Synthetic glycosylation of proteins using *N*-(β -saccharide) iodoacetamides: applications in site-specific glycosylation and solid-phase enzymic oligosaccharide synthesis

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A simple and efficient synthetic glycosylation method suitable for use in solid-phase enzymic oligosaccharide synthesis and site-specific glycosylation of recombinant proteins to produce defined glycoforms is described. This strategy utilizes *N*-(β -saccharide) haloacetamides for attaching oligosaccharides specifically to cysteine residues of proteins in solution to form neoglycoproteins. The alkylation reaction was tested using *N*-(β -chitotriose) bromoacetamide and an unprotected synthetic hexapeptide containing a single cysteine residue. The glycosylated product was confirmed by amino acid and hexosamine analyses as well as laser desorption mass spectrometry. Similarly *N*-(β -chitotriose) iodoacetamide was covalently linked to non-reduced BSA to produce a defined glycoform of this protein. The specific attachment of chitotriose at the single cysteine residue in non-reduced serum albumin was suggested by Ellman's assay for free thiols. This was verified by amino acid sequencing of a tryptic glycopeptide derived from this neoglycoprotein. Multiple sugar attachment was ac-

complished using fully reduced serum albumin as demonstrated by the formation of two neoglycoproteins using iodoacetamide derivatives of galactose β 1-3-*N*-acetylgalactosamine (Gal β 1-3GalNAc) and the major xylose/fucose-class plant-type oligosaccharide of horseradish peroxidase. These two neoglycoproteins with an average of 18–21 sugar residues attached were assayed positively for binding to peanut agglutinin and a sugar-specific anti-(horseradish peroxidase) monoclonal antibody YZ1/2.23 respectively. Sialylation of the neoglycoprotein containing Gal β 1-3GalNAc was accomplished using α -2,3-sialyltransferase and radiolabelled CMP-*N*-acetylneuraminic acid. Significantly, glycan attachment using this conjugation method is reversible as demonstrated by the release of oligosaccharides from these two neoglycoproteins using hydrazinolysis. Therefore this method could provide invaluable reagents for many glycobiological studies.

INTRODUCTION

Carbohydrates play important roles in many biological events. The structures of oligosaccharides in biological glycoconjugates are as complex and diverse as their functions. A large body of evidence has suggested that glycans can influence conformation and stability of proteins, modulate protein functions and alter immunogenicity and circulatory half-life of proteins and cells (Olden et al., 1982; Rademacher et al., 1988; Varki, 1993). They can also serve as recognition elements for receptors such as those involved in the transport of lysosomal enzymes (Kornfeld, 1992), the endocytosis of glycoproteins and pathogens (Weiss and Ashwell, 1989; Taylor et al., 1990) and the recruitment of leucocytes to sites of inflammation (Brandley et al., 1990; Lasky, 1992). The discovery of an increasing number of proteins with similar amino acid sequences to known lectins (Drickamer and Taylor, 1993) would suggest the existence of many more as yet undefined biological processes that are mediated by carbohydrates.

Synthetic glycoconjugates are valuable tools for elucidating the various biological roles of oligosaccharides. Neoglycolipids and neoglycoproteins in particular are very useful for identifying carbohydrate–lectin interactions and defining the fine structural requirements of oligosaccharide ligands for lectin recognition (Childs et al., 1989; Larkin et al., 1992; Lee, 1992). However, other investigations into the role of carbohydrates in glycoproteins are often complicated by the fact that glycoproteins exist as glycoforms and these glycosylation variants of the same

polypeptide chain can exhibit different *in vitro* or *in vivo* biochemical, physiological and immunological properties (Parekh, 1991; Varki, 1993). A major challenge in glycobiology is to obtain pure glycoforms for structural and functional studies. Although synthetic glycosylation strategies are available for producing N- and O-linked glycopeptides, they cannot be applied to proteins as they require the use of organic solvents. Current methods, including reductive amination (Gray, 1974), for attaching sugars to proteins usually involve the amino functions of free N-terminus and lysine side chains of the protein (Stowell and Lee, 1980). Consequently the net charge of the protein is changed dramatically and the sugar is randomly attached, and therefore the linkage between glycan and protein is very different from either N- or O-linkages. This may be insignificant if the protein is used solely as a medium to present multiple sugar moieties, as is the case in numerous *in vitro* binding studies. However, if the protein integrity is of importance and needs to be preserved, alternative chemical methods are required.

