Structural analysis of the asparagine-linked oligosaccharides of human complement component C3

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The asparagine-linked oligosaccharides of human C3 were characterized. The C3 oligosaccharides were released by endo-β-N-acetylglucosaminidase H and were analysed by lectin affinity chromatography and h.p.l.c. The released oligosaccharides bound tightly to concanavalin A–Sepharose and were not retained by agarose-bound wheat-germ agglutinin, indicating that they were only of high-mannose type. Two major oligosaccharide structures were separated from both the α- and β-chains of C3 by h.p.l.c. on Micropak AX-5, calibrated with high-mannose-type oligosaccharides of known structures. The oligosaccharide structures on the α-chain have the compositions $(Man)_9(GlcNAc)_2$-Asn and $(Man)_8(GlcNAc)_2$-Asn, and those on the β-chain have the compositions $(Man)_6(GlcNAc)_2$-Asn and $(Man)_5(GlcNAc)_2$-Asn.

INTRODUCTION

The third component of human complement (C3) is a 185000 Da glycoprotein that is composed of two polypeptide chains with molecular masses of 115000 (α-chain) and 75000 Da (β-chain) [1, 2]. C3 is synthesized as a single-chain precursor (pro-C3), which is processed by proteolytic cleavage into the α- and β-chains, which are linked by disulphide bonds [3]. The complete cDNA coding sequence and the derived amino acid sequence of human C3 have been reported [4].

Approx. 2% of the total mass of human C3 is due to carbohydrate [2, 5]. By using g.l.c. and m.s. methods, C3 has been found to contain only N-acetylglucosamine and mannose (approximate molar ratio 1:4) [5]. Both the α- and β-chains are glycosylated [6–8], and the sites of asparagine glycosylation have been determined as residues 67 (β-chain) and 917 (α-chain) of the C3 amino acid sequence [4, 8]. The carbohydrate moieties on the C3 α- and β-chains are susceptible to endo H and are therefore either of the high-mannose type or of the hybrid type [8].

The carbohydrate moiety on the α-chain has been found to be the binding site for the bovine plasma protein conglutinin [8]. Binding of conglutinin to the C3 molecule requires Ca^{2+} and is inhibitable by N-acetylglucosamine, chitobiose and mannose [8–10]. In the present paper we describe the structural analysis of the N-linked oligosaccharides of human C3.

MATERIALS AND METHODS

Endo H digestion of C3

Human C3 was purified as previously described [11]. C3 (14 mg) was incubated with 1% (w/w) trypsin (Copper Biochemical Co.) for 2 min at 37 °C and then immediately passed over an L-alanyl-L-alanyl-L-alanine column (Pierce Chemical Co.) to remove the trypsin. The column (0.8 cm × 2.5 cm) was washed with 5 bed volumes

of 10 mM-Tris/HCl buffer, pH 8.0, and the C3b was dialysed against 50 mM-sodium acetate buffer, pH 5.5 (endo H buffer), at 4 °C. We had previously observed that endo H was more efficient in removing the oligosaccharides from C3b rather than from native C3. The trypsin-treated C3 was then exhaustively treated with endo H (0.3 unit; 48 h at 37 °C) (Miles Scientific), bioled for 2 min and centrifuged (at 4000 g). The pellet was washed with endo H buffer, then re-centrifuged, and the supernatants were combined. The combined supernatant received 2 vol. of ice-cold ethanol and then centrifuged as before. The supernatant contained the endo-H-released oligosaccharides.

NaB^3H_4 reduction of released oligosaccharides

Oligosaccharides were labelled with a 10-fold molar excess of NaB^3H_4 (10 Ci/mmol; Amersham) in 0.3 ml of 50 mM-NaOH for 4 h at 30 °C. Termination of the reaction and subsequent procedures to isolate ^3H-labelled oligosaccharides from the free NaB^3H_4 and other contaminants were followed as previously described [12].

Lectin affinity chromatography

Chromatography on Con A–Sepharose (Pharmacia) [13] and agarose-bound WGA (Vector Laboratories) [14] was performed as described previously.

