Carbohydrate Research, 90 (1981) 203–214
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

USE OF THE SMITH DEGRADATION IN THE STUDY OF THE BRANCHING PATTERN IN THE COMPLEX-TYPE CARBOHYDRATE UNITS OF GLYCOPROTEINS

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(Received May 2nd, 1980, accepted for publication in revised form, August 3rd, 1980)

ABSTRACT

The site of substitution of the peripheral branches on the core mannose-residues in the complex-type carbohydrate units of glycoproteins has been studied by Smith degradation. By using glycopeptides of known structure, it is shown that the site of attachment of the (1→4)-linked, third branch to the mannose core in tri- and tetra-antennary structures may be conveniently demonstrated by this technique. A critical factor was found to be the concentration of acid used for cleavage of the periodate-oxidized product. This finding could explain discrepancies concerning the previously published structures of fetuin glycopeptides. The technique was applied for study of the branching pattern in human transferrin, porcine thyroglobulin, and rat plasma and brain glycoproteins. The results support the concept that, in glycoproteins from most sources, the third (1→4)-linked branch is attached to the (1→3)-linked mannose residue of the core portion in the complex-type structures. An indication for a novel type of branching pattern in human transferrin was, however, also obtained.

INTRODUCTION

The complex-type carbohydrate units of glycoproteins are composed of a D-mannose–2-acetamido-2-deoxy-D-glucose core, to which two, three, or four peripheral branches or "antennas" are attached[1,2]. Whilst similar biantennary structures have been described from several sources[1,2], there has been disagreement as to the structure of the more branched tri- and tetra-antennary structures. The disagreement concerns the branching pattern, that is, the site of attachment of third and fourth branch. In α_1-acid glycoprotein[3], urinary oligosaccharides[4,5] and ovotransferrin[6], the third branch has been reported to be bound to the outer mannose (1→3)-linked to the internal mannose residue, whereas in VSV glycoprotein[7] it was reported to be bound to the (1→6)-linked mannose residue. Both of these two alternatives have been suggested for fetuin[8,9].

We have used Smith degradation to study the branching in such carbohydrate units, as the branching pattern can be readily concluded from the products by using

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methylation analysis A difficulty arises, however, in the unexpected acid-resistance of the periodate-oxidized product, which has led to discrepancies concerning the structure of fetuin glycopeptides[8,9] We found that this difficulty can be overcome by optimizing the conditions of the mild acid-hydrolysis step Using this technique we have analyzed a number of glycoproteins The results indicate that there is a common preferential branching pattern is the tri- and tetra-antennary structures of glycoproteins from several sources suggesting similar common pathways of biosynthesis

EXPERIMENTAL

Materials — Fetuin (Type IV), transferrin ovalbumin (Grade V), porcine thyroglobulin (Type II), pronase (protease Type VI) and a mixture of *O*-(*N*-acetylneuraminyl)-(2→3)-lactose and *O*-(*N*-acetylneuraminyl)-(2→6)-lactose were obtained from Sigma Chemical Company α_1-Acid glycoprotein was a generous gift from Dr. G Myllyla of the Finnish Red Cross Blood Transfusion Service, Helsinki *O*-(*N*-Acetylneuraminyl)-(2→6)-lactose and lacto-*N*-fucopentaose I were kindly supplied by Dr A Gauhe, Heidelberg [^{3}H]Acetic anhydride (500 mCi/mmol) was purchased from the Radiochemical Centre, Con A–Sepharose from Pharmacia Fine Chemicals and *Vibrio cholerae* neuraminidase (500 U/mL) from Calbiochem

Preparation of glycopeptides — Glycopeptides were prepared from glycoproteins (200 mg each) by extensive digestion with pronase[10] α_1-Acid glycoprotein was treated with *Vibrio cholerae* neuraminidase[11] prior to protease digestion to increase its susceptibility to proteolytic treatment[12] The glycopeptides produced were purified by gel filtration on Sephadex G-25 Fine[13] and *N*-[^{3}H]acetylated in their peptide moiety with [^{3}H]acetic anhydride[11]

