Analysis of carbohydrate–protein interactions with synthetic N-linked neoglycoconjugate probes

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Recently we have described a simple efficient chemical method of generating an asparagine side-chain linker with β -stereochemistry at the anomeric position of neutral oligosaccharides. We now report the 1-*N*-glycyl β -derivatization of sialylated saccharides. Several neoglycoconjugates formed using these N-linked intermediates were investigated for their usefulness in probing carbohydrate–protein interactions. First, biotinyl derivatives of two xylose/fucose class plant-type oligosaccharides purified from horseradish peroxidase were effective in demonstrating the carbohydrate specificity of polyclonal anti-(horseradish peroxidase) antibodies. Secondly, a fluorescein-labelled asialo- and digalactosylated biantennary complex sugar was synthesized and shown to bind to a *Ricinus communis* agglutinin column. This galactose-specific recognition was abolished by treating this fluorescein-labelled oligosaccharide with jack bean β -galactos-

idase. Finally, two 1-*N*-glycyl β -saccharide derivatives were modified with thiophosgene to form their corresponding isothiocyanate derivatives. Coupling of these isothiocyanate derivatives of sugars to BSA, amino-derivatized polystyrene plates and glass-fibre discs resulted in multiple sugar presentation. The binding of an anti-*N*-acetylglucosamine monoclonal antibody to *N,N'*-diacetylchitobiose residues presented on BSA and solid supports was shown by e.l.i.s.a. Similarly the binding of concanavalin A to asialo-, agalactosylated biantennary complex oligosaccharide residues attached to BSA was demonstrated by a competitive e.l.i.s.a. Our results demonstrate that N-linked neoglycoconjugates could be made readily available and they are valuable tools for the detailed analyses of carbohydrates and carbohydrate-binding proteins.

INTRODUCTION

There is a diverse number of highly branched and structurally complex oligosaccharides attached to secreted and membrane-bound proteins. Glycans are important because they can influence not only the intrinsic properties of a glycoprotein, but also its bioactivity, immunogenicity, circulatory life-time and biodistribution (Olden et al., 1982; Rademacher et al., 1988; Varki, 1993). Cellular recognition events mediated by specific carbohydrate ligands and their receptors have been established by such studies as the uptake of circulatory asialoglycoproteins by the hepatocyte asialoglycoprotein receptor (Weiss and Ashwell, 1989), the transport of lysosomal enzymes by mannose-6-phosphate receptors (Kornfeld, 1992), and the adhesive interactions between leucocytes and endothelial cells by selectin receptors (Brandley et al., 1990; Lasky, 1992). The recent discovery of a number of genes encoding proteins belonging to either the S- or C-type lectin superfamily further suggests important roles for carbohydrates in a variety of biological processes (Drickamer, 1993; Drickamer and Taylor, 1993). Delineating these various roles of carbohydrates is a major focus of glycobiological research.

Attachment of oligosaccharides to chemical and biological compounds could provide valuable neoglycoconjugate probes for analysing carbohydrates and carbohydrate–lectin interactions (Ginsburg, 1987; Lee, 1992). Immobilization of oligosaccharides could facilitate the identification, characterization and purification of lectins, enzymes and anti-carbohydrate antibodies. There exists a variety of chemical methods for derivatizing sugars (Stowell and Lee, 1980). Unfortunately most methods often require many synthetic steps with unpredictable yields and

unavoidable losses at each step; thus making them unsuitable for derivatizing oligosaccharides from biological sources which are available only in limited quantities. Some simpler methods introduce non-biological linkers such as aromatic rings (McBroom et al., 1972). The most commonly used method is reductive amination (Gray, 1974). A number of neoglycoproteins and neoglycolipids formed using this method have been used in assays *in vitro* for measuring lectin activities; these include the mannose-binding proteins and the selectins (Childs et al., 1989; Larkin et al., 1992). Although this method is simple, the cyclic structure of the reducing core residue of the oligosaccharide is destroyed. Undesirable immunogenicity of these derivatives and the loss of structural and biological information at the reducing end, are two major drawbacks associated with this coupling procedure. This method is also restrictive in that the reagent to be attached must have an amino group to react with the acyclic aldehyde form of the sugar.

Since a variety of reagents are available for labelling the amino group of amino acids, we have investigated the formation of oligosaccharide β -glycosylamines and N-acylation of β -glycosylamines with heterobifunctional reagents (Manger et al., 1992a). Recently, we have reported high yields (70–85%) of neutral oligosaccharide β -glycosylamines using a simple condensation method (Manger et al., 1992a) and have shown acylation of β -glycosylamines with small reactants such as chloroacetic anhydride to be fast and almost quantitative. However, for more bulky reactants such as fluorescein and dansyl chloride, it was necessary to generate 1-*N*-glycyl β -saccharide derivatives to achieve acceptable yields of 55–70% and to maintain β -stereochemistry of the desired product. We have suggested the use of 1-*N*-glycyl β -oligosaccharide derivatives

Abbreviations used: Con A, concanavalin A; FITC, fluorescein isothiocyanate; Fuc, fucose; HRP, horseradish peroxidase; l.d.m.s., laser-desorption m.s.; NeuNAc, sialic acid; RCA, *Ricinus communis* agglutinin.

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as intermediates in the formation of a variety of glycobiological probes because the resulting neoglycoconjugates have several highly desirable characteristics not found in neoglycoconjugates formed by other methods including reductive amination (Manger et al., 1992a).

