAc residue and will also prevent the action of GlcNAc-transferase III (Schachter et al., 1983).

The structure GG(Gn) (Figure 1) has been found in several glycoproteins, e.g., rat kidney γ-glutamyltranspeptidase (Yamashita et al., 1983b), human IgD (Mellis & Baenziger, 1983), human IgG (Savvidou et al., 1984), human yolk sac tumor α-fetoprotein (Yamashita et al., 1983c), and human erythrocyte membrane band 3 (Tsuji et al., 1981). The structure SS(Gn) (Figure 1) has been found in human IgG (Baenziger & Kornfield, 1974; Savvidou et al., 1984; Mizuochi et al., 1982), bovine IgG (Baenziger & Fiete, 1979a), rat α-fetoprotein (Bayard et al., 1983), and human yolk sac tumor α-fetoprotein (Yamashita et al., 1983c). Since the presence of a Gal residue on the GlcNAcβ1,2Manα1,3 arm prevents the action of GlcNAc-transferase III, the synthetic path toward

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[1] Abbreviations: Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Fuc, L-fucose; Asn, asparagine; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; NMR, nuclear magnetic resonance; ACS, aqueous counting scintillant; OCS, organic counting scintillant; Con A, concanavalin A; E-PHA, erythroagglutinating phytohemagglutinin; RCA-120, *Ricinus communis* agglutinin 120. Complex Asn-linked oligosaccharides are named according to the sugar residues at the nonreducing termini by using the following abbreviations: M, Man; Gn, GlcNAc; G, Gal; S, sialyl residues; (Gn), a bisecting GlcNAc residue linked β1,4 to the β-linked Man of the core (Figure 1).

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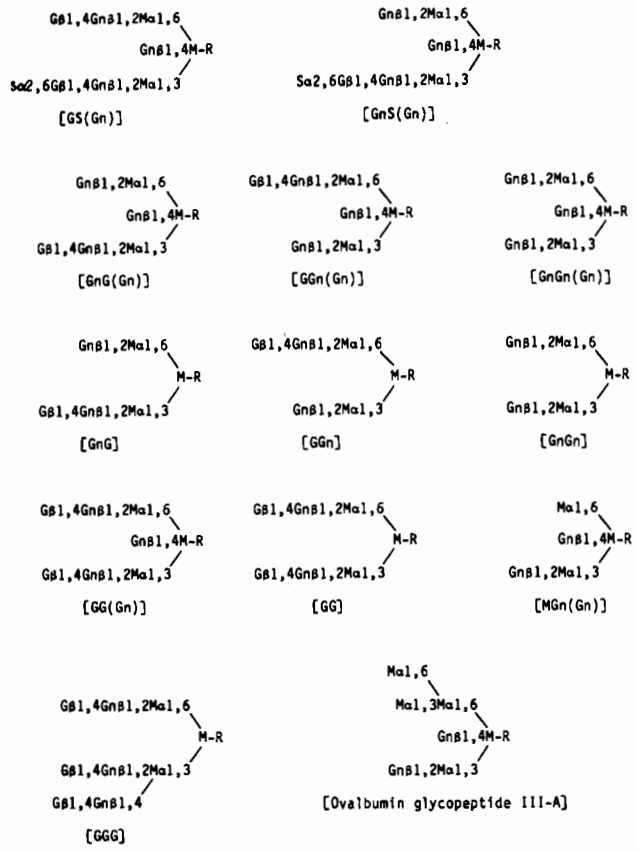

FIGURE 1: Structures and nomenclature of glycopeptides used in this study. The nomenclature has been published previously (Narasimhan et al., 1979, 1980; Grey et al., 1982; Carver & Grey, 1981; Narasimhan, 1982). Complex *N*-glycosyl glycopeptides are named according to the sugar residues at the nonreducing termini, the sugar on the Manα1,6 arm being named first. S, sialic acid; G, galactose; Gn, *N*-acetylglucosamine; M, mannose; (Gn), a bisecting GlcNAc linked β1,4 to the β-linked Man; R, GlcNAcβ1,4GlcNAc-Asn or GlcNAcβ1,4(Fucα1,6)GlcNAc-Asn.

GG(Gn) and SS(Gn) requires the insertion of a bisecting GlcNAc prior to the action of UDPGal:GlcNAc β-4-Gal-transferase.

We have therefore investigated the effect in vitro of a bisecting GlcNAc on the action of bovine milk β-4-Gal-transferase, as indicated in Figure 2a. We have previously reported (Pâquet et al., 1984) that rat liver Golgi β-4-Gal-transferase shows branch specificity toward the Manα1,3 arm of nonbisected biantennary substrate ($k_5/k_6 = 5$; Figure 2b). We now report that insertion of a bisecting GlcNAc reduces the rate of galactosylation of the Manα1,3 arm by about 78% ($k_5/k_1 = 4.6$; Figure 2) presumably due to the same steric factors responsible for the other inhibitory effects of a bisecting GlcNAc (Schachter et al., 1983; Brisson & Carver, 1983). However, even in the presence of a bisecting GlcNAc residue, there is specificity of galactosylation toward the Manα1,3 arm ($k_1/k_2 = 2.8$; Figure 2a), and both arms are eventually galactosylated to form GG(Gn).