We have recently described the use of oligosaccharide β -glycosylamines as intermediates for neoglycoconjugate formation. We have shown that N-acylation of β -glycosylamines with heterobifunctional reagents is a simple and efficient derivatization method which, unlike reductive amination, results in oligosaccharide derivatives that retain the closed ring structure and β -stereochemistry of asparagine-linked glycans (Manger et al., 1992a,b). Significantly the asparagine side chain linker introduced in these oligosaccharide derivatives would be cleavable by hydrazinolysis, a chemical method for releasing N-linked glycans

Abbreviations used: HRP, horseradish peroxidase; l.d.m.s., laser desorption mass spectrometry; TFA, trifluoroacetic acid.

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(Bayard and Montreuil, 1974; Takasaki et al., 1982) as well as O-linked glycans under certain conditions (Patel et al., 1993), thus making this sugar derivatization process reversible. We have subsequently illustrated several applications for these types of neoglycoconjugates including those synthesized using 1-*N*-glycyl- β -saccharide derivatives as intermediates (Wong et al., 1993). In the present paper, we report the formation of *N*-(β -saccharide) haloacetamides and their selective coupling to cysteine side chains of reduced and non-reduced serum albumin. The applications of this derivatization scheme in site-specific glycosylation and solid-phase enzymic oligosaccharide synthesis are demonstrated.

EXPERIMENTAL

Materials

NN'N''-Triacetylchitotriose was purified by Bio-Gel P4 gel-permeation chromatography from a partial acid hydrolysate of chitin as described previously (Rupley, 1964). The major N-linked glycan of horseradish peroxidase (HRP), Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc, was also purified by Bio-Gel P4 chromatography from a hydrazinolysate of HRP glycoprotein (Sigma, Poole, Dorset, U.K.). The disaccharide Gal β 1-3GalNAc was purchased from BioCarb (Lund, Sweden). Sugars were analysed by laser desorption mass spectrometry (l.d.m.s.) using LASERMAT (FinneganMat, Hemel Hempstead, Herts., U.K.) according to the manufacturer's instructions.

Galactosyltransferase (lactose synthase; UDP-galactose-D-glucose 4- β -galactosyltransferase; EC 2.4.1.22) from bovine milk (10 units/mg as lactose synthase) was purchased from Sigma. Porcine liver α -2,3-sialyltransferase (CMP-*N*-acetylneuraminate- β -D-galactosyl-1,3-*N*-acetyl- β -D-galactosamine α -2,3-*N*-acetylneuraminyltransferase; EC 2.4.99.4) at 200 munits/ml was purchased from Genzyme Diagnostics (West Malling, Kent, U.K.).

UDP-D-[U- 14 C]galactose (329 mCi/mmol; 10 μ Ci/400 μ l) and CMP-[14 C]sialic acid (289 mCi/mmol; 10 μ Ci/400 μ l) were purchased from Amersham International (Amersham, Bucks., U.K.).

Elderberry bark lectin, *Maackia amurensis* lectin I and peanut agglutinin were purchased from Vector Laboratories (Bretton, Peterborough, Northants., U.K.).

Analyses

T.l.c. was performed using Merck 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% diamino-butane. Amines and hexoses were visualized using ninhydrin and orcinol sprays respectively. H.p.l.c. was performed as described previously (Manger et al., 1992a), but the types of column and gradient conditions used in the present study are individually described where appropriate. Amino acid and hexosamine analysis using the PicoTag method has been previously described (Zamze et al., 1991).

Preparation of β -glycosylamines and *N*-(β -saccharide) haloacetamides

The formation of β -glycosylamines of neutral and acidic oligo-

saccharides and haloacetylation of β -glycosylamines were performed as previously described (Manger et al., 1992a; Wong et al., 1993). Samples of saccharide and saccharide derivatives were analysed by t.l.c. as described above. It was not necessary to separate the *N*-(β -saccharide) haloacetamides (approx. 85% of the total sugar) from unchanged saccharides as the latter do not react with thiol groups.