The fact that a number of mammalian lectins, including the selectins, have specificities for sialylated sugars (Varki, 1992; Lasky, 1992; Foxall et al., 1992) prompted us to investigate the formation of neoglycoconjugates involving these carbohydrates using β -glycosylamine intermediates. In this paper we report similar high yields and efficiencies of derivatizing sialyl-lactosamines using our original method designed for neutral oligosaccharides. We also show that these types of sugar derivatives can readily be conjugated to biotin, proteins and fluorescein to provide a wide range of neoglycoconjugate probes. In addition these oligosaccharide derivatives can be immobilized on amino-derivatized e.l.i.s.a. plates and glass-fibre discs. More importantly these neoglycoconjugates can be recognized by lectins, enzymes and anti-carbohydrate antibodies. Our results illustrate some of the many potential applications for these types of oligosaccharide derivatives in glycobiology.

EXPERIMENTAL

Sugar samples

N,N'-Diacetylchitobiose was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). The following oligosaccharides were used in this study: G(0), GlcNAc β 1-2Man α 1-3(GlcNAc β 1-2Man α 1-6)Man β 1-4GlcNAc β 1-4GlcNAc; G2, Gal β 1-4GlcNAc β 1-2Man α 1-3(Gal β 1-4GlcNAc β 1-2Man α 1-6)Man β 1-4GlcNAc β 1-4GlcNAc; G2F, Gal β 1-4GlcNAc β 1-2Man α 1-3(Gal β 1-4GlcNAc β 1-2Man α 1-6)Man β 1-4GlcNAc β 1-4(Fuc α 1-6)GlcNAc; HRP 8.9, Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc; and HRP 5.8, Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc. Oligosaccharide G(0), NeuNAc α 2-3Gal β 1-4GlcNAc and NeuNAc α 2-6Gal β 1-4GlcNAc were purchased from Oxford Glycosystems (Oxford, U.K.). Oligosaccharides G2 and G2F were obtained from human serotransferrin and pig thyroglobulin respectively using hydrazinolysis and were purified using Bio-Gel P-4 gel-filtration chromatography. The expected structures of purified G2 and G2F oligosaccharides were confirmed using a combination of ^1H -n.m.r. and methylation-analysis methods as previously described (Ashford et al., 1987). HRP 5.8 and HRP 8.9 oligosaccharides were prepared from horseradish peroxidase (HRP) (Sigma) as described for G2 and G2F. HRP 5.8 and HRP 8.9 were analysed by laser-desorption m.s. (l.d.m.s.) using LASERMAT (FinneganMat, Hemel Hempstead, Bucks., U.K.) according to the manufacturer's instructions.

Analyses

T.l.c. was performed using Merck 10 cm $\times$ 20 cm glass-backed Silica 60 F254 or 5 cm $\times$ 7.5 cm aluminium-backed Silica 60 h.p.t.l.c. plates obtained from BDH (Poole, Dorset, U.K.). For saccharides, Silica 60 t.l.c. plates were developed in acetonitrile/water (7:4, v/v) and 0.05% diaminobutane (Solvent A). Visualization of plates was performed using ninhydrin and orcinol sprays for amines and hexoses respectively. Fluorescein (FITC)-isothiocyanate oligosaccharide conjugates were viewed under u.v. light (254 and 366 nm). H.p.l.c. was performed as described previously (Manger et al., 1992a); however, the types of columns and gradient conditions used in this study are described where appropriate. Amino-acid and hexosamine analysis has been described previously (Zamze et al., 1991).

SDS/PAGE

Samples of *N,N'*-diacetylchitobiose-BSA conjugate and BSA (4 mg/ml) were mixed with an equal volume of SDS/PAGE sample buffer containing 6% (w/v) SDS, 2.5 mM Tris/HCl (pH 8.0) and 0.45 mM 2-mercaptoethanol. SDS/PAGE was performed using a Phastgel Gradient 10–15% (Pharmacia). After electrophoresis, the gel was stained with Coomassie Blue (R350) according to the manufacturer's instructions. Molecular masses of BSA and *N,N'*-diacetylchitobiose-BSA were estimated using RAINBOW protein molecular mass markers consisting of myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (69.0 kDa), ovalbumin (46.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa), purchased from Amersham International (Amersham, Bucks., U.K.).

Preparation of β -glycosylamines and 1-*N*-glycyl β -saccharide derivatives

The formation of β -glycosylamines of neutral oligosaccharides and their subsequent chloroacetylation and ammonolysis to generate 1-*N*-glycyl β -saccharide derivatives was performed as described previously (Manger et al., 1992a). For anionic sugars such as NeuNAc α 2-6Gal β 1-4GlcNAc, it was necessary to omit the use of ion-exchange chromatography for desalting the mixture after chloroacetylation. The following procedure was used: after chloroacetylation, one vol. of ethanol and four vol. of *n*-butanol were added to the mixture. This mixture was then passed through a 1-ml cellulose column prewashed with distilled water and pre-equilibrated with *n*-butanol/ethanol/water (4:1:1, by vol.). The column was then washed with 3 ml of the same solvent before elution of sugar with 3 ml of water. Fractions (1 ml) of the eluates were collected, rotoevaporated to dryness and resuspended in 100 μ l of water. Aliquots (1 μ l) of each sample were analysed by t.l.c. using solvent A. After ammonolysis and lyophilization, the 1-*N*-glycyl β -derivative of NeuNAc α 2-6Gal β 1-4GlcNAc was further analysed by t.l.c. and l.d.m.s.