Liberation of oligosaccharides from C3 α- and β-chains

C3 α- and β-chains were separated by electrophoresis on preparative SDS/polyacrylamide slab gels. Protein bands were located with cold 1 M-KCl, cut from the gels, washed in distilled water and sliced. The separated α- and β-chains were then treated with Pronase (Calbiochem) (4%, w/w) in 0.1 M-Tris/HCl buffer, pH 8.0, containing 1 mM-Ca^{2+} for 48 h at 60 °C. Released glycopeptides were separated from the gel pieces, and before being freeze-dried were boiled for 10 min to destroy any Pronase activity. The separated glycopeptides were treated with endo H (0.1 unit) for 48 h at 37 °C.

Abbreviations used: GlcNAcol, N-acetylglucosaminitol; Con A, concanavalin A; WGA, wheat-germ agglutinin; endo H, endo-β-N-acetylglucosaminidase H.

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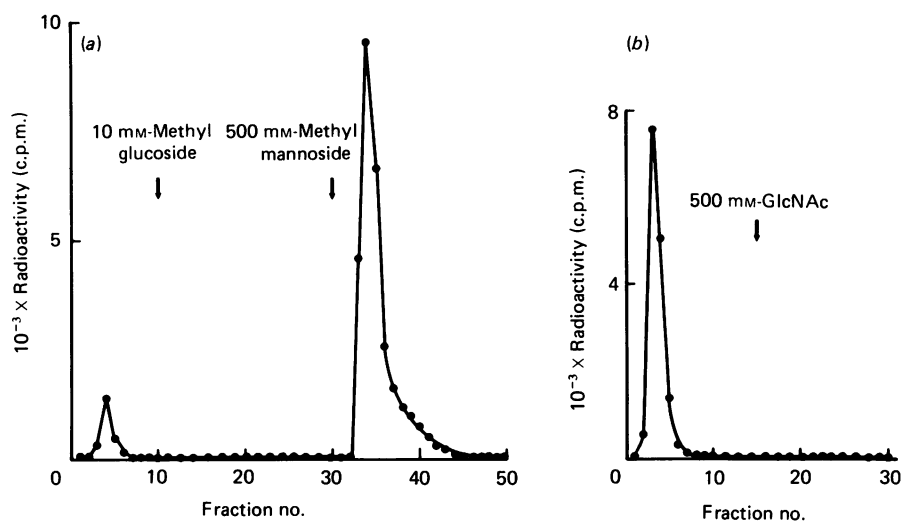
Fig. 1. Affinity chromatography of C3 oligosaccharides

3H-labelled oligosaccharides were applied to a column of (a) Con A–Sepharose and (b) agarose-bound WGA. The columns were washed with equilibration buffers (for Con A–Sepharose, 10 mM-Tris buffer, pH 8.0, containing 1 mM-Ca^{2+}, 1 mM-Mg^{2+} and 150 mM-NaCl; for WGA–agarose, 10 mM-sodium phosphate buffer, pH 7.3, containing 150 mM-NaCl), and elution was continued with various sugars as indicated.

Incubation was terminated by boiling for 2 min, and the material was applied to a column (0.8 cm × 4 cm) of Con A–Sepharose that had been prewashed with 0.1 M-HCl and re-equilibrated with 10 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-Ca^{2+}, 1 mM-Mg^{2+} and 150 mM-NaCl. Bound oligosaccharides were eluted with 0.1 M-HCl [15], adjusted to pH 7 and freeze-dried. The oligosaccharides were then reduced with NaB^3H_4 as before.

H.p.l.c.

The reduced oligosaccharides were analysed by h.p.l.c. on a MicroPak AX-5 (Varian Associates) column (4 mm × 30 cm) as described previously [16] except that fractions were collected at 0.6 min intervals. High-mannose-type oligosaccharides of known structures, kindly provided by Dr. H. Freeze, were used to establish precise elution positions. Double peaks are due to an alkali-induced epimerization of reducing terminus that ensued during the reduction of oligosaccharides in 50 mM-NaOH [16].