Tri- and tetra-antennary glycopeptides were separated from biantennary complex-type and high-mannose-type structures by affinity chromatography on concanavalin A-Sepharose as described previously[14,15] Glycopeptides from fetuin, rat brain, and rat plasma, which were not bound to concanavalin A were subjected to mild alkaline-borohydride treatment to liberate the *O*-glycosylic oligosaccharides[16] The alkali-stable *N*-glycosylic glycopeptides were separated from the released *O*-glycosidic oligosaccharides by gel filtration on Sephadex G-50 as described previously[17]

The tri- and tetra-antennary glycopeptides from α_1-acid glycoprotein were separated by gel filtration on Sephadex G-50 Fine The column (2 cm × 75 cm) was eluted with 0 1M pyridine–acetic acid buffer (pH 5 0). Fractions of 4 5 mL were collected and counted for radioactivity The glycopeptide peak was pooled in five parts The earliest eluting pool, containing the tetra-antennary glycans (as indicated by methylation analysis) was used for Smith degradation

Plasma of adult Albino Wistar rats was prepared as described previously [8] Plasma and whole brains were delipidated[13,18] and the lipid-free residue was subjected to extensive digestion by pronase[10] After the digestion, the glycosaminoglycans were precipitated with cetylpyridinium chloride[13] and the glycopeptides purified by gel

filtration[13] The purified glycopeptides were *N*-[^{3}H]acetylated and fractionated as already described

Smith degradation — Glycopeptides (0.7–1.0 μmol total sugars) were oxidized with 0.05M sodium metaperiodate in 2 mL of 0.05M sodium acetate buffer (pH 4.4) for 3 days at 4° in the dark. The excess of periodate was decomposed with 35 μL of ethylene glycol. The solution was brought to pH 7 with M sodium hydroxide and the oxidized sugars were immediately reduced with 0.5 mL of M sodium borohydride for 3 h at 20°. Borohydride was decomposed by addition of acetic acid to pH 5 and the glycopeptides were purified by gel filtration on Sephadex G-25 Fine. The column (2 × 45 cm) was eluted with 10mM pyridine–acetic acid buffer (pH 5.0). Fractions of 5.5 mL were collected and aliquots counted for radioactivity. Fractions containing the glycopeptides were pooled and lyophilized. After relyophilization from 2 mL of water, the glycopeptides were hydrolyzed in 2 mL of 0.1M hydrochloric acid for 1 h at 80°.

Methylation analysis — Glycopeptides were methylated by the method of Hakomori[19]. Additional samples were also methylated by using a methylation reagent made from potassium *tert*-butoxide in order to decrease the extent of interfering noncarbohydrate peaks[20]. The methylated glycopeptides were degraded by acetolysis and acid hydrolysis[21]. The samples were treated with 0.5 mL of M sulfuric acid in 95% acetic acid for 16 h at 80°. The mixture was then mixed with an equal volume of water and heated for an additional 5 h at 80°.

Partially methylated alditol acetates were analyzed by gas–liquid chromatography and mass spectrometry. Alditol acetates of neutral sugars (except monomethylated hexoses) were chromatographed on 1% OV-225 or 3% OV-210 at 180 or 190°, and corresponding derivatives of amino sugars on 2.2% OV-101 at 205°. Monomethylated hexoses were determined on 1% OV-225 at 200°. Detection was performed by total ionization current and by mass fragmentography at *m/z* values 161, 189, and 233 for neutral sugars and 158 for amino sugars as described previously[17]. Response factors for various differentially substituted sugar-residues in mass-fragmentographic detection at different *m/z* values were obtained from reference glycopeptides and oligosaccharides having known structures: the biantennary glycan from transferrin[22], fetuin[9], the tetraantennary glycan from α_1-acid glycoprotein[3], *O*-(*N*-acetylneuraminyl)-(2→3)-lactose, *O*-(*N*-acetylneuraminyl)-(2→6)-lactose, and lacto-*N*-fucopentaose I. As no homogeneous reference compound containing 3,4,6-tri-*O*-substituted mannose was available, this sugar derivative was quantitated by total ionization current.