Biotinylation of oligosaccharide β -glycosylamine derivatives

The β -glycosylamine derivatives of HRP 5.8, HRP 8.9 and G2F oligosaccharides were biotinylated by *N*-acylation of the amino function with sulphosuccinimidyl-6-(biotinamido) hexanoate (Pierce and Warriner, Cheshire, U.K.) as described previously (Manger et al., 1992b). The biotinylated sugar derivatives were analysed by l.d.m.s. The *m/z* values of 1201, 1550 and 2150 were found and these correspond to $[\text{M} + \text{H}]^+$, $[\text{M} + \text{Na}]^+$ and $[\text{M} + \text{Na}]^+$ ions of the biotinylated HRP 5.8, HRP 8.9 and G2F oligosaccharides respectively.

Binding of biotinylated oligosaccharide derivatives to immobilized anti-HRP polyclonal antibodies

Rabbit anti-HRP polyclonal antibodies (Sigma) were adsorbed on to an e.l.i.s.a. plate (20 μ g of protein/well). The plate was washed with 10 mM Tris/HCl and 0.15 M NaCl, pH 7.4, (TBS) and blocked with 4% (w/v) BSA in TBS. Aliquots (100 μ l) of serial 2-fold dilutions of biotinylated HRP 5.8, HRP 8.9, and G2F oligosaccharides, at an initial concentration of 1 μ mol/ml, were added to the plate. After incubation for 2 h, the plate was washed with TBS. Addition of streptavidin-biotinylated alkaline phosphatase complex (Boehringer-Mannheim, Germany) to detect bound biotinylated sugars was performed according to the manufacturer's instructions. The plate was finally developed using *p*-nitrophenyl phosphate as substrate at 37 °C for 20 min and absorbance at 405 nm was measured using a Multiskan MCC340 plate reader.

Figure 1 Reaction scheme for generating β -glycosylamines and 1- N -glycyl β -derivatives of oligosaccharides

Oligosaccharide (1) is converted into its β -glycosylamine (2) by incubation with ammonium bicarbonate. Reaction of β -glycosylamine with chloroacetic anhydride forms the N -chloroacetamido derivative (3). Condensation of this derivative (3) with ammonium bicarbonate results in a 1- N -glycyl β -derivative (4). Reaction of (4) with thiophosgene produces an isothiocyanate 1- N -glycyl β -derivative (5) which can then be used to conjugate to various amine-containing compounds and supports (6). R = saccharides linked to the reducing terminal N -acetylglucosamine residue. X = BSA, aminopropyl glass-fibre disc, or amino-derivatized e.l.i.s.a. plate.

Synthesis and purification of fluorescent G(0) oligosaccharide conjugate

The 1- N -glycyl β -derivative of G(0) oligosaccharide (100 μg) in 100 μl of 0.1 M NaHCO_3 , pH 10, was mixed with a 10-fold molar excess of FITC isomer I (Fluka, Derbyshire, U.K.) dissolved in methanol. The reaction was allowed to proceed overnight at room temperature. The mixture was analysed by t.l.c. using solvent A. R_f values for FITC, FITC-G(0), and the 1- N -glycyl β -derivative of G(0) were 0.76, 0.57 and 0.03 respectively. The band representing FITC-G(0) was wetted with a small quantity of water and scraped off into a microcentrifuge tube. Silica was washed with water four or five times and centrifuged at 15000 rev./min (15200 g) for 5 min after each washing. Supernatant was dried and resuspended in 100 μl of water. FITC-G(0) was further purified by reverse-phase h.p.l.c. on an 8 mm $\times$ 250 mm S50DS2 C18 column (Hichrom, Reading, Berks., U.K.) using a 0–50% gradient of water and acetonitrile containing 0.1% trifluoroacetic acid for 30 min at a flow rate of 2 ml/min. The m/z value of the purified FITC-G(0) was determined by l.d.m.s. and the amount was determined by absorbance measurement of FITC-G(0) conjugate using ϵ 42000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for FITC.

Synthesis and purification of fluorescent G2 oligosaccharide conjugates

Oligosaccharide G2 (0.5 mg or 300 nmol) was converted into its β -glycosylamine derivative and dissolved in 50 μl of 50 mM triethylamine acetate buffer, pH 10. To this was added 50 μl of FITC dissolved in methanol to a concentration of 10 mg/ml. After stirring overnight at room temperature, the mixture was extracted with diethyl ether, rotoevaporated to dryness and resuspended in 200 μl of water. An aliquot (15 nmol of sugar) of this extract was dried, resuspended in 50 μl of 50 mM sodium acetate buffer, pH 6.0, and applied to a *Ricinus communis* agglutinin (RCA)-agarose column. Another aliquot (20 nmol of sugar) was subjected to β -galactosidase digestion as described below.

The remaining sample was further purified by the application of aqueous Bio-Gel P-4 and salt P-6 gel-filtration chromatography. The conditions for P-4-column chromatography have been described previously (Ashford et al., 1987). Unlabelled G2 oligosaccharide eluted at 13.0 glucose units and was not used

further. The fluorescent material was collected from the void volume of a P-4 column run, dried and resuspended in 200 μl of water. This was then applied to a Bio-Gel P-6 jacketed column (2.5 cm $\times$ 1 m) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.5, containing 0.1% chlorbutol. The column was maintained at 55 $^\circ\text{C}$. The flow rate was 0.2 ml/min and the eluant was monitored by absorbance at 230 nm using a Beckman 163 variable-wavelength detector, and by fluorescence using a Perkin-Elmer LS-1 fluorescence monitor (excitation wavelength = 450 nm and detection wavelength = 521 nm). The fractions containing the fluorescent G2 conjugate (retention time between 250 and 350 min) were well-separated from the carbohydrate-free fluorescent material which was eluted close to the included volume of the column (a retention time of 500 min). Fluorescent G2 oligosaccharide was desalted by passage over a 1-ml Dowex AG502-X12 (H^+) cation-exchange column and was eluted with water. The yield of purified fluorescent G2 oligosaccharide was determined by measuring the absorbance at 488 nm of an aliquot dissolved in 0.1 M sodium hydroxide, pH 11, using a Beckman DU-8 spectrophotometer.

