Carbohydrate Research, 138 (1985) 91–98
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

METHYLATION ANALYSIS IN GLYCOPROTEIN CHEMISTRY: A COMPARATIVE STUDY OF THE CARBOHYDRATE STRUCTURES OF THREE HORMONE-BINDING GLYCOPROTEINS FROM HUMAN SERUM

GEORGE V. AVVAKUMOV AND OLEG A. STREL'CHYONOK

Laboratory of Protein Hormone Chemistry, Institute of Bio-organic Chemistry, Academy of Sciences of the Byelorussian S.S.R., Minsk 220600 (U.S.S.R.)

(Received June 21st, 1984; accepted for publication, September 18th, 1984)

ABSTRACT

The structures of the carbohydrate moieties of three hormone-binding glycoproteins from human serum, namely, thyroxine-binding globulin, transcortin, and sex hormone-binding globulin, have been characterised using quantitative g.l.c. of the methylated monosaccharide derivatives obtained after methanolysis of the methylated glycoproteins.

INTRODUCTION

The carbohydrate moieties of the serum hormone-binding glycoproteins are not directly involved in the formation of hormone–glycoprotein complexes[1]. However, the oligosaccharide chains of glycoconjugates often play a key role in the biological recognition processes at the cellular and molecular levels[2]. This is the reason for the interest attached to investigations of the carbohydrate structures of the serum hormone-binding glycoproteins as possible determinants for the recognition of the hormone–glycoprotein complexes by the corresponding target cells[3].

Methylation analysis is one of the most powerful tools available for elucidating the carbohydrate structures of glycoproteins[4,5]. G.l.c. or g.l.c.–m.s. is now used conventionally for the analysis of the methylated monosaccharide derivatives formed after solvolysis of methylated glycoprotein oligosaccharide-chains[6].

The general principles of structural organisation of the glycoprotein carbohydrate moieties are now established[5]. This knowledge provides an opportunity for obtaining information on the structure of sugar chains of a glycoprotein by applying the methods of carbohydrate chemistry directly to the intact biopolymer and thereby avoiding fragmentation and tedious isolation of individual sugar chains, which is an advantage when studying glycoproteins that occur in minor amounts in human or animal organisms. However, due to the microheterogeneity inherent in the glycoprotein carbohydrate moieties[5], the scope and reliability of this information will depend on the accuracy of the analytical techniques used.

Akhrem *et al.*[7,8] have reported a technique for the quantification of methylated monosaccharides as the corresponding trimethylsilylated alditol and 2-deoxy-

0008-6215/85/$ 03.30

2-(*N*-methylacetamido)alditol derivatives. This technique involves high-performance g.l.c., using columns packed with a surface-modified silica carrying, as stationary phase, the polar silicone OV-225 at a low concentration. This technique was intended not only for the study of glycopeptides or oligosaccharides but also for the methylation analysis of intact glycoproteins. The latter possibility has been demonstrated with ovalbumin[8] and human transcortin[9], and we now report applications to the hormone-binding glycoproteins from human blood serum, namely, thyroxine-binding globulin (TBG), transcortin, and sex hormone-binding globulin (SHBG).

EXPERIMENTAL

For isolation of TBG, transcortin, and SHBG from human post-partum blood-serum, the techniques previously described[10–12] were used. The glycoprotein preparations obtained were homogeneous according to gel electrophoresis and analysis of *N*-terminal amino acid; their basic physicochemical properties and parameters of hormone binding were similar to those earlier determined[10–12].

Methylation of the glycoproteins by the Hakomori method[13] was performed essentially as previously described[8]. The conditions for the methanolysis of methylated glycoproteins, transformation of the methylated methyl glycosides into the corresponding trimethylsilylated alditol derivatives, and g.l.c. have been described elsewhere[7,8]. Standard methylated derivatives of 2-acetamido-2-deoxyglucose were synthesised by conventional techniques[14]. The methyl ethers of manno- and galacto-pyranosides used as standards were prepared using column chromatography[15].