Retention times of partially methylated alditol acetates were compared with those obtained from reference oligosaccharides and glycopeptides and with values reported previously[23]. The methyl substitution-patterns were confirmed by mass-spectral analysis[23,24]. Mass spectra were recorded with a Varian MAT CH-7 mass spectrometer equipped with SpectroSystem 100 MS data-processing system. The ionization potential was 70 eV and the ionization current 300 μA. An Altema AL 5 multiple-ion detector was used for mass fragmentographic detection.

RESULTS AND DISCUSSION

Determination of the branching pattern by Smith degradation and methylation analysis — In the structures proposed for fetuin and other glycoproteins, a disagreement concerns the site of attachment of the third peripheral branch or "antenna"[3-9] As shown in Fig 1, it is possible to differentiate between these two alternatives by using Smith degradation followed by methylation analysis of the product In structure *A*, the 3,6-disubstituted mannose will be converted to a 3-monosubstituted residue, whereas structure *B* would give rise to a 6-monosubstituted mannose residue As the 2-acetamido-2-deoxy-D-glucose residues in this type of glycopeptides is usually periodate-resistant, the 2,4-disubstituted mannose residue remains unchanged

The approach is also suitable for analysis of the site of attachment of the (1→4)-linked third branch in tetraantennary structures In such glycans, the fourth branch is bound by a (1→6)-linkage to the 2-*O*-substituted mannose residue As the 2,6-di-*O*-substituted mannose is periodate-labile, a similar change in the substitution pattern of the internal, 3,6-di-*O*-substituted mannose residue should be seen after Smith degradation, as in the case for the triantennary carbohydrate units

The method was tested by using glycopeptides of known structure As shown in Table I for the tetraantennary glycan of α_1-acid glycoprotein, the results of methylation analysis after Smith degradation are, as expected[3], consistent with structure *A* in Fig 1

As a further control of the experimental procedure, ovalbumin glycopeptide were analyzed The "high-mannose' and "hybrid" type carbohydrate units of ovalbumin contain a periodate-stable mannose residue bound (1→6) to the internal mannose[25,26] Fig 2 shows the results of Smith degradation of total ovalbumin glycopeptides Taking into account the response factors, the ratio of the 6-*O*-substituted mannose to that of the 3,6-di-*O*-substituted mannose was found to be 6 1, which is in good agreement with the expected value of ~4 1 as calculated from the

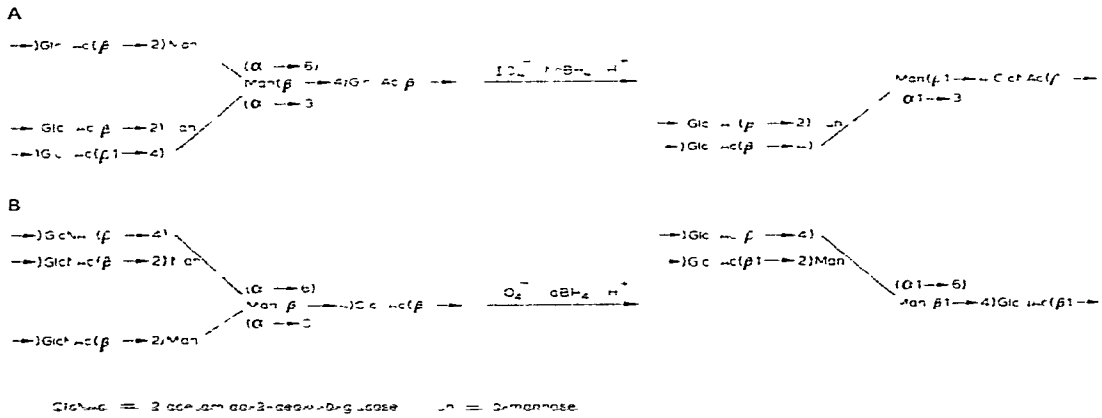

Fig 1 Two alternative possibilities for the branching pattern in triantennary glycans and the products expected after one cycle of Smith degradation