Enzyme digestion and RCA lectin-affinity chromatography of fluorescent G2 oligosaccharide conjugate

An aliquot (10 μl or 15 nmol) of the crude material consisting of fluorescent material, unlabelled and fluorescein-labelled G2 oligosaccharide (see above), was applied to a RCA-120-agarose column (6 mm $\times$ 4 cm) containing a total of 5 mg of lectin. The column was run at a flow rate of 6 ml/h in 50 mM sodium acetate buffer, pH 6, containing 0.05% sodium azide at 4 $^\circ\text{C}$. The column was calibrated using ^3H -labelled reduced G2 and G(0) oligosaccharide standards. Elution of fluorescent sugars in 400 μl fractions was monitored as described above. A gradient of 0–20 mM galactose prepared in the starting buffer was applied immediately after injection. The column was washed between runs with five column vol. of the starting buffer containing 0.1 M galactose. Another aliquot of the above crude mixture (20 nmol of sugar) was dried before digestion with 10 μl of 6 units/ml sequence-grade jack bean β -galactosidase (a gift of T. Butters) in 0.05 M sodium citrate buffer, pH 3.5. The mixture was incubated overnight with 10 μl of toluene (as bactericide) in a vial capped with Teflon. The digested mixture was subjected to a sequential combination of P-4 and P-6 gel-filtration chromatography as

described above. This enzyme-treated fluorescent oligosaccharide preparation was subsequently analysed by RCA lectin affinity chromatography.

Synthesis of isothiocyanate-1-*N*-glycyl β -saccharide derivatives and neoglycoprotein formation

The derivatization of 1-*N*-glycyl derivatives of *N,N'*-diacetylchitobiose, G(0) oligosaccharide and NeuNAc α 2-6Gal β 1-4GlcNAc (approx. 1 mg of each) with thiophosgene (Aldrich Chemical Co., Gillingham, Dorset, U.K.) to form the corresponding isothiocyanate derivatives, and the subsequent coupling of these derivatives to BSA were performed according to Smith et al. (1978). Neoglycoproteins were analysed by SDS/PAGE and cation-exchange h.p.l.c. using a 0.46 cm $\times$ 20 cm PolyCAT A column (PolyLC, Columbia, MD, U.S.A.) and a combined pH and ionic strength gradient over a 60 min period. Initial conditions were 50 mM ammonium acetate, pH 5.1, and final conditions were 250 mM ammonium acetate, pH 7.9. The molar ratios of sugar to BSA were determined by amino acid and hexosamine analysis as described above.

Concanavalin A (Con A) and anti-carbohydrate antibody binding to neoglycoproteins

Standard e.l.i.s.a.s were performed to test the binding of an anti-GlcNAc monoclonal antibody and Con A to *N,N'*-diacetylchitobiose and G(0) oligosaccharide-derivatized neoglycoproteins respectively. Aliquots (100 μ l) of 12 serial 3-fold dilutions of *N,N'*-diacetylchitobiose-BSA conjugate at an initial concentration of 1 mg/ml were used to coat the wells of a Micro-e.l.i.s.a. plate (Dynatech Laboratories, Billingshurst, West Sussex, U.K.) overnight at 40 °C. The plate was washed with 10 mM sodium phosphate and 0.15 M NaCl, pH 7.4, (PBS) and quenched with 4% (w/v) BSA in PBS for 2 h at room temperature. The wells were then incubated with either GN7, an anti-GlcNAc mouse monoclonal antibody (a gift of Dr. G. Rook), at a 1:1000 dilution of ascites fluid or PBS for 1 h at room temperature. After extensive washing the wells were incubated with a goat anti-(mouse μ chain) antibody-HRP conjugate (Boehringer-Mannheim) diluted 1:500 in PBS/1% (w/v) BSA buffer for 1 h. The plate was developed using 50 μ l of the substrate 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) at 10 mg/ml (Sigma). Absorbance was measured at 405 nm. For Con A binding, 100 μ l aliquots of G(0) oligosaccharide-derivatized neoglycoprotein (1 mg/ml) were used to coat the wells. After quenching with BSA, the wells were incubated with 100 μ l of Con A (4 μ g/ml in TBS with 1 mM MgCl₂ and 1 mM CaCl₂) preincubated with serial 5-fold dilutions of a competitive inhibitor α -methylmannoside at an initial concentration of 0.25 M. The wells were incubated with HRP to detect bound Con A. After extensive washings, the plate was developed using *o*-phenylenediamine substrate (Sigma). Absorbance was read at 492 nm.