RESULTS

Fig. 1. shows the chromatograms of the mixtures of the methylated monosaccharide derivatives obtained after methanolysis of methylated TBG, transcortin, and SHBG. In effect, these chromatograms are fingerprints of the carbohydrate moieties and permit certain conclusions on structures to be made.

Thus, the chromatograms indicate that the carbohydrate chains of each of the three glycoproteins appear to be *N*-linked oligosaccharides of the *N*-acetyllactosamine type (**1** and **2**). The formation of 2,4-di-*O*-methyl and 3,4,6-tri-*O*-methyl derivatives of mannose and the 3,6-di-*O*-methyl derivative of 2-acetamido-2-deoxyglucose indicates the presence of the typical mannotriosido-di-*N*-acetylchitobiose core **3**.

The formation of 3,6-di-*O*-methylmannose suggests that, in some sugar chains of TBG and transcortin, there are additional branch-points at the mannosyl residues, *i.e.*, along with biantennary isoglycans **1**, these two glycoproteins contain (in lesser amounts) tri- (**2**) and/or tetra-antennary isoglycans. Two types of linkage between the terminal sialyl residues and penultimate residues of galactose, namely,

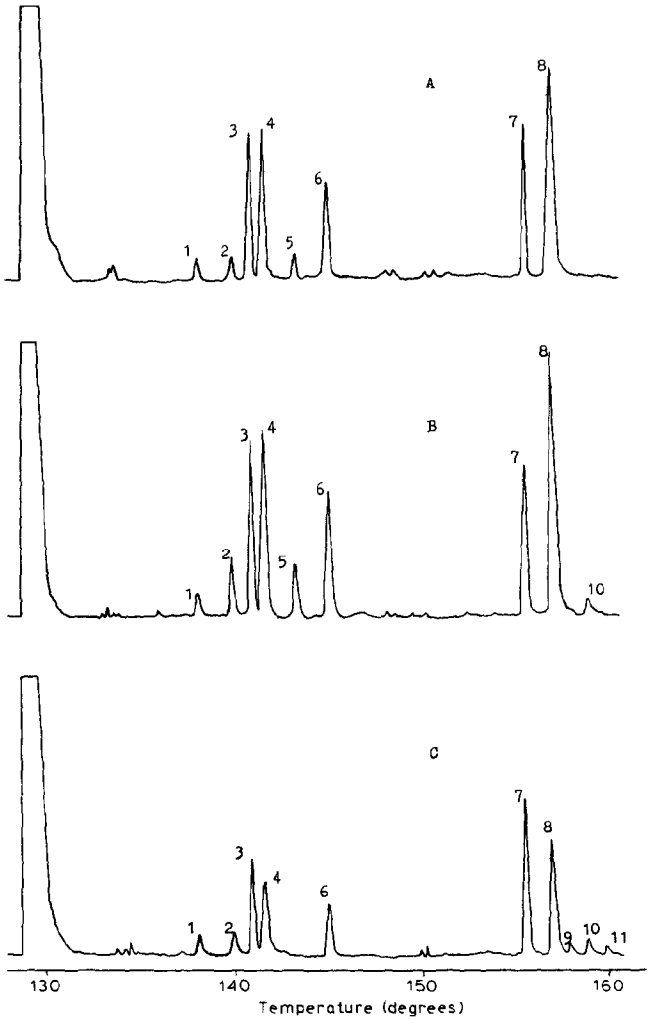
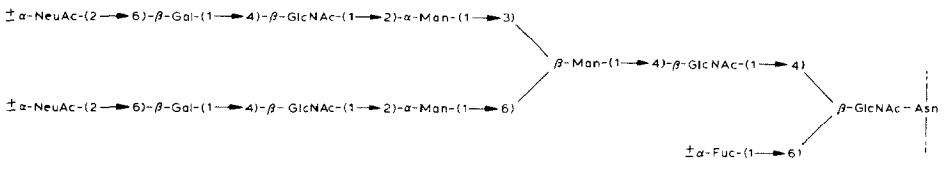