Alkylation of peptides and proteins

Peptide (1 μ mol) or unreduced BSA (1.7 μ mol) was dissolved in 1 ml of 50 mM (NH $_4$) $_2$ CO $_3$, pH 8.1. *N*-(β -saccharide) bromo- or iodo-acetamide in 1 ml of the same buffer was added to the peptide or BSA solution and incubated at room temperature. The mixture was stirred in a Pierce vial in the dark for 8–24 h. Alkylated peptide was purified by reversed-phase h.p.l.c. and analysed by l.d.m.s. The thiol contents of BSA and sugar-derivatized BSA were measured indirectly using Ellman's reagent (Pierce and Warriner, Chester, U.K.) or 5,5'-dithiobis-(2-nitrobenzoic acid) according to the manufacturer's instructions. The release of 5-thio-2-nitrobenzoic acid, which shows strong absorption at 412 nm, is proportional to the amount of free thiol present. Using cysteine as standard and $\epsilon_{412} = 14\,500$, the absorbance was linear from 49.4 nmol to 1 μ mol of thiol content in the assay.

Determination of synthetic glycosylation site on unreduced BSA

BSA (10 mg) was allowed to react with a 100-fold molar excess of the *N*-(β -chitotriose) iodoacetamide as described above. The mixture was dialysed exhaustively against distilled water at 4 °C to remove unbound sugar. Neoglycoprotein was redissolved in 0.8 ml of 0.1 M Tris/HCl, pH 7.0, containing 0.5 M NaCl, 20 mM MnCl $_2$ and 0.5 mM ATP. 14 C-labelled galactose was incorporated into this neoglycoprotein substrate using galactosyltransferase (100 μ l) and UDP-[14 C]galactose (100 μ l) as donor. This enzyme reaction was evaluated using ovalbumin (chicken egg albumin, Grade VI; Sigma Ltd) as a standard glycoprotein acceptor substrate. Reversed-phase h.p.l.c. on a Vydac C4 column was used to purify radiolabelled neoglycoprotein. Gradient conditions were as follows [solvent A was 0.1% trifluoroacetic acid (TFA) (Aldrich) in water, solvent B was 0.1% TFA in acetonitrile]: initial conditions of 95% solvent A/5% solvent B were maintained for 10 min, then a linear gradient from 5 to 100% solvent B was applied over 20 min; the flow rate was 1 ml/min and 2 ml fractions were collected. Ultima Gold scintillation fluid (Canberra Packard, Pangbourne, Berks., U.K.) (3 ml) and water (490 μ l) were added to 10 μ l portions of the eluate. These samples were counted for radioactivity using a Beckman LS 3801 liquid-scintillation counter. Approx. 31% of the total radioactivity was recovered in the neoglycoprotein fractions. The radiolabelled neoglycoprotein was reduced and alkylated before digestion with trypsin. The resultant tryptic peptides were separated by reversed-phase h.p.l.c. using the conditions described above. Radiolabelled fractions were further purified by reversed-phase h.p.l.c. using an Aquapore OD-300 microbore (100 mm $\times$ 2 mm) column (Applied Biosystems Ltd., Warrington, Cheshire, U.K.). Gradient conditions were as follows (solvent A was 0.1% TFA in water, solvent B was 0.09% TFA in 80% acetonitrile): initial conditions of 90% solvent A/10% solvent B were maintained for 5 min, then linear gradients from 10 to 60% solvent B and 60 to 90% solvent B were applied over 70 and 15 min respectively at a flow rate of 150 μ l/min using a Severn Analytical microbore h.p.l.c. system. A single radiolabelled peak was subjected to amino acid se-

quencing using an Applied Biosystems 470A automated sequencer.

Alkylation of reduced BSA

BSA was reduced in 0.2 M Tris/HCl, pH 8.0, containing 0.1 M dithiothreitol, 6 M guanidium chloride and 1 mM EDTA for 2 h at 25 °C. After reduction, the pH of the protein solution (10 mg/ml) was adjusted to 3 by dropwise addition of acetic acid. Reduced BSA was subsequently separated from the denaturing and reducing agents by dialysis at 4 °C against 0.1 M acetic acid. The dialysate was lyophilized and stored under nitrogen until use. Lyophilized reduced BSA was resuspended in 50 mM (NH₄)₂CO₃, pH 8.1, containing 5 M guanidium chloride and filtered through a 0.45 µm pore-diam. filter. Reduced BSA was alkylated as described above. A tenfold molar excess of iodoacetamide derivatives of 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 Gal β 1-3GalNAc was used to alkylate reduced BSA. Both neoglycoproteins were concentrated and washed with water using a Centricon C30 protein concentrator (Amicon, Danvers, MA, U.S.A.). Neoglycoproteins were lyophilized and then subjected to analysis of amino acid and hexosamine. The number of sugar-attachment sites was determined by quantifying CM-cysteine and hexosamine residues in the samples. Typically 70% of total cysteine residues in reduced BSA were coupled.