TABLE I

RELATIVE AMOUNTS OF PARTIALLY METHYLATED MANNITOL ACETATES OF NATIVE AND SMITH-DEGRADED GLYCOPEPTIDES. VALUES OF UNTREATED GLYCOPEPTIDES ARE EXPRESSED AS MOLES PER THREE MOLES OF MANNOSE, AND VALUES OF DEGRADED GLYCOPEPTIDES RELATIVE TO 3,6 DI-*O*-METHYL MANNOSE

Component *Glycosidic linkage*	*3,4,6-tri-Me* *2*	*2,4,6 tri O-Me* *3*	*2,3,4-tri-O-Me* *6*	*3,6-di-O-Me* *2 and 4*	*3,4-di O Me* *2 and 6*	*2,4 di O Me* *3 and 6*	*2-Mono O Me* *3, 4, and 6*
α_1-Acid glycoprotein							
native	0 1	0 0[a]	0 0	0 9	1 0	1 0	0 0
degraded	0 0	0 8	0 0	0 9	0 1	0 1	0 0
Fetuin							
native	1 0	0 0	0 0	1 0	0 0	1 0	0 0
degraded	0 1	1 0	0 0	1 0	0 0	0 1	0 0
Transferrin (fraction A)							
native	1 1	0 0	0 1	0 5	0 4	0 9	0 0
degraded	0 0	0 5	0 1	0 5	0 0	0 0	0 0
Thyroglobulin (fraction A)							
native	0 4	0 0	0 0	0 8	0 6	1 2	0 0
degraded	0 0	0 7	0 0	0 8	0 0	0 2	0 0
Plasma (fraction A)							
native	1 2	0 0	0 1	0 6	0 2	0 9	0 0
degraded	0 0	0 5	0 1	0 6	0 0	0 1	0 0
Brain (fraction A)							
native	0 7	0 0	0 0	0 8	0 6	0 7	0 2
degraded	0 1	0 8	0 2	0 8	0 0	0 2	0 0