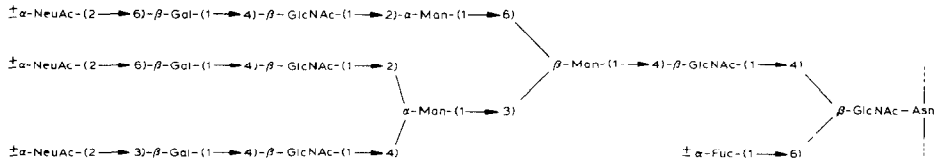
Fig. 1. Chromatograms of the methylated monosaccharide mixtures obtained after methanolysis of methylated TBG (A), transcortin (B), and SHBG (C). Peaks: 1, 2,3,4,6-tetra-*O*-methyl; 2, 2,4,6-tri-*O*-methyl; and 4, 2,3,4-tri-*O*-methyl derivatives of galactose; 3, 3,4,6-tri-*O*-methyl; 5, 3,6-di-*O*-methyl; and 6, 2,4-di-*O*-methyl derivatives of mannose; 8, 3,6-di-*O*-methyl and 10, 3-*O*-methyl derivatives of 2-deoxy-2-(*N*-methylacetamido)glucose; 9 and 11, unidentified; 7, mannitol (internal standard).

(2→3) and (2→6), are present in the carbohydrate chains of each glycoprotein, as indicated by the formation of 2,4,6- and 2,3,4-tri-*O*-methyl derivatives of galactose. The former linkages are characteristic of tri- and tetra-antennary *N*-linked oligosaccharides[5].

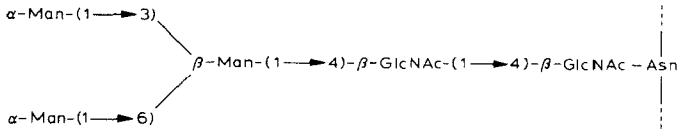
The formation of 2,3,4,6-tetra-*O*-methylgalactose, which corresponds to the terminal residues, clearly reflects partial desialylation of the glycoproteins in the blood[16] or during their purification[17].



1 (biantennary)



2 (triantennary)



3

TABLE I

MONOSACCHARIDE COMPOSITIONS OF HUMAN-SERUM HORMON-BINDING GLYCOPROTEINS AND METHYLATED MONOSACCHARIDES DERIVED THEREFORM[a]

Monosaccharide	*Glycoprotein*		
	TBG	*Transcortin*	*SHBG*
Fucose	0[b]	1.2 ±0.02	1.1 ±0.02
Mannose	11.7 ±0.2	15.0 ±0.2	6.1 ±0.2
3,4,6-tri-*O*-methyl	6.7 ±0 6	8.0 ±0.6	4.1 ±0.4
3,6-di-*O*-methyl	0.9 ±0.1	2.0 ±0.1	0
2,4-di-*O*-methyl	3.9 ±0.4	4.9 ±0.5	1.8 ±0.1
Galactose	8.8 ±0.1	12.1 ±0.2	5.0 ±0.1
2,3,4,6-tetra-*O*-methyl	1.3 ±0.2	1.0 ±0.1	0 7 ±0.05
2,4,6-tri-*O*-methyl	1.0 ±0.1	2.6 ±0 3	0.8 ±0.1
2,3,4-tri-*O*-methyl	6.8 ±0.7	8.4 ±0.7	3.4 ±0.3
2-Acetamido-2-deoxyglucose	16.5 ±0.6	22.1 ±0.6	7.8 ±0.3
3,6-di-*O*-methyl	16.3 ±1.7	21.0 ±1.6	6.8 ±0.7
3-*O*-methyl	0	0.9 ±0 1	0.8 ±0.1
2-Acetamido-2-deoxygalactose	0	0	1.1 ±0.05
N-Acetylneuraminic acid	8.3 ±0.2	10.3 ±0.2	5.2 ±0.3

[a]Values represent means ±s.e.m. of a minimum of four determinations. [b]Mol/mol of glycoprotein.