Analyses of neoglycoproteins using antibody and lectins

E.l.i.s.a.s were used to confirm the integrity of the conjugated sugar structures. Briefly, neoglycoprotein containing Gal β 1-3GalNAc (1.5 nmol of sugar/ml) was adsorbed on to an e.l.i.s.a. plate (100 µl/well). The plate was washed with 10 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl (TBS) and blocked with 2% (w/v) BSA in TBS. Portions (75 µl) of serial twofold dilutions of biotinylated lectins at an initial concentration of 10 µg/ml in 10 mM Hepes, pH 7.5, containing 0.15 M NaCl and 0.1 mM CaCl₂ were added to the plate. After incubation for 2 h, the plate was washed with TBS. Streptavidin-biotinylated alkaline phosphatase complex (Boehringer, Mannheim, Germany), to detect bound biotinylated lectins, was added according to the manufacturer's instructions. The plate was finally developed using *p*-nitrophenyl phosphate as substrate at 37 °C for 20 min, and *A*₄₀₅ was measured using a Multiskan MCC340 plate reader. For neoglycoprotein containing Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc, a similar e.l.i.s.a. was performed using serial two-fold dilutions of an anti-(HRP carbohydrate) monoclonal antibody termed YZ1/2.23 (McManus et al., 1988) at an initial concentration of 2.5 µg/ml and alkaline phosphatase-conjugated goat anti-rat immunoglobulin (Sigma).

Enzymic synthesis of NeuNAc α 2-3Gal β 1-3GalNAc and release of sugars from neoglycoproteins

Neoglycoprotein containing Gal β 1-3GalNAc was used as an acceptor substrate for α -2,3-sialyltransferase. The reaction mixture contained 10 µl of BSA (5 mg/ml), 10 µl of 0.5 M sodium cacodylate, pH 6.0, 5% Triton X-100, 10 µl of neoglycoprotein (20 mg/ml), 5 µl of enzyme (200 munits/ml) and 5 µl of CMP-[¹⁴C]sialic acid (289 mCi/mmol; 10 µCi/400 µl). As a positive control for this reaction, asialofetuin was also added in a separate experiment which was performed concurrently. The mixture was incubated at 37 °C overnight and subsequently passed through a PD10 gel-filtration column (Bio-Rad). Radiolabelled protein

fractions (2 ml) were pooled and dialysed against 0.1% TFA and lyophilized. Samples were redissolved with 1 ml of freshly prepared 0.1% TFA in a GlycoPrep 1000 reactor vial (Oxford Glycosystems, Oxford, Oxfordshire, U.K.). Automated hydrazinolysis of these samples was subsequently performed in a GlycoPrep 1000 machine using the manufacturer's program for the release of N- and O-linked sugars from glycoproteins. Each glycan pool was filtered through a 0.22 µm poly(tetrafluoroethylene) filter, taken to dryness by rotary evaporation with the temperature maintained at less than 30 °C, rinsed with 1 ml of water and finally dissolved in 500 µl of water. A small portion (5 µl) was measured for radioactivity and the remaining samples were analysed by weak anion-exchange h.p.l.c. on a Vydac 301PLX575 column (Hichrom, Reading, Berks., U.K.). Gradient conditions were as follows (solvent A was 0.5 M ammonium acetate, pH 5.3, and solvent B was water): initial conditions of 100% solvent B were maintained for 5 min, then a concave gradient (Waters curve number 7) from 0 to 80% solvent A was applied over 45 min, followed by a linear gradient from 80 to 100% solvent A over 5 min. The flow rate was 1 ml/min and 0.5 ml fractions were collected; sample was detected by liquid-scintillation counting of the entire set of fractions. In contrast, neoglycoprotein containing Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc was subjected to manual hydrazinolysis as previously described (Ashford et al., 1987). Briefly, the conjugated oligosaccharides were released from salt-free and cryogenically dried neoglycoprotein (0.5–1 mg) by treatment with anhydrous hydrazine, re-N-acetylated and reduced with NaB[³H]₄ (370 GBq/mmol) from DuPont (Stevenage, Herts., U.K.). The released radiolabelled sugar was analysed by Bio-Gel P4 gel chromatography as previously described (Ashford et al., 1987).