[a] 0 0 = less than 0 05

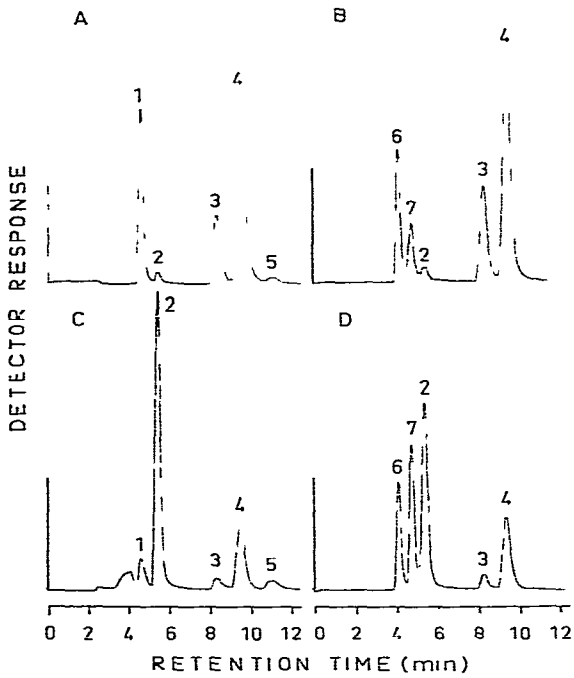

Fig 2 Mass fragmentograms of partially methylated alditol acetates obtained from total ovalbumin glycopeptides Upper traces (A and B), untreated glycopeptides, lower traces (C and D) Smith degraded glycopeptides Peak 1, 3 4 6-tri-*O*-methylmannitol, peak 2, 2 3,4-tri-*O*-methylmannitol, peak 3 3 6-di-*O*-methylmannitol, peak 4, 2 4-di-*O*-methylmannitol, peak 5, 3,4-di-*O*-methylmannitol, peak 6, 2 4 6-tri-*O*-methylmannitol, and peak 7 2,3,6-tri-*O*-methylmannitol Conditions 3% OV-210, 190° detection by mass fragmentography (A and C) *m/z* 189, (B and D) *m/z* 233

relative proportions of the ovalbumin glycopeptides and their known structures[25 26] This result indicates that if a periodate-stable sugar residue is (1→6)-linked to the internal mannose (structure *B*) it is detected by the method used

Influence of the conditions of mild acid hydrolysis — In accordance with the proposed structures of fetuin[8 9], one mole each of 2-*O*-substituted, 2 4-di-*O*-substituted and 3,6-di-*O*-substituted mannose residues were found in the methylation analysis of the fetuin glycopeptide (Table I and Fig 3) As expected periodate treatment decomposed the 2-*O*-substituted mannosyl nearly quantitatively, whereas the relative proportions of 2,4-di-*O*-substituted and 3,6-di-*O*-substituted mannose residues were unchanged. After periodate treatment and mild acid hydrolysis, the relative amount of 3,6-di-*O*-substituted mannose was decreased to 0 1 mol (Table I) In gas–liquid chromatography, one major new peak was observed (Fig 3, peak 8) Based on its mass spectrum and relative retention-time, this component was identified as the partially methylated derivative arising from 3-*O*-substituted mannose The result is in agreement with structure *A* (Fig 1), as recently proposed by Nilsson *et al* [9]

In another recent study, the branching pattern of fetuin glycopeptides was reported[8] to correspond to structure *B* (Fig 1) The studies were performed by

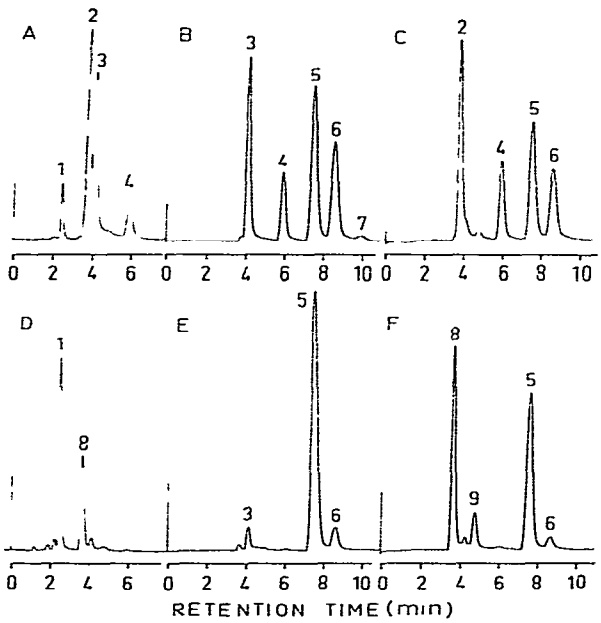

Fig 3 Mass fragmentograms of partially methylated alditol acetates obtained from fetuin glycopeptides Upper traces (A–C) untreated glycopeptides lower traces (D–F) Smith-degraded glycopeptides Peak *1*, 2,3,4,6-tetra-*O*-methylgalactitol, peak *2*, 2,4,6-tri-*O*-methylgalactitol peak *3* 3,4,6-tri-*O*-methylmannitol, peak *4*, 2,3,4-tri-*O*-methylgalactitol, peak *5* 3,6-di-*O*-methylmannitol peak *6* 2,4-di-*O*-methylmannitol, peak *7* 3,4-di-*O*-methylmannitol peak *8* 2,4,6-tri-*O*-methylmannitol, and peak *9*, 2,3,6-tri-*O*-methylglucitol Conditions 3% OV-210, 180 (A and D) and 190 (B, C E and F) Detection by mass fragmentography (A and D) *m/z* 161 (B and E) *m/z* 189 and (C and F) *m/z* 233