Thus, the interpretation of methylation analysis fingerprints of the glycoprotein carbohydrate moieties allows certain assumptions on the structures of the sugar chains to be advanced. Moreover, quantitative analysis of the data on the monosaccharide compositions of glycoproteins and the compositions of the methylated monosaccharide mixtures derived therefrom (Table I) provides more detailed information on their carbohydrate structures as illustrated below.

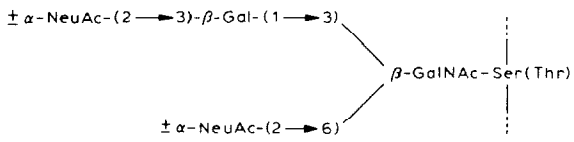
Thyroxine-binding globulin. — Each oligosaccharide of the *N*-acetyl-lactosamine type contains three mannosyl residues, one of which is substituted at positions 3 and 6 (see **1** and **2**). TBG contains ~12 mol of mannose per mol of glycoprotein and, after methylation, ~4 mol of this monosaccharide was recovered as the 2,4-di-*O*-methyl derivative (Table I). Consequently, there are four *N*-linked sugar chains in the TBG molecule. The formation of ~1 mol of 3,6-di-*O*-methylmannose and ~1 mol of 2,4,6-tri-*O*-methylgalactose per mol of TBG indicated one of the sugar chains to be a triantennary oligosaccharide.

The above conclusions are in good agreement with the data reported by Zinn *et al.*[18], and obtained after fragmentation of TBG and isolation and determination of the structure of the individual oligosaccharide chains. However, we did not find evidence for the presence of the reported[18] unusual (1→6)-linkage between the residues of 2-acetamido-2-deoxyglucose and mannose in some carbohydrate chains of TBG, since neither 2,3,4-tri-*O*-methyl nor 2,3-di-*O*-methyl derivatives of mannose were detected amongst the products of methanolysis of methylated TBG.

Transcortin. — Analysis of the data in Table I suggests that, unlike TBG, transcortin contains five *N*-linked oligosaccharides of the *N*-acetyl-lactosamine type. This is indicated by the following facts. Transcortin contains ~15 mol of mannose per mol of glycoprotein, and ~5 mol of this monosaccharide have been found as the 2,4-di-*O*-methyl derivative after methylation. The formation of ~2 mol of 3,6-di-*O*-methylmannose per mol of transcortin indicates that, together with biantennary oligosaccharides, this glycoprotein contains either two triantennary oligosaccharides or one tetra-antennary oligosaccharide per molecule. The carbohydrate chains of transcortin are also heterogeneous with respect to the content of fucose. The attachment of fucosyl residues to the residues of 2-acetamido-2-deoxyglucose in some oligosaccharide chains appears to result in the formation of the 3-*O*-methyl derivative of the latter monosaccharide (see Table I). Such microheterogeneity has been found in other serum glycoproteins[4].

The above conclusions on the structure of the transcortin carbohydrate have been confirmed by the structural investigation of individual asparaginyl-oligosaccharides isolated from the pronase digest of the glycoprotein[19], which revealed that three of the five carbohydrate chains are biantennary oligosaccharides of the *N*-acetyl-lactosamine type, the others being triantennary.

Sex hormone-binding globulin. — The presence of 2-acetamido-2-deoxygalactose residues in SHBG (Table I) suggests that, in contrast to TBG and transcortin, this glycoprotein contains *O*-linked oligosaccharides together with *N*-linked sugar chains. The residues of 2-acetamido-2-deoxygalactose are known[4] to



4

form *O*-glycosylic linkages (**4**) with seryl or threonyl residues in polypeptide chains of glycoproteins.