RESULTS

Formation and coupling of *N*-(β -saccharide) haloacetamides

Scheme 1 illustrates generation of *N*-(β -saccharide) haloacetamides from β -glycosylamines and their alkylation to thiol-containing molecules including peptides and proteins. Treatment of β -glycosylamine of *NN'N''*-triacylchitotriose with bromoacetic anhydride produced its bromoacetamide derivative (results not shown). This reaction was found to be as rapid (2–4 h) and as quantitative (85% yield) as *N*-chloroacetylation of β -glycosylamine, which we have described previously (Manger et al., 1992a). Alkylation of a synthetic hexapeptide with the sequence Ac-Gly-Arg-Gly-Asp-Cys-Phe-amide with the *N*-(β -chitotriose) bromoacetamide resulted in the formation of a neoglycopeptide as shown by reversed-phase h.p.l.c. (Figure 1a). An additional h.p.l.c. peak appeared after 8 h of the alkylation reaction [Figure 1a (ii)]. This peak has a slightly longer retention time (23.6 min) than that of the starting peptide [Figure 1a (i)]. Fractions spanning this new peak were pooled and subjected to l.d.m.s. (Figure 1b). The *m/z* values of 1321 and 1343 correspond exactly to the proton and sodium adducts respectively of the putative neoglycopeptide [Figure 1b (ii)]. In contrast, *m/z* values of 653 and 675, which correspond to the proton and sodium adducts respectively of the peptide, were obtained for the purified synthetic peptide. A peak of much lower intensity (*m/z* 1308) was also detected in the peptide sample. This is probably due to dimerization of the peptide by disulphide bond formation [Figure 1b (i)].

Thiol-specific glycosylation of serum albumin

Similar amounts of β -glycosylamine of chitotriose were *N*-

Scheme 1 Reaction scheme for generating β -glycosylamines and *N*-(β -saccharide) haloacetamides

Oligosaccharide (1) is converted into its β -glycosylamine (2) by incubation with NH_4HCO_3 . Reaction of β -glycosylamine with bromo- or iodo-acetic anhydride produces its haloacetamide derivative (3). This derivative (3) alkylates thiol groups of peptides and proteins to form neoglycopeptides or neoglycoproteins (4). R', saccharides linked to the reducing terminal *N*-acetylglucosamine residue; R'', cysteine-containing peptide or protein.

Figure 1 Alkylation of peptides with *N*-(β -saccharide) haloacetamides

(a) H.p.l.c. analysis of a synthetic hexapeptide, Ac-Gly-Arg-Gly-Asp-Cys-Phe-amide, before and after reaction with the bromoacetamide derivative of *NN'**N''*-triacetylchitotriose (i and ii respectively). (b) L.d.m.s. analysis of purified synthetic hexapeptide and peak II indicates the m/z values for the proton and sodium adducts of the peptide (i) and the glycopeptide (ii). au, arbitrary unit of intensity.

acetylated with chloroacetic and iodoacetic anhydride to form the corresponding haloacetamide derivatives of this sugar. These derivatives were subsequently tested for their reactivity toward unreduced BSA, which contains a single free cysteine residue (Cys-58). The remaining 34 cysteine residues are disulphide linked. The alkylation reaction was monitored indirectly by Ellman's assay for free thiols. No 5-thio-2-nitrobenzoic acid was released from unreduced BSA allowed to react with the iodo-

acetamide derivative of chitotriose, which suggests the absence of free thiols. However, the opposite was observed for BSA incubated with the chloroacetamide derivative of the same sugar. Compared with untreated BSA, 10% of free thiol had been removed from BSA treated with the chloroacetamide derivative of chitotriose (results not shown). This demonstrates the specificity of the alkylation reaction and the greater reactivity of iodoacetamide than chloroacetamide derivatives.

Figure 2 Purification of [^{14}C]galactose-labelled neoglycoprotein and tryptic peptides by reversed-phase h.p.l.c.