treatments with glycosidase and by methylation analysis It was reported that the periodate oxidized and subsequently reduced substituent bound to the 3,6-di-*O*-substituted mannose was resistant to hydrolysis, and Smith degradation could therefore not be used for the structural study[8] In order to study whether the different results could arise from differences in experimental conditions glycopeptides were also subjected to Smith degradation exactly as reported in the foregoing study Under these conditions, and using mild acid treatment (25mM sulfuric acid, 1 h 80°), only small amounts of 3-*O*-substituted mannose were produced (Fig 4D, peak 8) However, when the periodate-oxidized and reduced glycopeptides were hydrolyzed with 50 and 100mM sulfuric acid, the proportion of 3-*O*-substituted mannose was increased (Fig 4F and H), and was found in amounts similar to those found after the standard conditions of hydrolysis (0 1M, 1 h, 80°) used in the present study (Fig 3)

In order to study whether production of the 3-*O*-substituted mannose could be due to "nonspecific" acid degradation, fetuin glycopeptides that had not been subjected to periodate oxidation were directly hydrolyzed by 100mM hydrochloric acid No changes in the relative proportions of the differentially substituted mannose residues were detected, indicating that the production of the 3-*O*-substituted mannose

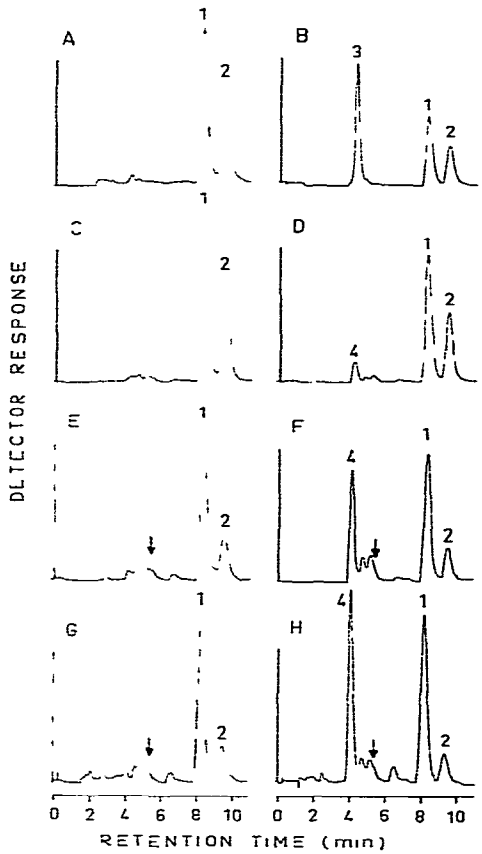

Fig 4 Mass fragmentograms of partially methylated alditol acetates obtained from periodate-oxidized and subsequently reduced fetuin glycopeptides (A and B), not hydrolyzed, (C and D), hydrolyzed by 25mM sulfuric acid, (E and F), hydrolyzed by 50mM sulfuric acid, and (G and H), hydrolyzed by 100mM sulfuric acid Peak 1, 3,6-di-*O*-methylmannitol, peak 2, 2,4-di-*O*-methylmannitol, peak 3, 2,4,6-tri-*O*-methylgalactitol, and peak 4, 2,4,6-tri-*O*-methylmannitol The retention time of 2,3,4-tri-*O*-methylmannitol is indicated by an arrow Conditions 3% OV-210, 190°, detection by mass fragmentography (A, C, E, and G), *m/z* 189, and (B, D, F and H) *m/z* 233

was due specifically to periodate oxidation The results are thereafter in agreement only with structure *A* (Fig 1) The previous suggestion[8] of structure *B* can most likely be ascribed to the inherent problems in the approach of using glycosidase digestion for structural analysis[27] Possible differences between fetuin preparations obtained from different sources have, however, also to be considered