Columns of immobilized lectins (Con A, E-PHA, and RCA-120) have been used to separate, identify, and quantitate the three products of galactosylation of both bisected [GnG-(Gn), GGn(Gn), and GG(Gn)] and nonbisected (GnG, GGn, and GG) biantennary glycopeptide (Figure 2). Preliminary reports of this work have appeared (Narasimhan & Freed, 1984; Freed & Narasimhan, 1984).

Experimental Procedures

Materials. UDP-D-[U-^{14}C]galactose (302 mCi/mmol) and [1-^{14}C]acetic anhydride (10 mCi/mmol) were from New

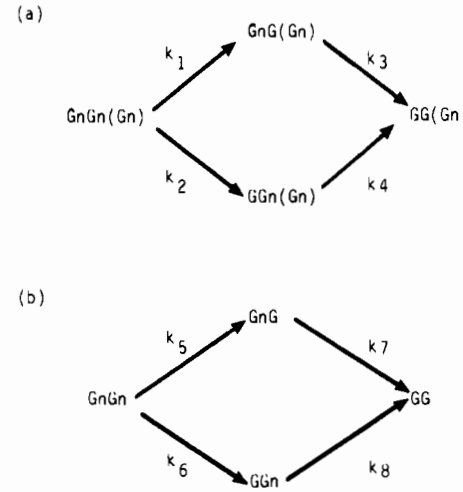

FIGURE 2: (a) Galactosylation of GnGn(Gn). (b) Galactosylation of GnGn.

England Nuclear, and UDP-D-[6-^{3}H]galactose (16.3 Ci/mmol) was from Amersham. Purified bovine milk UDPGal:GlcNAc β-4-galactosyltransferase (G-5507, 4.3 units/mg of protein) and jack bean β-galactosidase (19 units/mg of protein) were from Sigma. *Cl. perfringens* culture supernatant was used to prepare a mixture of β-D-galactosidase (1.5 units/mL) and β-*N*-acetyl-D-glucosaminidase (2 units/mL) (McGuire et al., 1972). An enzyme unit is defined as causing the conversion of 1 μmol of substrate/min. Sephadex G-25 and Con A/Sepharose 4B (lot no. HC24782, 10 mg of protein/mL of gel) were from Pharmacia, E-PHA/agarose (lot no. 0201F, 5 mg of protein/mL of gel) was from EY Labs, and RCA-120/agarose (lot no. AR 27, 1.2 mg of protein/mL of gel) was from Miles-Yeda. MES, HEPES, Pronase, and human fibrinogen (lot no. 102009) were from Calbiochem-Behring. Tris, UDP-galactose, GlcNAc, and Triton X-100 were from Sigma. Methyl α-D-glucopyranoside was from Nutritional Biochemical Corp. or Aldrich Chemical Corp. Mannose was from Pfanstiehl Labs. Lactose was from BDH. Bio-Gel P-2 (100–200 and 200–400 mesh), P-4 (–400 mesh, extra fine), P-6DG (80–170 mesh, <40-μm bead size), Chelex-100 (100–200 mesh, Na^+ form) and AG 1-X8 (100–200 mesh, Cl^- form) were from Bio-Rad. Deuterium oxide was from Aldrich Chemical Corp. (99.8% and 100%) or Merck Sharp & Dohme (99.7% and 100%). ACS and OCS were from Amersham. The β-*N*-acetylglucosaminidase inhibitor 2-acetamidoglucal was a kind gift from Dr. W. Korytnyk, Roswell Park Memorial Institute, Buffalo, NY. All other chemicals were reagent grade and used without further treatment.

Preparation of GnGn. Human fibrinogen (20 g) was incubated in 1 L of 0.1 N H_2SO_4 at 80 °C for 1.5 h to remove sialic acid. The protein was digested with Pronase (Narasimhan et al., 1979) and subjected to gel filtration on Sephadex G-25 in water. The glycopeptide fraction was subjected to a second Pronase digestion and gel filtration and was treated with jack bean β-galactosidase (Gleeson & Schachter, 1983). GnGn (Figure 1) was purified from this digest by gel filtration on Bio-Gel P-2 and high-voltage electrophoresis (Gleeson & Schachter, 1983). About 42 μmol of purified GnGn was obtained. The final preparation ran as a single band on high-voltage paper electrophoresis in 1% borate (Narasimhan et al., 1980) and had a proton NMR spectrum at 360 MHz characteristic of GnGn lacking a core fucose residue (Gleeson & Schachter, 1983).