a-Mannosidase digestion

Digestion with jack-bean α-mannosidase (Sigma Chemical Co.) was performed in 0.1 M-citrate/phosphate buffer, pH 4.0, in the presence of 2-acetamido-2-deoxygluconolactone (to inhibit any traces of β-hexosaminidase). One unit of enzyme was added initially, and another unit after 24 h at 37 °C. A small volume of toluene was added as a bacteriostatic agent. The digestions were terminated after 72 h by boiling for 2 min and desalted by passing over a small column of Amberlite MB-3 (1.5 ml) (Mallinckrodt) in water. The digests were analysed on MicroPak AX-5 as before, except that the program was started at 25% water in acetonitrile [17]. Cellobiose and maltose that had been reduced with $NaBH_4$ were used as disaccharide standards, and the phenol/H_2SO_4 method scaled down 10-fold [18] was used to detect them. 2-Acetamido-2-deoxygluconolactone was synthesized as described previously [19].

RESULTS AND DISCUSSION

The oligosaccharides released from C3b by endo H were subjected to affinity chromatography on Con A–Sepharose and agarose-bound WGA as shown in Fig. 1. Over 90% of the radioactivity applied bound to Con A and was eluted with 500 mM-methyl mannoside (Fig. 1a). As shown previously [13, 20, 21], N-linked glycopeptides can be separated into three fractions on Con A–Sepharose, on the basis of different affinities for the lectin. Tri- and tetra-antennary complex-type oligosaccharides pass through unretarded by the column, whereas biantennary complex-type oligosaccharides bind to Con A but can be eluted with low concentrations of competing sugar. With high-mannose- or hybrid-type oligosaccharides the binding is stronger, and higher concentrations of sugar are needed for elution. Yamamoto et al. [14] have demonstrated that certain glycopeptides with hybrid-type structures and a 'bisecting' N-acetylglucosamine residue bind to WGA–agarose, providing a way of separating these species from the typical high-mannose-type molecules that fail to bind to this lectin. The C3 oligosaccharides were not retained on a column of WGA–agarose (Fig. 1b). Their susceptibility to endo H and the behaviour of these oligosaccharides on the lectin affinity columns indicates that the C3 oligosaccharides are only of the high-mannose type.

The 3H-labelled oligosaccharides isolated from C3 and its constituent chains were analysed by anion-exchange h.p.l.c. on a MicroPak AX-5. The chromatographic profiles displayed in Fig. 2 show that two major oligosaccharide structures are present on both the α- and β-chains (Figs. 2b and 2c). The α-chain oligosaccharides co-migrated with authentic high-mannose-type oligosaccharides bearing eight and nine mannose residues, whereas the β-chain oligosaccharides co-migrated with those bearing five and six mannose residues. These structures were also evident when the C3 oligosaccharide mixture was fractionated (Fig. 2a).
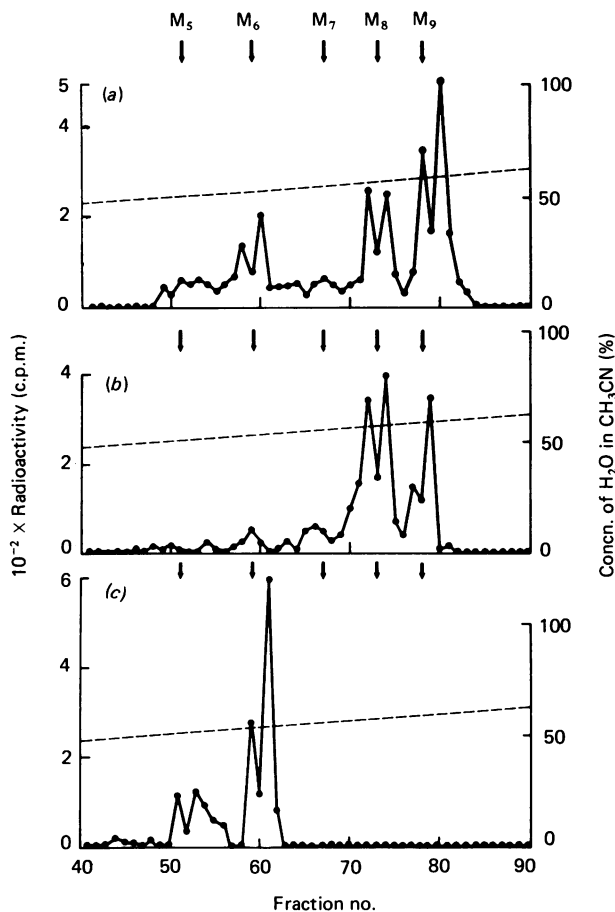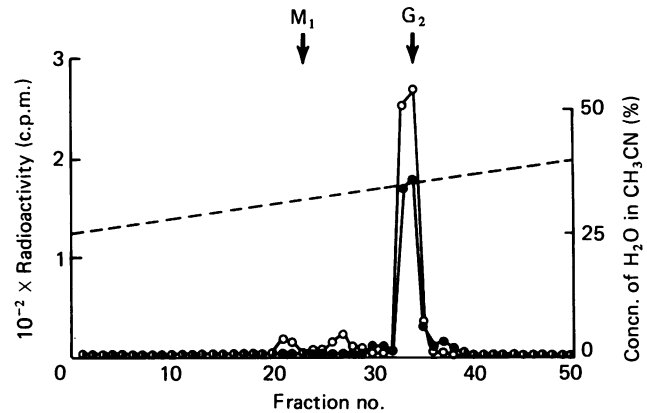
Fig. 2. Analysis of C3 oligosaccharides by h.p.l.c. on MicroPak AX-5