The unidentified peaks in the region of the peaks of 2-deoxy-*O*-methyl-2-(*N*-methylacetamido)-*O*-trimethylsilylglucitols in the chromatogram of the methylated monosaccharides derived from SHBG (Fig. 1C) apparently correspond to the methylated derivatives of 2-acetamido-2-deoxygalactose.

The methylated derivatives of mannose, galactose, and 2-acetamido-2-deoxyglucose obtained from SHBG (Fig. 1C, Table I) are consistent with the presence of biantennary oligosaccharides of the *N*-acetyl-lactosamine type (**1**) in SHBG. The formation of 3,4,6-tri-*O*-methylmannose and 2,4-di-*O*-methylmannose in the ratio 2:1 and the absence of other derivatives of this monosaccharide clearly indicate that there are no extra branch-points in the core mannotriose component of the SHBG oligosaccharide chains. The total content of mannose and the amount of 2,4-di-*O*-methylmannose formed (Table I) indicate that the molecule of SHBG contains two biantennary oligosaccharides. However, two biantennary isoglycans would contain four galactosyl residues, whereas SHBG contains ~5 mol of galactose per mol of glycoprotein. This is additional evidence for the presence of *O*-linked oligosaccharides in SHBG. This assumption is also supported by two other observations. First, the formation of 2,4,6-tri-*O*-methylgalactose, after methylation of SHBG, reveals the presence of NeuAc-(2→3)-Gal linkages, which generally occur in *O*-linked and not in biantennary *N*-linked oligosaccharides (*cf.* **1** with **4**). Second, two biantennary *N*-linked oligosaccharides would contain ≤4 sialyl residues, whereas SHBG contains >5 mol of this monosaccharide per mol of glycoprotein.

The investigation of individual glycopeptides isolated from a pronase digest of SHBG[20] confirms that the glycoprotein contains two biantennary oligosaccharides of the *N*-acetyl-lactosamine type and one *O*-linked oligosaccharide.

DISCUSSION

The conventional approach for investigating the structure of the carbohydrate components of glycoproteins involves enzymic or chemical fragmentation, to give a mixture of oligosaccharides or glycopeptides, followed by isolation of the individual oligosaccharides (glycopeptides). This approach is both labour- and time-consuming and usually requires relatively large amounts of glycoprotein.

Moreover, some selective losses, especially of minor components, may occur during the isolation of the individual oligosaccharides.

Elucidation of the general principles of the structural organisation of glycoproteins[5] now allows the structure of the carbohydrate moiety of the intact glycoprotein to be studied using the methods of carbohydrate chemistry, and methylation analysis in particular. The preliminary information obtained by the methylation analysis of a glycoprotein (which requires only a few milligrams of a biopolymer) may indicate the procedure which will be optimal for isolating individual oligosaccharides and techniques which will be appropriate for the determination of structure. Also, such information is of interest in comparative studies of related glycoproteins, *e.g.*, isolated from different sources or exhibiting similar physiological functions such as pregnancy-associated variants of human transcortin and TBG[21,22].

We have now applied the approach involving the methylation analysis of glycoproteins in a comparative investigation of the carbohydrate structures of three hormone-binding glycoproteins from human blood serum. The results indicate that TBG, transcortin, and SHBG contain mainly biantennary oligosaccharides of the *N*-acetyl-lactosamine type. *N*-Linked oligosaccharides with extra branch-points are also encountered in transcortin and TBG. In contrast, SHBG also contains *O*-linked oligosaccharides. Pronounced microheterogeneity is characteristic of the carbohydrate moieties of all three glycoproteins, but, even so, each has a specific structural organisation.

This information is important, particularly in the light of the hypothesis[3] that suggests that the oligosaccharide chains of the serum hormone-binding glycoproteins function as specific determinants for the recognition of the hormone–glycoprotein complexes by the corresponding target cells. Individual features of the three hormone-binding glycoproteins studied here offer a chemical basis for this hypothesis.