Preparation of GnGn(Gn). The bisected glycopeptide

GnGn(Gn)(−F) [Figure 1; (−F) indicates the preparation to be free of core fucose] was prepared from hen ovalbumin (fraction V-1) and identified by proton NMR spectrometry at 360 MHz (Longmore & Schachter, 1982; Narasimhan, 1982). The preparation was passed through a Bio-Gel P-4 (−400 mesh) column (1.5 × 115 cm) in water to remove minor impurities.

Four preparations of [^{14}C]GlcNAc-labeled GnGn(Gn) were used in this study, as follows: (i) *N*-[^{14}C]Acetyl-GnGn-(Gn)(−F) was obtained by acetylation (Narasimhan et al., 1980) of GnGn(Gn)(−F) prepared from hen ovalbumin. (ii) GnGn(Gn*)(+F) glycopeptide contained core fucose and a [^{14}C]GlcNAc-labeled bisecting GlcNAc residue, prepared by the action of hen oviduct GlcNAc-transferase III on GnGn-(+F) (Narasimhan, 1982). (iii) GnGn(Gn*)(−F) glycopeptide was a similar preparation except that the substrate used for GlcNAc-transferase III action was GnGn(−F) free of fucose prepared from human transferrin (Gleeson & Schachter, 1983). (iv) GnGn(Gn*)(−F) oligosaccharide formed during the above incubation with GnGn(−F) due to the presence in hen oviduct membranes of an asparaginase which cleaved 53% of the Asn–GlcNAc bonds (Gleeson & Schachter, 1983). Since hen oviduct membranes contain both GlcNAc-transferases III and IV, preparative Con A/Sepharose chromatography (Narasimhan, 1982; Gleeson & Schachter, 1983) was used to separate the products of GlcNAc-transferases III and IV. Products were desalted on Bio-Gel P-2 columns (200–400 mesh, 2.5 × 44 cm) in water. GnGn(Gn*)(−F) glycopeptide and oligosaccharide were separated by gel filtration on Bio-Gel P-4 (−400 mesh, 1.5 × 115 cm).

Preparation of SS(Gn), GS(Gn), GG(Gn), and GGn(Gn). The bisected glycopeptides SS(Gn), GS(Gn), GG(Gn), and GGn(Gn) (Figure 1), all containing core fucose residues, were kind gifts of Dr. J. P. Carver, Department of Medical Genetics, University of Toronto, and were prepared from a Pronase digest of IgG (Hom) obtained from a human multiple myeloma patient, as previously described (Savvidou et al., 1981, 1984). All fractions were identified by proton NMR spectrometry at 360 MHz.

Preparation of ^{14}C-Labeled N-Acetylated GnS(Gn) and GnG(Gn). The bisected glycopeptides GnS(Gn) and GnG-(Gn), containing a core fucose residue, were prepared by sequential degradation of GS(Gn) from IgG (Hom). GS(Gn), 140 nmol, was acetylated with [^{14}C]acetic anhydride and purified (Narasimhan et al., 1979, 1980). Galactose was removed by incubation for 24 h with β-galactosidase from *Cl. perfringenes* in the presence of 10 mM 2-acetamidoglucal to inhibit β-*N*-acetylglucosaminidase. The resulting glycopeptide, GnS(Gn), was purified by gel filtration on Bio-Gel P-2 in water. GnS(Gn), 18 nmol or 98 000 dpm, was hydrolyzed in 0.5 mL of 2 N acetic acid at 100 °C for 15 min (Baenziger & Fiete, 1982) to remove sialic acid, and GnG(Gn) was purified on Bio-Gel P-2 in water. As a control experiment to ensure that our conditions of mild acid hydrolysis did not remove the bisecting GlcNAc residue, SS(Gn) was hydrolyzed to GG(Gn) under similar conditions. The conversions of GS(Gn) to GnS(Gn), of GnS(Gn) to GnG(Gn), and of SS-(Gn) to GG(Gn) were monitored by high-voltage paper electrophoresis in 1% borate (Narasimhan et al., 1980) and by lectin column chromatography using radioactivity to detect the positions of the various glycopeptides.

Preparation of GnG. Glycopeptide GnG, containing a core fucose residue, was prepared by sequential degradation of GS purified from a Pronase digest of IgG (Tem) from a human multiple myeloma patient (Narasimhan et al., 1979, 1980; Grey et al., 1982). GS (9 μmol) was converted to GnS by treatment with *Cl. perfringens* β-galactosidase in the presence of 10 mM 2-acetamidoglucal, purified on Bio-Gel P-2 in water, and hydrolyzed in 1 mL of 0.05 N HCl at 80 °C for 1 h to remove sialic acid. The digest was neutralized with 1 mL of 0.05 N $NaHCO_3$, and GnG was purified by preparative high-voltage paper electrophoresis in 1% borate (Narasimhan et al., 1980). This preparation of GnG gave a characteristic proton NMR spectrum at 360 MHz (Grey et al., 1982).