The iodoacetamide derivative of NN' -triacetylchitotriose was used to alkylate unreduced BSA. Radiolabelled galactose residues were added to the neoglycoprotein using galactosyltransferase and UDP- D -[^{14}C]galactose. (a) This radiolabelled neoglycoprotein was purified by reversed-phase h.p.l.c. and fractions were measured for radioactivity ($\circ$) and absorbance ($\bullet$). (b) Purified reduced and alkylated neoglycoprotein was digested with trypsin. Tryptic peptides were separated by reversed-phase h.p.l.c. Fractions containing radioactivity (*) were pooled and analysed. (c) Further purification of radiolabelled tryptic peptide from this pool by high-resolution microbore reversed-phase h.p.l.c. was performed before amino acid sequencing. All h.p.l.c. fractions were monitored for A_{280} and radioactivity. Only peak 8 contains radioactivity.

Localization of the alkylated site on BSA

It was necessary to add a radiolabelled monosaccharide to facilitate the detection of the synthetic glycosylation site on BSA. Galactosyltransferase was used to incorporate ^{14}C -labelled galactose residues into chitotriose conjugated to BSA. After the transferase reaction, the neoglycoprotein was purified by reversed-phase h.p.l.c. As shown in Figure 2(a), radioactivity is associated with the protein fraction. The radiolabelled neoglycoprotein was subsequently digested with trypsin and the resultant tryptic peptides were separated by reversed-phase h.p.l.c. (Figure

Figure 3 (a) Antibody binding to a neoglycoprotein and (b) Bio-Gel P4 size-exclusion chromatography of glycans released from the neoglycoprotein

(a) Reduced BSA alkylated with the iodoacetamide derivative of an oligosaccharide derived from HRP, $\text{Man}\alpha 1\text{-}6(\text{Man}\alpha 1\text{-}3)(\text{Xyl}\beta 1\text{-}2)\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4(\text{Fuc}\alpha 1\text{-}3)\text{GlcNAc}$, was tested for binding to a rat monoclonal antibody with this sugar specificity, YZ1/2.23. Twofold serial dilutions of YZ1/2.23 antibody at an initial concentration of $2.5 \mu\text{g/ml}$ were incubated with equal amounts of immobilized neoglycoprotein containing HRP oligosaccharide ($\bullet$), neoglycoprotein containing a similar density of N -acetylglucosamine ($\circ$) and BSA ($\blacksquare$). Bound antibodies were detected by rabbit anti-rat immunoglobulin–HRP conjugates. The plate was then developed with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) or ABTS substrate. A_{405} was measured. (b) The neoglycoprotein containing HRP oligosaccharide was subjected to hydrazinolysis. The released glycans were reduced, tritium-labelled and separated on a Bio-Gel P4 column. Radioactive peaks A and B represent glycans with hydrodynamic volumes of 8.8 and 5.8 glucose units (gu) respectively. Unreduced HRP oligosaccharide standard has a hydrodynamic volume of 8.3 glucose units (double-headed arrow).

2b). Radioactive fractions were analysed by the PicoTag method and l.d.m.s. before amino acid sequencing. The ratio of CM-cysteine to glucosamine was 1:3 as expected from the alkylation of one cysteine residue with chitotriose which contains three N -acetylglucosamine residues. However, l.d.m.s. analysis has indicated that several peptides are co-eluted with the putative neoglycopeptide (results not shown). Therefore this sample was further purified by high-resolution microbore h.p.l.c. (Figure 2c). Only a single radiolabelled peak (peak 8) was resolved and subjected to amino acid sequencing. The amino acid sequence obtained was G-L-V-L-I-A-F-S-Q-Y-L-Q-Q-X-P-F-D-E-H-V-K which corresponds exactly to a tryptic fragment of BSA from Gly-45 to Lys-65 with the exception of the single non-disulphide-linked cysteine residue at position 58. Attachment of chitotriose to this cysteine residue resulted in a glycosylated cysteine residue (X) which could not be identified by the amino acid sequencer. In contrast, unconjugated cysteine in another tryptic peptide (peak 6), M-P-C-T-E-D-Y-L-S-L-I-L-N-R (amino acid positions 468–481), was accurately identified.