ACKNOWLEDGMENT

We thank Mrs. N. L. Petrova for her assistance in the preparation of the manuscript.

REFERENCES

1 K. E. Mickelson, G. B. Harding, M. Forsthoefal, and U. Westphal, *Biochemistry*, 21 (1982) 654–660.
2 J. Montreuil and J. F. G. Vliegenthart, in J. D. Gregory and R. W. Jeanloz (Eds.), *Glycoconjugate Research*, Vol. 1, Academic Press, New York, 1979, pp. 35–78.
3 P. J. Winterburn and C. F. Phelps, *Nature (London)*, 236 (1972) 147–151.
4 J. Montreuil, *Pure Appl. Chem.*, 42 (1975) 431–477.
5 R. Kornfeld and S. Kornfeld, *Annu. Rev. Biochem.*, 45 (1976) 217–237.
6 H. Rauvala, J. Finne, T. Krusius, J. Karkkainen, and J. Jarnefelt, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 389–416.

7 A. A. AKHREM, G. V. AVVAKUMOV, AND O. A. STREL'CHYONOK, *J. Chromatogr*, 176 (1979) 207–216.
8 A. A. AKHREM, G. V. AVVAKUMOV, I. V. SIDOROVA, AND O. A. STREL'CHYONOK, *J Chromatogr.*, 180 (1979) 69–82.
9 A. A. AKHREM, G. V AVVAKUMOV, L. V. AKHREM, I. V. SIDOROVA, AND O. A. STREL'CHYONOK, *Biochim. Biophys. Acta*, 714 (1982) 177–180.
10 J. PENSKY AND J. S. MARSHALL, *Arch. Biochem. Biophys.*, 135 (1969) 304–310.
11 A. A. AKHREM, G. V. AVVAKUMOV, I. I. KUKUSHKINA, O. V SVIRIDOV, O. A. STREL'CHYONOK, L. I. SURVILO, AND V. L. CHASHCHIN, *Vestsi Akad. Navuk BSSR, Ser. Khim. Navuk*, 6 (1977) 111–115.
12 O. A. STREL'CHYONOK, L. I. SURVILO, G. Z. TSAPELIK, AND O. V. SVIRIDOV, *Biokhimiya*, 48 (1983) 756–762.
13 S. HAKOMORI, *J. Biochem (Tokyo)*, 55 (1964) 205–208.
14 G. O. H. SCHWARZMANN AND R. W. JEANLOZ, *Carbohydr. Res.*, 34 (1974) 161–168.
15 U.S.S.R Inventor's Certificate No. 765278 (1980).
16 G. W. JOURDIAN, G G. SAHAGIAN, AND J DISTLER, *Biochem. Soc. Trans.*, 9 (1981) 510–512.
17 K.-L WONG AND E. REGOECZI, *Int. J. Pept Protein Res.*, 9 (1977) 214–248
18 A. B. ZINN, J. S. MARSHALL, AND D. M. CARLSON, *J. Biol. Chem.*, 253 (1978) 6768–6773.
19 O. A. STREL'CHYONOK, G. V. AVVAKUMOV, I. V MATVEENTSEVA, L. V. AKHREM, AND A. A. AKHREM, *Biochim. Biophys. Acta*, 705 (1982) 167–173.
20 G. V. AVVAKUMOV, I V. MATVEENTSEVA, L. V. AKHREM, O A. STREL'CHYONOK, AND A A. AKHREM, *Biochim. Biophys. Acta*, 760 (1983) 104–110.
21 O. A STREL'CHYONOK, G. V. AVVAKUMOV, AND A. A. AKHREM, *Dokl. Akad. Nauk SSSR*, 272 (1983) 233–236.
22 O A. STREL'CHYONOK, G. V AVVAKUMOV, AND A. A. AKHREM, *Carbohydr. Res.*, 134 (1984) 133–140.