Other Glycopeptides. Glycopeptides GG (Narasimhan et al., 1979), MGn(Gn) (Narasimhan et al., 1980) and GGn (Grey et al., 1982), containing core fucose residues, were prepared from a Pronase digest of human IgG (Tem) as previously described. The glycopeptides were identified by their characteristic proton NMR spectra at 360 MHz (Grey et al., 1982). Glycopeptide GGG (Figure 1) from human α_1-acid glycoprotein was a kind gift from Dr. K. Schmid, Boston University. Glycopeptide III-A from a Pronase digest of hen ovalbumin (Tai et al., 1977) was isolated as previously described (Narasimhan et al., 1980) and identified by proton NMR spectrometry at 360 MHz.

Galactosyltransferase Incubations. The incubations contained the following in a final volume of 0.050 mL: 0.05–0.75 mM GnGn(Gn) or 0.04–0.53 mM GnGn, 0.1 M MES, pH 7.4, 10 mM $MnCl_2$, 0.5% (v/v) Triton X-100, 1.2 mM UDP-[^{14}C]Gal (1550 dpm/nmol), and 1.25 milliunits of bovine milk β-4-Gal-transferase. Incubations were from 15 to 60 min at 37 °C. The reaction was stopped by addition of 0.010 mL of 2% borate–0.25 M EDTA and the digest subjected to high-voltage paper electrophoresis in 1% borate (Narasimhan et al., 1980). Product was detected by counting strips of the electrophoretogram paper in OCS.

Time Course Study. Pseudo-first-order rate constants (Figure 2) were determined by measuring the products formed after different times of incubation (15, 30, 45, and 60 min). Initial substrate concentrations were chosen [0.5 mM GnGn(Gn) and 0.53 mM GnGn] such that the reaction velocity was proportional to substrate concentration during the time course. Incubation conditions were as above. Several incubations were carried out at each time point and were pooled to yield sufficient product for subsequent characterization. At every time point total incorporation of Gal was determined as above. The papers were then removed from the counting vials, product was extracted, borate was removed, and the glycopeptides were acetylated with nonradioactive acetic anhydride and were purified by gel filtration on Bio-Gel P-2 (100–200 mesh, 2.5 × 44 cm), all as previously described (Narasimhan et al., 1980; Pâquet et al., 1984). The acetylated glycopeptides were then analyzed on lectin columns as described below.

Exhaustive Galactosylation. GnGn(Gn) (6.3 and 12.5 nmol) was incubated for a total of 8 h under standard conditions but with relatively large amounts of galactosyltransferase (5 milliunits added at zero time and at 2.5 and 6 h) and UDP-[^{14}C]Gal. Incorporation of Gal into glycopeptide was determined at 1, 2.5, 4, 6, and 8 h. Incorporation reached a maximum between 2.5 and 4 h and did not change thereafter. This preliminary experiment suggested the following conditions for exhaustive galactosylation; i.e., [^{14}C]GlcNAc-labeled GnGn(Gn) (0.21–7.3 nmol; see Results) was incubated with 2.1 mM UDP-[^{3}H]Gal (14 400 dpm/nmol) and 5 milliunits β4-Gal-transferase under standard conditions at 37 °C for 2.5 h. A further 5 milliunits of Gal-transferase was added, and incubation was continued for a total of 4 h. Reaction was stopped by addition of 1 mL of ice-cold water, and the solution

was passed through a column (0.6 × 5 cm) of AG 1-X8 (100–200 mesh, Cl^- form). The column was washed with 2 mL of water, and the total eluate (3.06 mL) was collected. Both ^{14}C and ^3H were measured on an aliquot of this eluate; control incubations in the absence of exogenous acceptor were also carried out, and the values obtained were subtracted. The eluates from the AG 1-X8 columns were flash evaporated to dryness, nonacetylated samples were acetylated with nonradioactive acetic anhydride (Narasimhan et al., 1980; Pâquet et al., 1984), and all samples were passed through Bio-Gel P-2 columns (100–200 mesh; 1.2 × 48 cm) in water to remove salts and any free [^3H]galactose formed during the incubations. The purified double-labeled products were analyzed on lectin columns. Exhaustive galactosylation was carried out under similar conditions on ovalbumin glycopeptide III-A and IgG glycopeptide MGn(Gn) (see Figure 1 for structures).

Con A/Sepharose Chromatography. Conditions were modified from those described by Narasimhan et al. (1979). The products of Gal incorporation into GnGn(Gn) were fractionated on 0.7 × 16 cm columns run at room temperature. Small aliquots of every fraction were counted, and the glycopeptide peaks were desalted on Bio-Gel P-2 (1 × 39 cm) in water.

E-PHA/Agarose Chromatography. Conditions were derived from Cummings & Kornfeld (1982). Columns (0.5 × 60 cm) were run at room temperature at 2.6 mL/h in PBS (6.7 mM KH_2PO_4, pH 7.4, and 0.15 M NaCl) containing 0.02% sodium azide.

RCA-120/Agarose Chromatography. Conditions were modified from Gleeson & Schachter (1983). Column size was 0.5 × 44 cm, and runs were carried out at room temperature at 4.2 mL/h in 0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.02% sodium azide. The samples were allowed to interact with the gel for 1 h before elution. The column was eluted with 90 mL of the above buffer and then with 20 mL of 0.1 M lactose in the same buffer.