Sugar release from neoglycoproteins

The major N-linked oligosaccharide structure of HRP with a

Figure 4 Lectin binding to a neoglycoprotein

Serial twofold dilutions of biotinylated elderberry bark lectin (○), *Maackia amurensis* lectin (●) and peanut agglutinin (■) each at an initial concentration of 10 µg/ml were incubated with immobilized BSA conjugated with Gal β 1-3GalNAc residues (18 sugars/BSA). The plate was quenched, washed and incubated with streptavidin-biotinylated alkaline phosphatase complex. The plate was finally rinsed and developed with *p*-nitrophenyl phosphate substrate. Absorbance of *p*-nitrophenoxide was measured at 405 nm.

hydrodynamic volume of 8.3 (non-reduced form) was purified by Bio-Gel P4 size-exclusion chromatography (results not shown). The iodoacetamide derivative of this sugar was coupled to fully reduced BSA to form a neoglycoprotein for testing the possibility of sugar release from neoglycoconjugates with this type of linker. On average, 21 of the 35 available thiols were conjugated as determined by amino acid analysis. As in the case of other alkylated proteins, cysteine residues conjugated with sugars in this neoglycoprotein were converted into CM-cysteine after acid hydrolysis. Unlike cysteine, CM-cysteine is not partially destroyed by the acid hydrolysis conditions employed in the PicoTag method, thus making quantification more accurate. Figure 3(a) shows the specific binding of a monoclonal antibody against HRP oligosaccharides, YZ1/2.23, to this neoglycoprotein, and Figure 3(b) shows the Bio-Gel P4 gel-permeation chromatogram of reduced tritium-labelled sugars released from the above neoglycoprotein by hydrazinolysis. The major peak (70 % of total radioactivity) with a hydrodynamic volume of 8.8 glucose units represents the sugar structure [Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc] used for neoglycoprotein formation. The difference of 0.5 glucose unit between reduced and non-reduced sugars on Bio-Gel P4 gel-permeation chromatograms is always observed. The minor peak (30 % of total radioactivity) has a hydrodynamic volume of 5.8.

Enzymic sialylation of neoglycoprotein

A common O-linked disaccharide (Gal β 1-3GalNAc) was coupled to cysteine residues of fully reduced BSA (18 sugars per BSA molecule) using its iodoacetamide derivative. Peanut agglutinin, a lectin with Gal β 1-3GalNAc specificity, was found to bind strongly to this neoglycoprotein, whereas *Maackia amurensis* lectin and Elderberry bark lectin with sugar specificities for α -2,3- and α -2,6-linked sialic acids respectively did not (Figure 4). This demonstrates that the integrity of the disaccharide remained intact after its derivatization and conjugation to BSA. This neoglycoprotein was tested as an acceptor substrate for α -2,3-sialyltransferase using CMP-*N*-acetylneuraminic acid as donor. Sialylated neoglycoprotein bound to *Maackia amurensis* lectin but not Elderberry bark lectin (results not shown). Radiolabelled

Figure 5 Enzymic synthesis, release and analysis of an anionic trisaccharide on a neoglycoprotein

(a) Separation of radiolabelled glycans from fetuin by weak anion-exchange chromatography. Peak N is neutral saccharides, and peaks I to IV represent mono- to tetra-sialylated glycans. Similar analyses of glycans released from asialofetuin and BSA conjugated with Gal β 1-3GalNAc residues treated with α -2,3-sialyltransferase and 14 C-labelled CMP-*N*-acetylneuraminic acid are shown in (b) and (c) respectively.

donor was employed to trace the release of the newly formed trisaccharide from the neoglycoprotein by hydrazinolysis. Asialofetuin which also contains this disaccharide was used as a positive control for the transferase and hydrazinolysis reactions. The recoveries of radiolabelled glycans were 64 % and 32 % for transferase-treated asialofetuin and neoglycoprotein containing Gal β 1-3GalNAc respectively. The released sugars from transferase-treated asialofetuin and neoglycoprotein were eluted as single peaks on a weak anion-exchange column (Figures 5b and 5c respectively). These peaks have the same elution time as the monosialylated structures of a sugar mixture derived from fetuin (Figure 5a) which is known to contain mono- to tetra-

sialylated structures (Spiro and Bhoyroo, 1974; Green et al., 1988).