The triantennary glycans of transferrin — Human transferrin contains both biantennary and triantennary *N*-glycosylic carbohydrate units[28 29] The relative amount of triantennary glycans is ~15% of all carbohydrate[14 29] The tri- and bi-antennary glycans were isolated by affinity chromatography on Con-A-Sepharose[14]. The structure of the biantennary glycan has been previously described[22], whereas the structures of the triantennary glycans have not yet been fully elucidated[28 29]

Methylation analysis of the triantennary glycans revealed, as expected, that the ratio of monosubstituted to disubstituted mannose residues was 1 2 (Table I) An unexpected finding was, however, that these glycopeptides also contained 2 6-di-*O*-substituted mannose, and that the relative amount of 2,4-di-*O*-substituted mannose was only 0 5 mol per glycopeptide (Table I) As was the case with glycopeptides from fetuin and α_1-acid glycoprotein, Smith degradation converted the 3,6-di-*O*-substituted mannose into 3-*O*-substituted mannose, and in equimolar amount to 2,4-di-*O*-substituted mannose Trace amounts of 6-*O*-substituted mannose were possibly also present, both in the native and in the oxidized glycopeptide

Although several possibilities exist, the most probable explanation for the results is that transferrin contains two types of triantennary glycan, which differ in their mannose branching pattern In about half of the structures, the third branch would be attached by a (1→4) linkage to the outer mannose residue, as in fetuin and α_1-acid glycoprotein In the rest of the glycans, the third branch could be bound by a (1→6) linkage to one of the outer mannose residues The occurrence of two different triantennary glycans in transferrin was recently suggested by Regoeczi *et al* [30]

Glycopeptides from thyroglobulin — Methylation analysis of porcine thyroglobulin glycopeptides not bound to concanavalin A suggests that both tri- and tetraantennary glycans were present in this fraction (Table I) This interpretation is reflected as a proportion of 2-*O*-substituted mannose clearly below 1 0 and as a proportion of di-*O*-substituted mannose residues above 2 0 Smith degradation converted the 3,6-di-*O*-substituted mannose into 3-*O*-substituted mannose No 6-*O*-substituted mannose was detected Provided that the glycopeptides correspond to the common type of complex-type glycopeptides, the (1→4)-linked branch would be bound also in this glycoprotein according to structure *A*, Fig 1

The results are seemingly in disagreement with a previous report on the branching pattern of thyroglobulin glycopeptides[31] It was reported that one peripheral branch is bound by a (1→2) linkage, whereas the second and third branches are bound by (1→3) and (1→6) linkages to the mannose core This difference may be due to the fact that the glycopeptides characterized in that study accounted for only 6–7% of the total carbohydrate from thyroglobulin, whereas, in the present study, all triantennary and tetraantennary glycopeptides derived from thyroglobulin were analyzed as one fraction, in order to reveal the predominant structures

Glycopeptides from rat plasma and brain — As single, randomly chosen glycoproteins could carry a selection of carbohydrate chains that may not be representative of the general situation, glycopeptides from rat whole plasma were also analyzed Analysis of the whole, unfractionated mixture should thus give a quantitative picture of the branching pattern In addition, glycopeptides from rat brain were analyzed as they represent, in contrast to all of the soluble glycoproteins analyzed, mainly membrane-derived material[32]