Other Methods. Glycopeptide concentrations were determined by amino acid analysis for aspartic acid as described previously (Narasimhan et al., 1980). ^{14}C-Labeled N-acetylation of glycopeptides and purification of acetylated glycopeptides were carried out as previously described (Narasimhan et al., 1979, 1980; Pâquet et al., 1984). Radioactivity on paper was counted in OCS, and aqueous solutions were counted in 10 mL of ACS by using an LKB Rackbeta counter. Proton NMR spectrometry was carried out as previously described (Narasimhan et al., 1980) on samples which had been exchanged in 100% D_2O and on a Nicolet 360-MHz spectrometer (Carver & Grey, 1981). Glycopeptides were monitored on columns either by the phenol–sulfuric acid method (Dubois et al., 1956) or by counting. Sialic acid release was monitored by the method of Warren (1959), galactose release by the method of Finch et al. (1969), and GlcNAc release by the method of Reissig et al. (1955).

Results

Galactosylation of Biantennary Glycopeptides. Figure 3 shows that pseudo-first-order kinetics are obeyed for bovine milk β-4-galactosyltransferase at substrate concentrations below 0.7 mM for GnGn(Gn) and below 0.4 mM for GnGn; i.e., enzyme velocity is proportional to substrate concentration. The slope of the velocity vs. substrate concentration plot (approximately equal to V_{max}/K_m) is 0.0070 ± 0.0002 min^{-1} for GnGn(Gn) and 0.021 ± 0.0003 min^{-1} for GnGn. In terms of the schemes shown in Figure 2, $k_1 + k_2 = 0.0070$ and $k_5 + k_6 = 0.021$.

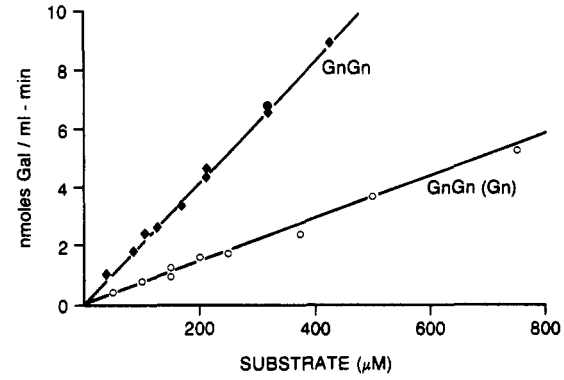

FIGURE 3: Velocity–substrate curves for the galactosyltransferase reaction under standard conditions with GnGn(Gn) (○) and GnGn (♦) as substrates.

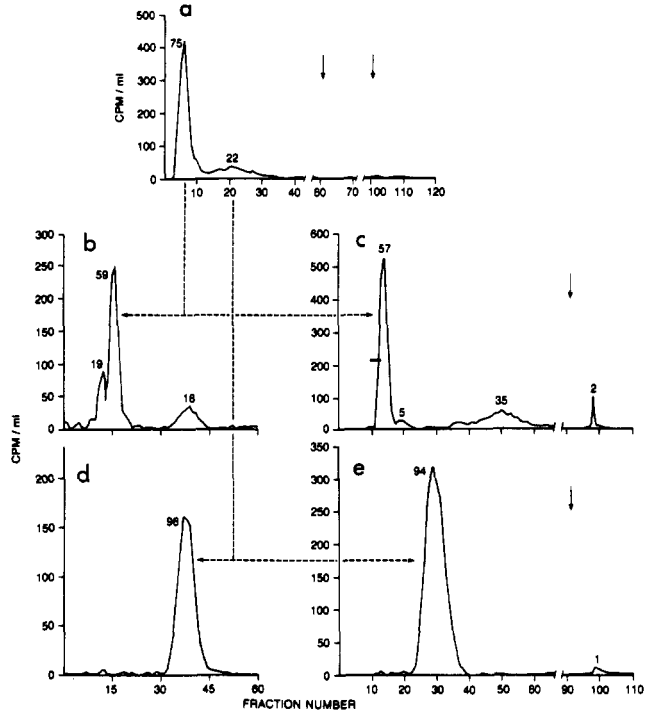

FIGURE 4: Analysis of the products of galactosylation of GnGn(Gn) after 45 min of incubation on lectin/agarose columns. The numbers near the peaks represent the percent radioactivity recovered in the peak. Part a shows the elution pattern obtained on a Con A/Sepharose column. The two arrows indicate the points of addition of 10 mM and 100 mM methyl-α-glucoside, respectively. The first Con A peak is analyzed on E-PHA/agarose (b) and RCA-120/agarose (c). The second Con A peak is analyzed on E-PHA/agarose (d) and RCA-120/agarose (e). The arrows in parts c and e indicate the points of addition of 0.1 M lactose. The radioactivity loaded on every column varied between 1030 and 2600 cpm, and the percent recovery varied between 97 and 100%.