Endo-H-released oligosaccharides from C3 and its separated chains were reduced with NaB^3H_4 and analysed on MicroPak AX-5. (a) C3 oligosaccharides; (b) α-chain oligosaccharides; (c) β-chain oligosaccharides. The standard markers are: M_9, $Man_9GlcNAcol$; M_8, $Man_8GlcNAcol$; M_7, $Man_7GlcNAcol$; M_6, $Man_6GlcNAcol$; M_5, $Man_5GlcNAcol$.

Fig. 3. α-Mannosidase digestion of oligosaccharides from α- and β-chains of C3

Purified oligosaccharides from the α-chain ($\bullet$) and β-chain ($\bigcirc$) were digested with jack-bean α-mannosidase, desalted and analysed by h.p.l.c. as described in the Materials and methods section. G_2 indicates elution of disaccharides cellobiose and maltose; M indicates elution of mannitol.

Digestion of the liberated α- and β-chain oligosaccharides with jack-bean α-mannosidase (Fig. 3) converted all the oligosaccharides into species that co-migrated with the disaccharides maltose and cellobiose. The expected product of digestion of a high-mannose-type oligosaccharide with this enzyme would be the disaccharide $Man\beta1-4GlcNAcol$. Thus the appearance of a disaccharide after α-mannosidase digestion would rule out the presence of a bisecting or a terminal N-acetylglucosamine residue in either chain. Furthermore, the data presented here are in good agreement with the carbohydrate composition analysis given by Tomana et al. [5], who have estimated four and sixteen residues of N-acetylglucosamine and mannose respectively per C3 molecule.

The removal of the oligosaccharide chains from native C3 by endo H requires the detergent SDS (results not shown). the detergent presumably alters the protein structure such that the susceptible linkage to enzymic cleavage is exposed. In contrast, when native C3 is converted into C3b, endo H is able to remove the oligosaccharide chains in the absence of detergent. These

observations suggest steric inaccessibility of the oligosaccharide in native C3. Hsieh et al. [22] have previously used endo H to probe the relative accessibility of asparagine-linked oligosaccharides of Sindbis virions, and their results strongly support the hypothesis that the extent of oligosaccharide processing at individual glycosylation sites is determined primarily by the physical accessibility of oligosaccharides. Steric inaccessibility of the oligosaccharide is a possible explanation for the retention of eight or nine mannose residues on the α-chain of C3, and also why conglutinin does not bind to native C3 but binds to C3b [8, 10]. The high-mannose-type oligosaccharides present on C3 have been found in other glycoproteins such as IgM [23], Chinese-hamster ovary cells [24], cathepsin D from pig spleen [25] and the β-chain of human complement protein C4 [26]. However, the exclusive presence of high-mannose-type oligosaccharides on C3 is a novel and striking characteristic for a serum glycoprotein.

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