Antibody binding to immobilized *N,N'*-diacetylchitobiose

The isothiocyanate-1-*N*-glycyl derivative of *N,N'*-diacetylchitobiose was directly coupled to several aminopropyl glass-fibre discs prepared according to the procedures of Aebersold et al. (1988). Ninhydrin reaction (Sarin et al., 1981) was used to quantify bound amino groups (approx. 28 nmol/mg of disc). The discs were incubated with the isothiocyanate-1-*N*-glycyl derivative of *N,N'*-diacetylchitobiose at approx. 12 μ mol/ml for

18 h. Immobilized sugar (approx. 12 nmol/mg of disc) was assayed for GN7 antibody-binding as described above. For a negative control another mouse IgM monoclonal antibody was used. This monoclonal antibody (630/7H1) has specificity for the trisaccharide Gal β 1-4(Fuc α 1-3)GlcNAc or Lewis^x antigen and was purchased from A.M.S. Biotechnology (Oxon, U.K.). The discs were air-dried for photography and absorbance values of the substrate solutions were measured following colour development. The isothiocyanate-1-*N*-glycyl β -derivative of *N,N'*-diacetylchitobiose was also directly coupled to an amino-derivatized e.l.i.s.a. plate (Becton Dickinson, Cowley, Oxon, U.K.). Briefly 50 μ l aliquots of 12 serial 2-fold dilutions of the activated sugar in 0.3 M NaCl/0.1 M NaHCO₃, pH 10, at an initial concentration of approx. 12 μ mol/ml were added to the wells. After overnight incubation at room temperature, the plate was blocked with 4% (w/v) BSA in PBS before addition of GN7 monoclonal antibody in the absence or presence of a competitive inhibitor, GlcNAc, at 60 mM. The detection of bound antibody thereafter is as described above.

Figure 2 Comparative analysis of the formation of β -glycosylamine and 1-*N*-glycyl β -derivatives of neutral and sialylated sugars

(a) The near quantitative conversion of *N,N'*-diacetylchitobiose (lane 1) and G2 oligosaccharide (lane 3) to their β -glycosylamines (lanes 2 and 4 respectively) was detected by t.l.c. (b) A similar result was obtained using NeuNAc α 2-6Gal β 1-4GlcNAc. Its β -glycosylamine derivative (lane 2) also migrated more slowly than the untreated sugar (lane 1). In contrast its *N*-chloroacetamido derivative (lane 4) migrated faster than the unmodified sugar (lane 3). The 1-*N*-glycyl derivatives of NeuNAc α 2-3Gal β 1-4GlcNAc (lane 5) and NeuNAc α 2-6Gal β 1-4GlcNAc (lane 6) had the slowest mobilities. All sugar samples were stained with orcinol except for the 1-*N*-glycyl derivatives which were stained with ninhydrin.

RESULTS

Formation of β -glycosylamine and 1-*N*-glycyl β -saccharide derivatives of neutral and sialylated sugars

The scheme for generating β -glycosylamines and 1-*N*-glycyl β -saccharide derivatives is illustrated in Figure 1. T.l.c. analysis of the β -glycosylamine preparations of *N,N'*-diacetylchitobiose and G2 oligosaccharide indicated that essentially all of the starting sugars had been converted into their slower-migrating glycosylamines (Figure 2a). Similar t.l.c. analysis also indicated the efficient formation of glycosylamine and chloroacetylated derivatives of negatively charged NeuNAc α 2-6Gal β 1-4GlcNAc (Figure 2b, lanes 1–4). Conversion of the chloroacetamido derivatives of NeuNAc α 2-6Gal β 1-4GlcNAc and NeuNAc α 2-3Gal β 1-4GlcNAc into their 1-*N*-glycyl derivatives resulted in the appearance of a slower-migrating component that is ninhydrin-sensitive (Figure 2b, lanes 5 and 6). The *m/z* values for the starting sugars and their 1-*N*-glycyl derivatives are $[M + H]^+ = 699$ and $[M + Na]^+ = 778$ respectively, as determined by l.d.m.s. (results not shown).

Figure 3 Binding of biotinylated HRP oligosaccharides to anti-HRP antibodies

Serial 2-fold dilutions of 1 mM solutions of biotinylated HRP 8.9 (●), HRP 5.8 (○), and G2F (×) oligosaccharides were tested for their binding to anti-HRP polyclonal antibodies adsorbed on to a microtitre plate. The plate was developed using a streptavidin–biotin–alkaline phosphatase complex and *p*-nitrophenyl phosphate as substrate.

Figure 4 Analysis of purified fluorescent G(0) oligosaccharide

The structure of FITC–G(0) conjugate and the reverse-phase h.p.l.c. profile of this conjugate purified to homogeneity are shown.

Figure 5 RCA-120 lectin-affinity chromatography of fluorescein-labelled G2 oligosaccharide

(a) An aliquot (approx. 20 nmol) of total sugar obtained after ether extraction of the FITC–G2 glycosylamine reaction mixture was applied to an RCA-120 lectin column. Bound fluorescent G2 was eluted with a 0–20 mM galactose gradient. Residual non-extracted fluorescein was not retained by the column and FITC–G2 was eluted with 10 mM galactose. (b) FITC–G2 conjugate after digestion with jack bean β -galactosidase and purification by Biogel P-4 and salt P-6 gel-filtration chromatography was applied to the same RCA-120 lectin column. Only one peak, which corresponds to the structure now terminating with *N*-acetylglucosamine residues (non-interactive), was detected. Elution positions of 3 H-labelled G(0) and G2 oligosaccharide standards are indicated by double arrows.