A time course was carried out to resolve the various pseudo-first-order rate constants shown in Figure 2. The incorporation of Gal into GnGn(Gn) and GnGn at various times is shown in Table I. Incorporation into GnGn was about 3 times as fast as into GnGn(Gn).

Figure 4 shows the resolution of the products of galactosylation of GnGn(Gn) after 45 min of incubation. Similar results were obtained for the other time points. The retarded peak on Con A/Sepharose (fraction 20, Figure 4a) elutes at the same position as a standard preparation of GGn(Gn) (data not shown). This fraction eluted at 40 mL on E-PHA/agarose (Figure 4d) and at 30 mL on RCA-120/agarose (Figure 4e), the elution positions of standard GGn(Gn) on these two lectin columns (data not shown).

Table I: Incorporation of Galactose into GnGn(Gn) and GnGn[a]

time of incubation (min)	total galactose incorporated (nmol/0.050 mL) GnGn(Gn)	GnGn
15	4.2	12.5
30	6.8	18.5
45	9.0	23.8
60	9.8	25.8

[a] Initial substrate concentrations were 0.5 mM GnGn(Gn) (25 nmol/0.050 mL) and 0.53 mM GnGn (26.5 nmol/0.050 mL).

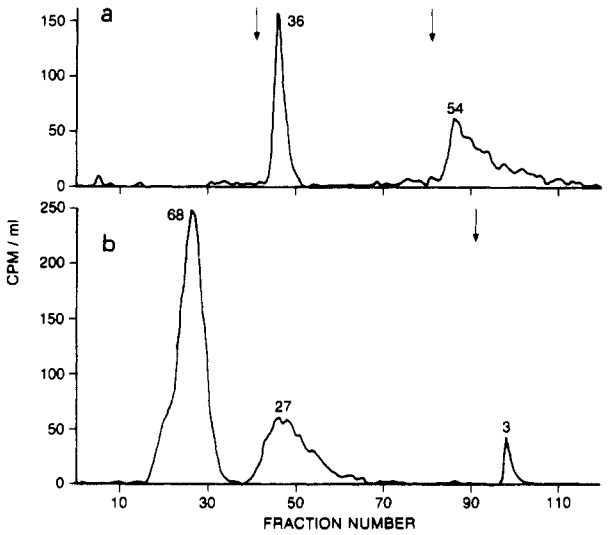

FIGURE 5: Analysis of the products of galactosylation of GnGn, after 45 min of incubation, on Con A/Sepharose (a) and RCA-120/agarose (b). The numbers near the peaks represent the percent radioactivity recovered in the peak. The arrows are as explained in the legend to Figure 4. The radioactivity loaded on every column varied between 2000 and 2660 cpm, and the percent recovery varied between 83 and 100%.

The unretarded peak on Con A/Sepharose (fraction 5, Figure 4a) resolved into two fractions on both E-PHA/agarose (Figure 4b) and RCA-120/agarose (Figure 4c). The major fraction interacted weakly with both E-PHA/agarose (fraction 15) and RCA-120/agarose (Fraction 12). A standard preparation of GnG(Gn) eluted at fractions 5, 15, and 12 on these three lectin columns, respectively (data not shown), and it is therefore concluded that the major Con A unretarded fraction is GnG(Gn).

The minor component of the Con A unretarded fraction interacted strongly with both E-PHA (fraction 40, Figure 4b) and RCA-120 (fraction 50, Figure 4c). Standard GG(Gn) was found to pass through Con A/Sepharose unretarded but eluted from E-PHA/agarose and RCA-120/agarose at fractions 40 and 55, respectively (data not shown). The minor component of the Con A unretarded fraction was therefore identified as GG(Gn).

Thus, a combination of lectin columns can be used to separate and quantitate GnG(Gn), GGn(Gn), and GG(Gn). This allows calculations of the four kinetic constants k_1–k_4 (Figure 2a).

It is possible by analogous methods to separate and quantitate GnG, GGn, and GG and thereby to calculate the rate constants k_5–k_8 (Figure 2b). Figure 5 shows the resolution of the products of galactosylation of GnGn after 45 min of incubation. Similar results were obtained for the other time points. Two fractions were obtained on Con A/Sepharose (Figure 5a). The fraction requiring 0.1 M methyl α-D-glucopyranoside for elution was identified as GnG since standard GnG behaves in an identical fashion (data not shown). The fraction eluted with 0.01 M methyl α-D-glucopyranoside was identified as a mixture of GGn and GG since standard preparations of these two glycopeptides elute in an identical manner (data not shown).