The complex-type glycopeptides derived from plasma that are not bound to concanavalin A resemble triantennary glycopeptides from fetuin and transferrin in molecular size, in sugar composition, and in the relative proportions of differentially

substituted mannose residues[18] (Table I) The corresponding glycopeptide fraction prepared from brain tissue is of similar or slightly larger size in gel filtration[17], and the results of the methylation analysis suggest that it could be constituted of both tri- and tetra-antennary glycans (Table I) The relative amount of 2-*O*-substituted mannose was below 1 0 mol and that of di-*O*-substituted mannose residues above 2 0 mol per mol of glycopeptide In contrast to the other glycopeptide fractions studied, brain glycopeptides also contained 3,4,6-tri-*O*-substituted mannose (Table I) Similarly substituted mannose residues have previously been described from immunoglobulins[33 34], ovalbumin[25 26] and ovotransferrin[6], where they constitute the internal 3,6-di-*O*-substituted mannose, to which in addition 2-acetamido-2-deoxy-D-glucose is bound at O-4 The sum of the relative proportions of 3,6-di-*O*-substituted and 3,4,6-tri-*O*-substituted mannose residues in brain glycopeptides was 0 9 mol per mol of glycopeptide which is in good agreement with the common core-pentasaccharide structure

Periodate oxidation and mild acid-hydrolysis of glycopeptides derived from brain and plasma decomposed nearly quantitatively their 2-*O*-substituted, 2,6-di-*O*-substituted, 3,6-di-*O*-substituted and 3,4,6-tri-*O*-substituted mannose residues (Table I) In both instances, the major, new partially methylated sugar was the derivative of 3-*O*-substituted mannose, whereas only small amounts of 6-*O*-substituted mannose were produced Provided the glycans of brain and plasma glycoproteins are composed of the same types of basic structure as are present in other glycoproteins, the results suggest that the mannose branching pattern of the (1→4)-linked branch is also similar (structure *A* Fig 1)

Final comments — In the samples studied representing tri- and tetra-antennary structures, the (1→4)-linked, third branch was found to be bound mainly to the outer mannose residue, (1→3)-linked to the internal mannose residue As the glycopeptides studied were from different animal species and tissue sources, and represent both soluble and membrane-bound glycoproteins, this specific branching-pattern may be a common structural property of glycoproteins This may indicate a common biosynthetic pathway for the addition of the third and fourth peripheral branches of 'antennas'

The occurrence of a common, predominant biosynthetic pathway would not, however, exclude the occurrence of other structures as well A proportion of the triantennary glycans of transferrin contained a (1→6)-linked, third branch This suggests that the synthesis of the (1→4)- and (1→6)-linked branches may be competing reactions in some cases. Another type of branching has also been suggested for the glycans of calf thymocytes[35]

The common triantennary structure shows resemblance to the so-called mixed-type glycans from ovalbumin[25 26] These glycans may be regarded as representing intermediates between neutral-type and complex-type carbohydrate units. The mixed-type glycans contain peripheral branches composed of 2-acetamido-2-deoxy-D-glucose and galactosyl-2-acetamido-2-deoxy-D-glucose bound by (1→2) and (1→4) linkages to the outer mannose residues of the core In every instance, the

(1→4)-linked branch is bound to the outer mannose linked (1→3) to the internal mannose residue[25,26], that is in the same sequence as in the complex-type glycans The presence of the (1→4)-linked branches in the mixed-type glycans may indicate that the synthesis of the third branch could sometimes begin already before the synthesis of the first two branches has been completed

The biantennary carbohydrate structure is being found in an increasing number of glycoproteins Most of these glycoproteins have, however, been isolated from plasma Analysis of glycopeptides from different tissue sources, representing mainly membrane-bound molecules has, in contrast, revealed that the majority of the complex-type glycans occur as more-branched structures[17,18] In view of the postulated biological functions of membrane-bound carbohydrates[36,37], it will be necessary to elucidate in detail the structures and biosynthetic pathways of these branched glycans This will also be of importance for understanding the transformation-associated glycosylation change, which seems to affect specifically this class of carbohydrate unit[38]

ACKNOWLEDGMENTS

We thank Mrs Maire Ojala Mrs Hilkka Ronkko, and Mrs Kirsti Salmela for skilful technical assistance This work was supported by the Sigrid Juselius Foundation

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