Analysis of carbohydrate epitopes of anti-HRP antibodies using biotinylated oligosaccharide conjugates

The specificity of anti-HRP polyclonal antibodies against HRP sugars was probed by e.i.s.a. using biotinylated HRP sugars. As shown in Figure 3, biotinylated HRP 8.9 and HRP 5.8, but not biotinylated G2F, did bind to immobilized antibodies. Also HRP 8.9 bound more strongly than HRP 5.8 sugar. Half-maximal binding for biotinylated HRP 8.9 occurred at approx. 3×10^{-8} M.

Comparison of the use of β -glycosylamine and 1-*N*-glycyl β -derivatives in the formation of FITC–oligosaccharide conjugates

FITC–G(0) and FITC–G2 conjugates were prepared from the β -

Figure 6 Gel electrophoresis and cation-exchange h.p.l.c. analyses of neoglycoproteins

(a) Neoglycoprotein containing *N,N'*-diacetylchitobiose (lane 1) had a slightly slower mobility than unmodified BSA (lane 2) on a 10–15% gradient gel following SDS/PAGE. RAINBOW protein molecular-mass standards consisting of myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (69.0 kDa), ovalbumin (46.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa) were used (lane 3). (b) Neoglycoprotein containing NeuNAc α 2-6Gal β 1-4GlcNAc was analysed by cation-exchange h.p.l.c. The neoglycoprotein (lower profile) was eluted earlier than the unmodified BSA (upper profile).

glycosylamine derivative of G2 and the 1-*N*-glycyl β -derivative of G(0). The yield of FITC-G(0) was approx. 72% of the starting sugar using ϵ 42000 M⁻¹·cm⁻¹ for FITC. In contrast the total yield of FITC-G2 was only 18%. These results indicate that 1-*N*-glycyl β -oligosaccharide derivatives are better intermediates than the β -glycosylamines in the formation of FITC glycoconjugates. FITC-G2 and FITC-G(0) conjugates were obtained in high purity by applying either a combination of P-4 and salt P-6 gel-filtration chromatography or t.l.c. and reverse-phase h.p.l.c. The h.p.l.c. profile of purified FITC-G(0) is shown

Figure 7 Anti-carbohydrate antibody binding to a disaccharide-containing neoglycoprotein

Serial 3-fold dilutions of a solution of neoglycoprotein containing *N,N'*-diacetylchitobiose (1 mg/ml) were adsorbed on to a microtitre plate. The plate was incubated with either GN7, an anti-GlcNAc antibody (●), or PBS buffer (x). The plate was developed using goat anti-(mouse μ chain) antibody-HRP conjugate and 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) as substrate.

in Figure 4. The expected *m/z* value of 1756 for FITC-G(0) was obtained by l.d.m.s. (results not shown).

Binding of FITC-G2 conjugate to an RCA lectin-affinity column

The specific interaction of FITC-G2 (prepared using the β -glycosylamine derivative of G2 oligosaccharide) with RCA was investigated. RCA lectin-affinity chromatography of the reaction mixture containing FITC-G2, G2 and fluorescent material showed two major fluorescent peaks (Figure 5a). The first peak was most likely due to residual non-extracted fluorescein not retained by the lectin column. The second peak, which eluted with 10 mM galactose, was due to FITC-G2 conjugate, since ³H-labelled G2 oligosaccharide standard was also eluted at the same position. The identity of the minor fluorescent peak which was eluted with 20 mM galactose is not known, but could represent FITC-G2 aggregates that have higher affinity for RCA lectin. Aggregates were observed during the purification of FITC-G2 by salt P-6 gel-filtration chromatography (results not shown).

The specific binding of FITC-G2 to RCA was indicated by a β -galactosidase digestion experiment. Purified FITC-G2 was digested with β -galactosidase to remove the two outer-arm galactose residues required for RCA-binding activity. Complete digestion of FITC-G2 to produce FITC-G(0) is indicated in Figure 5(b). Only a single fluorescent peak was eluted at a position very similar to where ³H-labelled G(0) was found (arrow). No fluorescent peak was eluted with 10 mM galactose.

Neoglycoprotein formation and analysis by lectin and anti-carbohydrate antibody

The 1-*N*-glycyl β -derivatives of *N,N'*-diacetylchitobiose, NeuNAc α 2-6Gal β 1-4GlcNAc and G(0) oligosaccharide were

Figure 8 Lectin binding to an oligosaccharide-containing neoglycoprotein

Con A in the presence of serial 5-fold dilutions of a 0.25 M α -methylmannoside solution was added to G(0)-BSA neoglycoprotein adsorbed on to a microtitre plate. The plate was developed using HRP which binds Con A and *o*-phenylenediamine as substrate.

Figure 9 Anti-carbohydrate antibody binding to glass-fibre discs with immobilized sugar

(a) Glass-fibre discs with covalently attached *N,N'*-diacetylchitobiose were incubated with either GN7 antibody (disc 2) or anti-Lewis^x antibody (disc 1). The discs were developed using goat anti-(mouse μ chain) antibody-HRP conjugate (both primary antibodies were mouse IgM molecules) and 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) as substrate. (b) The absorbance at 405 nm of the solution from each of the two developed discs was measured.

converted into their isothiocyanate derivatives in order to couple them to the lysine residues of BSA. Formation of these neoglycoproteins was confirmed by SDS/PAGE and cation-exchange h.p.l.c. SDS/PAGE showed that a neoglycoprotein containing *N,N'*-diacetylchitobiose had an apparent molecular mass of 67–68 kDa, whereas the unmodified BSA had an apparent