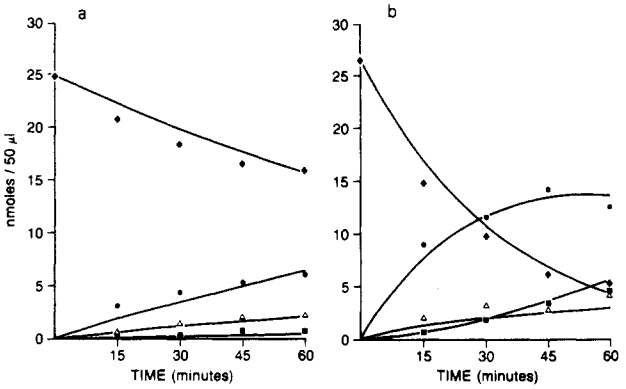

FIGURE 6: (a) Kinetics of galactosylation of GnGn(Gn). (b) Kinetics of galactosylation of GnGn. The ordinate shows nanomoles of Gal incorporated per 0.050 mL. The symbols are as follows: (♦) GnGn(Gn) and GnGn; (●) GnG(Gn) and GnG; (△) GGn(Gn) and GGn; (■) GG(Gn) and GG. The smooth lines were computer fitted by using the following k values: $k_1 = 0.0056$; $k_2 = 0.0020$; $k_3 = 0.0013$; $k_4 = 0.0043$, $k_5 = 0.026$; $k_6 = 0.004$; $k_7 = 0.009$; $k_8 = 0.0001$.

Table II: Pseudo-First-Order Rate Constants (min^{-1}) for Galactosylation

GnGn(Gn)	GnGn
$k_1 = 0.0056 \pm 0.0012$	$k_5 = 0.026 \pm 0.003$
$k_2 = 0.0020 \pm 0.0012$	$k_6 = 0.004 \pm 0.003$
$k_3 = 0.0013 \pm 0.0050$	$k_7 = 0.009 \pm 0.006$
$k_4 \simeq 0.004 \pm 0.025$	$k_8 < 0.015$

We have confirmed the findings of others (Cummings & Kornfeld, 1982; Mellis & Baenziger, 1983; Yamashita et al., 1983a) that nonbisected glycopeptides do not adhere to E-PHA/agarose. This lectin column is therefore of no use in the separation of nonbisected glycopeptides. It has been reported that RCA I interacts with GG more strongly than with GnG (Kornfeld et al., 1981; Baenziger & Fiete, 1979b). We have verified this finding and shown in addition that GGn interacts even more weakly than GnG. Thus, RCA-120/agarose was found to resolve GG (fraction 50) from GnG (fraction 30) and GGn (fraction 20) (data not shown). The product peak at fraction 48 on RCA-120/agarose (Figure 5b) is therefore identified as GG, and the major peak between fractions 18 and 30 is a mixture of GGn and GnG. The two lectin columns therefore allow separation and quantitation of GnG and GG. The amount of GGn was calculated by difference.

When the amounts of the three radioactive products of galactosylation at the four incubation times were known, the amounts of GnGn(Gn) and GnGn remaining were calculated by difference. The data are summarized in Figure 6. Galactosylation of the Manα1,3 arm proceeds more rapidly for both bisected and nonbisected compounds, but galactosylation is greatly inhibited by the presence of a bisecting GlcNAc.

Four equations relating the amount of each glycopeptide to the four pseudo-first-order rate constants shown in part a or b of Figure 2 have been presented in a previous publication (Pâquet et al., 1984). The experimental data were fitted to these equations to obtain estimates of the various rate constants (Table II). The fits for k_1, k_2, k_5, and k_6 had relatively small standard errors (Table II). Further, the values for $k_1 + k_2$ and $k_5 + k_6$ obtained from the time course were 0.0076 and 0.030 min^{-1}, respectively, which compare well with the corresponding values obtained from velocity vs. substrate con-

Table III: Exhaustive Galactosylation of GnGn(Gn)[a]

substrate	nmol/ 0.050 mL	$^3H/^{14}C$ molar ratio of galactosylation product: after P-2	$^3H/^{14}C$ molar ratio of galactosylation product: after E-PHA/ agarose
GnGn(Gn*)(+F)	5.2	1.9	2.0
GnGn(Gn*)(-F)	6.4	2.0	1.9
GnGn(Gn*) oligosaccharide	7.3	2.2	1.9
N-[^{14}C]acetyl-GnGn(Gn)(-F)	0.21	2.1	1.8

[a] The preparation of the four substrates and the nomenclature are explained under Experimental Procedures. All four substrates were labeled with ^{14}C (indicated by asterisk) and were incubated with UDP-[^{3}H]Gal and galactosyltransferase as described under Experimental Procedures. Double-labeled products were purified by succesive passage through AG 1-X8, Bio-Gel P-2, and E-PHA/agarose columns.

centration plots (Figure 3), 0.0070 and 0.021 min^{-1}, respectively.

The errors for the other four rate constants were large (Table II). A separate experiment was therefore carried out to determine k_4. GGn(Gn) was incubated with β-4-Gal-transferase under standard conditions at substrate concentrations ranging between 0.08 and 0.56 mM. A linear velocity vs. substrate concentration plot was obtained (data not shown) with a slope of 0.0043 ± 0.0004 min^{-1}, thereby giving a more accurate value for k_4.