DISCUSSION

In this study we have investigated the use of bromo- and iodoacetamide derivatives of oligosaccharides as alkylating reagents for the production of neoglycopeptides and neoglycoproteins (Scheme 1). The advantages of this method are: (1) the alkylation of protein thiols using iodoacetamide derivatives in aqueous buffers is simple and effective; (2) the attachment of iodoacetamide derivatives is selective to cysteine residues; (3) the resultant thioether linkage does not appear to change the conformation of the sugar (Davis and Flitsch, 1991); (4) thioethers are stable and have not been shown to be immunogenic or antigenic (Robey and Fields, 1989). The present results indicate that bromo- and iodoacetamide derivatives of oligosaccharides are readily formed from their β -glycosylamines and these derivatives are reactive and can be selectively coupled to cysteine-containing peptides (Figure 1) and proteins. Thiol-specific glycosylation of proteins by *N*-(β -saccharide) iodoacetamides was investigated using BSA. The attachment of chitotriose iodoacetamide derivative to the single free cysteine residue (Cys-58) in non-reduced BSA was demonstrated conclusively by amino acid sequencing of the putative neoglycopeptide purified from its tryptic digest (Figure 2).

This method of conjugating a defined oligosaccharide to cysteine side chains on a protein provides a finer-tuned strategy for synthetic glycosylation of proteins than conventional conjugation methods that utilize ϵ -amino functions of lysine residues, as the number of non-disulphide-linked cysteine residues in proteins is often far fewer than lysine residues. Also modification of cysteines should not alter significantly the net charge on proteins. In principle, it should be possible to replace natural N-linked glycosylation sites with synthetic cysteine-linked ones in recombinant proteins, thus enabling the synthesis of defined glycoforms of a glycoprotein for functional studies. There is as yet no simple biological or chemical means of obtaining glycoproteins with homogeneous carbohydrate structures attached.

Neoglycoproteins formed using this method can be used in lectin- and antibody-binding assays as shown by the specific binding of an anti-(HRP carbohydrate) monoclonal antibody (YZ1/2.23) and peanut agglutinin to neoglycoproteins presenting HRP oligosaccharides and Gal β 1-3GalNAc (Figures 3a and 4 respectively). This demonstrates that the integrity of the saccharide moieties has not been altered by the derivatization or coupling procedures. Multiple presentation of glycans in neoglycoproteins is advantageous in that affinity to sugar-binding proteins is often increased with multivalency. Neoglycoproteins produced by this method usually contain 15–21 oligosaccharide residues per BSA molecule. Not all 35 cysteine residues on fully reduced BSA were alkylated in the presence of excess *N*-(β -saccharide) iodoacetamide derivatives, which suggests that disulphide formation was occurring during the reaction. It may be possible to alkylate serum albumin fully in an oxygen-free atmosphere, although this was not further investigated.

Synthetic cysteine linkage mimics natural N-linkage as it is equivalent to N-linkage that is extended by an extra CH₂-S group. Importantly we have shown that cysteine-linked oligosaccharides can be released from two neoglycoproteins. To our knowledge there is currently no scheme for the release of unprotected sugars from neoglycoproteins. Hydrazinolysis of the neoglycoproteins containing the major HRP oligosaccharide unexpectedly yielded two oligosaccharides. The major HRP oligosaccharide structure has a hydrodynamic volume of 8.8

glucose units which corresponds to peak A in Figure 3b. The oligosaccharide with hydrodynamic volume of 5.8 glucose units (Figure 3b, peak B) was not identified, but may be the result of the loss of the core fucose and *N*-acetylglucosamine residues from the 8.8 glucose unit structure which would reduce its hydrodynamic volume to 5.8 glucose units. This result indicates that these sugars retained their reducing ends on cleavage from neoglycoproteins, and suggests a test to determine the stability of certain oligosaccharide structures to hydrazinolysis. Similarly radiolabelled glycans from transferase-treated asialofetuin and neoglycoprotein containing Gal β 1-3GalNAc were recovered after automated hydrazinolysis. The conditions for maximum release were not determined as the amount of each released glycan was more than sufficient for analysis by weak anion-exchange h.p.l.c. The released glycans have similar elution times to those of monosialylated sugars from fetuin on a weak anion-exchange column (Figure 5). In the case of the neoglycoprotein containing Gal β 1-3GalNAc, enzymic transfer of sialic acid created NeuNAc α 2-3Gal β 1-3GalNAc on BSA. This suggests the possibility of using this type of linkage in solid-phase enzymic oligosaccharide synthesis schemes.

In summary this study has illustrated the general use of *N*-(β -saccharide) iodoacetamides for conjugating sugars to cysteinyl side chains of peptides and proteins. Our results demonstrate its usefulness in applications such as site-specific glycosylation and solid-phase enzymic oligosaccharide synthesis.

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