Figure 10 Anti-carbohydrate antibody binding to sugar-derivatized microtitre plate

Serial 2-fold dilutions of a solution of isothiocyanate-1-*N*-glycyl β -derivative of *N,N'*-diacetylchitobiose (12 μ mol/ml) were directly coupled to an amino-derivatized microtitre plate. The binding of GN7 monoclonal antibody to the plate in the presence ($\times$) or absence ($\bullet$) of 60 mM GlcNAc inhibitor was tested. The plate was developed using goat anti-(mouse μ chain) antibody-HRP conjugate and 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) as substrate.

molecular mass of 66 kDa (Figure 6a). The cation-exchange h.p.l.c. profile of a neoglycoprotein containing NeuNAc α 2-6Gal β 1-4GlcNAc is shown in Figure 6(b). The neoglycoprotein (bottom) is less positively charged than the unmodified BSA (top) and thus was eluted earlier on this column. In addition it did not resolve as a single sharp peak, as did the unmodified BSA. This reflected the presence of a population of BSA molecules with different numbers of sugar residues attached. The average number of sugar residues attached was determined by quantitative amino acid and hexosamine analysis. The mole ratios of *N,N'*-diacetylchitobiose, NeuNAc α 2-6Gal β 1-4GlcNAc and G(0) oligosaccharide to BSA were 13:1, 8:1 and 10:1 respectively.

Antibody and lectin analyses of neoglycoproteins

The binding of GN7, an anti-GlcNAc monoclonal antibody, to a Petri dish containing adsorbed BSA-*N,N'*-diacetylchitobiose is shown in Figure 7. Decreasing the amount of immobilized neoglycoprotein resulted in binding of a lower amount of the GN7 antibody. In the absence of GN7 antibody, no detectable binding of secondary antibody-HRP to neoglycoprotein was observed. Also GN7 binding was abolished in the presence of an excess concentration of GlcNAc (results not shown). The binding of Con A in the presence of various concentrations of the competitive inhibitor, α -methylmannoside, to immobilized BSA-G(0) is shown in Figure 8. As the concentration of this inhibitor decreased, Con A binding increased. At very low inhibitor concentrations (approx. 1.28×10^{-7} M), Con A binding appeared to have reached a maximum.

GN7 binding to *N,N'*-diacetylchitobiose directly immobilized on to glass-fibre discs and e.l.i.s.a. plates

Figure 9(a) shows the labelling of two fibre discs containing immobilized *N,N'*-diacetylchitobiose with an anti-Lewis^x antibody (disc 1) and the anti-GlcNAc monoclonal GN7 antibody (disc 2). Disc 2, but not disc 1, stained green with the HRP substrate, indicating specific binding of GN7 to the immobilized sugar. The absorbance at 405 nm of the substrate solution from disc 2 was about four times that of disc 1 (Figure 9b). A similar GN7 binding study was performed on *N,N'*-diacetylchitobiose which was directly immobilized on to an amino-derivatized e.l.i.s.a. plate. As shown in Figure 10, the amount of GN7 bound to the plate decreased with decreasing amounts of immobilized sugar. This binding was totally inhibited by 60 mM GlcNAc.

DISCUSSION

The study of the functions of carbohydrates could be greatly facilitated by the availability of neoglycoconjugate probes. We have recently demonstrated that β -glycosylamines of purified neutral complex oligosaccharides can readily be obtained in high yields. We have also suggested that the acylation of the 1-amino function of oligosaccharylamine should provide a wide range of oligosaccharide derivatives and probes for structural and biochemical analyses (Manger et al., 1992a). The chemical simplicity and efficiency of this derivatization method (Figure 1) make it more practical than most published procedures for biochemists and cell biologists, who often deal with small quantities (μ mol to nmol amounts) of oligosaccharides from biological sources. Importantly oligosaccharide β -glycosylamine derivatives have structural integrity and chemical reactivity that are not found in the commonly used reductive aminated derivatives. In this report we investigate the applicability of this method for obtaining β -glycosylamine derivatives of sialylated sugars and then demonstrate the usefulness of these types of derivatives in studies involving lectins, enzymes and anti-carbohydrate antibodies.

The formation of the β -glycosylamine of NeuNAc α 2-6Gal β 1-4GlcNAc was as efficient as those of *N,N'*-diacetylchitobiose and G2 oligosaccharide based on the intensities of the orcinol staining of the underivatized sugars and their respective glycosylamines (Figures 2a and 2b). Previously we have performed n.m.r. analyses to quantify the yields of β -glycosylamines of *N,N'*-diacetylchitobiose and G2 oligosaccharide and to confirm the closed ring structure of the reducing terminal GlcNAc residue and β anomeric configuration (Manger et al., 1992a). However, insufficient amounts of the sialylated derivatives prevented similar detailed analyses. The 1-*N*-glycyl β -saccharide derivatives of NeuNAc α 2-3Gal β 1-4GlcNAc and NeuNAc α 2-6Gal β 1-4GlcNAc were formed by chloroacetylation and ammonolysis procedures using conditions identical to those for neutral oligosaccharides except that cellulose column chromatography was used instead of mixed-bed ion-exchange chromatography to desalt the sugar preparations. Chloroacetylation of the 1-amino group appears to be more favourable than that of the carboxyl function of the sialic acid, as high yields (65–70% of the total sugar) of the desired 1-*N*-glycyl β -saccharide derivatives were obtained as estimated by orcinol staining and m.s. respectively (Figure 2b). These results demonstrate that sialylated sugars without protecting groups can also be derivatized to form β -glycosylamine and 1-*N*-glycyl β -saccharide intermediates as previously shown for neutral sugars. It is also likely that this method could be used for derivatizing phosphorylated and sulphated sugars as they contain less reactive anionic groups.