[^{3}H]Gal Incorporation into ^{14}C-Labeled GnGn(Gn). Four different preparations of ^{14}C-labeled GnGn(Gn) (0.21–7.3 nmol in 0.050 mL; Table III) were incubated with 2.1 mM UDP-[^{3}H]Gal and a total of 10 milliunits of β-4-Gal-transferase for 4 h at 37 °C. Preliminary experiments (see Experimental Procedures) showed that galactosylation had leveled off under these conditions. In all four incubations, the elution of GnGn(Gn) from E-PHA/agarose was altered from 15–16 mL to 39–40 mL [the position of standard GG(Gn)] by exhaustive galactosylation (data not shown), and the molar $^3H/^{14}C$ ratio was 1.8–2.0 (Table III). The elutions on RCA-120/agarose after exhaustive galactosylation also coincided with standards of GG(Gn) (about 55 mL) whereas standard GGG eluted from our RCA-120/agarose column in a broad peak at about 68 mL (data not shown).

It is concluded that only two Gal residues per mole can be incorporated into GnGn(Gn) and that this incorporation is identical for glycopeptides and oligosaccharides and occurs both in the presence and absence of a core fucose residue. It is also concluded that the behavior of GnGn(Gn) and GG(Gn) on E-PHA/agarose and RCA-120/agarose is not influenced by the presence or absence of a core fucose.

Other Glycopeptide Substrates. Galactosylation was carried out, as above, on ovalbumin glycopeptide III-A (Figure 1). Both III-A and its galactosylated product (major peak) eluted from E-PHA/agarose at 12 mL, and the product (major peak) eluted from RCA-120/agarose at 21 mL (data not shown), suggesting incorporation of only one Gal per mole. A similar experiment was performed with MGn(Gn) (Figure 1). Both MGn(Gn) and galactosylated MGn(Gn) eluted from E-PHA/agarose at 12 mL, and galactosylated MGn(Gn) eluted from RCA-120/agarose at 14 mL (data not shown), suggesting incorporation of only a single Gal residue per mole of glycopeptide.

Discussion

The purpose of this study was to determine the effect of a bisecting GlcNAc residue on the galactosylation by bovine milk β-4-galactosyltransferase of biantennary complex oligosaccharides. Lectin affinity chromatography was used to resolve the products of galactosylation when bovine milk β-4-Gal-transferase acts on GnGn(Gn) or GnGn (Figure 2). We have verified previous work using purified β-4-Gal-transferases from calf thymus (Blanken et al., 1983) and rat liver Golgi (Pâquet et al., 1984) that there is preferential galactosylation of the Manα1,3 arm of GnGn (Figure 2b). The ratio k_5/k_6 was 6.5 in the present work and 5.4 in the report of Pâquet et al. (1984).

The presence of a bisecting GlcNAc in GnGn(Gn) inhibits the activity of β-4-Gal-transferase by 78% ($k_1/k_5 = 0.22$). The enzyme still shows preference for the Manα1,3 arm but at a reduced rate ($k_1/k_2 = 2.8$). It has been suggested (Brisson & Carver, 1983; H. Van Halbeek and J. F. G. Vliegenthart, personal communication) that the Manα1,3 arm of both bisected and nonbisected biantennary oligosaccharides is freely available to the environment whereas the Manα1,6 arm is folded back toward the core. This probably explains the preferential action of Gal-transferase on the Manα1,3 arm. Brisson & Carver (1983) have also pointed out that the bisecting GlcNAc covers the Manα1,3 arm, thereby inhibiting the action of four enzymes involved in glycoprotein synthesis (Schachter et al., 1983). This same steric hindrance probably explains the inhibition of Gal-transferase action on the Manα1,3 arm of bisected oligosaccharides.

We have shown that complete galactosylation of GnGn(Gn) leads to the incorporation of only two Gal residues. Lectin column studies of the galactosylated products strongly suggest that these two Gal residues are on the antennae and that the bisecting GlcNAc is not galactosylated. Similar conclusions were drawn from galactosylation of ovalbumin glycopeptide III-A and MGn(Gn). Indeed, a galactosylated bisecting GlcNAc residue has not been reported. The same steric factors by which the bisecting GlcNAc covers the Manα1,3 arm probably act in reverse to make the bisecting GlcNAc unavailable to galactosylation.

It has been reported that swine mesentery lymph node β-4-Gal-transferase had a lower K_m toward GnGn than toward monogalactosylated product (Rao & Mendicino, 1978). Paquet et al. (1984) reported that k_7 was less than k_5 and that k_8 was less than k_6 (Figure 2b). We have verified this finding for nonbisected oligosaccharides.

Our studies have provided in vitro evidence that the bisected biantennary oligosaccharide structures present in many glycoproteins (see the introduction) are synthesized by sequential action of GlcNAc-transferase III, which inserts a bisecting GlcNAc residue followed by the action of Gal-transferase on the bisected structures. The latter show a greatly reduced rate of galactosylation relative to nonbisected oligosaccharides.

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