Carbohydrate epitopes, such as those recognized by anti-HRP

antiserum, can be expressed in a cell- or tissue-specific manner and defining these epitopes may provide insights into the significance of these glycosylation patterns in biological systems. Kurosaka et al. (1991) have used competitive inhibition assays to define a sugar structure on HRP which was recognized by an anti-HRP antiserum. The use of pyridylamino oligosaccharide derivatives to inhibit the binding of an anti-HRP antiserum to immobilized HRP glycopeptides conjugated to BSA was effective in this case. However, this method may not be practical in other situations as it requires the isolation of sufficient quantities of glycopeptides and sugars for conjugation, derivatization and inhibition studies. Our approach was to immobilize the anti-HRP antibodies and assay directly for binding to biotinylated HRP sugars using e.l.i.s.a. Our results show that biotinylated HRP 8.9 oligosaccharide binds to anti-HRP antibodies (Figure 3), which is consistent with the finding obtained by competitive inhibition assays (Kurosaka et al., 1991). The specificity of this recognition was suggested by the lack of binding activity of G2F and weaker binding activity of HRP 5.8 oligosaccharides. These findings suggest that a combination of a xylose residue on the tri-mannosyl core and the α 1-3-linked fucose residue in the HRP 8.9 sugar is critical for strong binding to anti-HRP antibodies. This approach combines the advantages of using e.l.i.s.a. to increase the sensitivity of detection of protein-carbohydrate interactions and the use of the more readily available proteins (lectins, enzyme and antibodies) rather than precious oligosaccharides for immobilization. The biotinylation of β -glycosylamines of oligosaccharides is simple and quantitative and n.m.r. analysis of biotinylated G2F oligosaccharide showed a closed ring structure with β -stereochemistry (results not shown) which was also found in 1-*N*-glycyl β -saccharide derivatives. We and other workers have described the use of biotinyl oligosaccharide derivatives in the formation of pseudoglycoproteins for biochemical analyses (Shao and Wold, 1987; Manger et al., 1992b), but a number of bioanalytical applications are possible using biotinyl saccharide derivatives (Wilchek and Bayer, 1988).

Fluorescent or chromophoric derivatives of oligosaccharides have many potential applications including the identification of cell-surface carbohydrate-binding components using flow cytometry and fluorescence microscopy and the detection and analyses of oligosaccharides (Hase et al., 1984; Her et al., 1987). Previously we have shown that direct coupling of a monosaccharide β -glycosylamine with fluorescein and dansyl chloride resulted in low yields. In contrast a 1-*N*-glycyl β -derivative of the same monosaccharide conjugated readily to these probes to produce yields of 55–70% (Manger et al., 1992a). This finding also applies to oligosaccharides, as demonstrated by the much higher yield (72%) of FITC-G(0) using its 1-*N*-glycyl β -derivative compared with the formation of FITC-G2 (18% yield) using its β -glycosylamine derivative. Fluorescent G(0) and G2 sugars were purified to homogeneity by a combination of t.l.c. and reverse-phase h.p.l.c., and Biogel P-4 and salt P-6 gel-filtration chromatography respectively (Figure 4 and results not shown). The solubility of fluorescent oligosaccharides in aqueous buffer enables enzyme digestion and lectin-affinity chromatography to be performed. This was shown by the conversion of FITC-G2 into FITC-G(0) by β -galactosidase and the subsequent loss of its ability to bind to RCA lectin (Figure 5). These results demonstrated that the derivatization of oligosaccharides using their β -glycosylamines or 1-*N*-glycyl β -derivatives did not alter their recognition by lectins and enzymes.

Neoglycoproteins (Stowell and Lee, 1980; Aplin and Wriston, 1981) and immobilized sugars (Honda et al., 1988) should enable the rapid and direct assessment of carbohydrate function independent of the protein or lipid moieties to which they are

normally attached, as well as the purification of carbohydrate-binding molecules. Furthermore the potentially higher avidity of these clustered oligosaccharides over their monovalent counterparts may be advantageous in carbohydrate-receptor studies (Lee, 1989). The use of isothiocyanate-1-*N*-glycyl β -oligosaccharide derivatives allows the coupling of sialylated sugars without protection of their carboxyl groups (Figure 6). Our studies clearly demonstrated that the formation of neoglycoproteins and the immobilization of sugars using 1-*N*-glycyl β -derivatives were effective, as shown by their uses in determining specific binding properties of antibodies and lectins (Figures 7–10). It remains to be determined whether immobilized sugars can act as substrates or acceptors for glycosidases or glycosyltransferases, respectively. Nevertheless the potential for solid-phase enzymic synthesis of oligosaccharides exists using 1-*N*-glycyl β -derivatives because the linker is identical to the side chain of asparagine; therefore immobilized oligosaccharides could be released from the solid supports as this linkage would be cleavable by hydrazine.

In summary we have demonstrated the usefulness of β -glycosylamine derivatives of oligosaccharides as intermediates in the formation of neoglycoconjugates for detecting, analysing and purifying carbohydrates, and for studying enzymes, lectins and anti-carbohydrate antibodies. The functional relevance of carbohydrates in various biological processes could now feasibly be investigated using biologically derived oligosaccharides rather than monosaccharides.

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