Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1192-1199

A NOVEL CARBOHYDRATE STRUCTURE IN BOVINE AND OVINE LUTEINIZING HORMONES

Om P. Bahl, Molakala S. Reddy and Gurrinder S. Bedi

Department of Biological Sciences, Division of Cell & Molecular Biology
State University of New York at Buffalo, 347 Cooke Hall, Amherst
Buffalo, N.Y. 14260

Received August 26,1980

SUMMARY: Bovine and ovine lutropins (bLH and oLH) have three similar asparagine-linked carbohydrate units made up of Fuc, Gal (present only in oLH), Man, GlcNAc and GalNAc. The structural analyses of these carbohydrate units were performed on the oligosaccharides obtained by the alkaline borohydride treatment of the hormones and on the native hormones. The determination of intersugar and anomeric linkages, monosaccharide sequences and the polypeptide-carbohydrate linkage was carried out by methylation, periodate oxidation and deamination techniques and treatment with exoglycosidases. Based on these studies the structure for the oligosaccharide of bLH and oLH is proposed.

While the amino acid sequences of human chorionic gonadotropin (hCG), lutropin (LH), follitropin (FSH) and thyrotropin (TSH) (1-6), have been completed, the carbohydrate structures of these glycoprotein hormones, with the exception of hCG (7,8), still remain to be established. One unique feature of the carbohydrate units of LH, FSH and TSH is the presence of N-acetylgalactosamine in their N-glycosidically linked carbohydrates (1). Although N-acetylgalactosamine is the commonly present linkage monosaccharide in O-glycosidically linked carbohydrate units, its presence in the N-glycosidic carbohydrates has been discovered only recently. This communication describes the proposed structures for the carbohydrates of ovine and bovine LH, in particular the location of N-acetylgalactosamine. The structural characterization of the carbohydrates was performed by methylation, periodate

---

Abbreviations: oLH & bLH, ovine and bovine lutropin; FSH, follitropin; TSH, thyrotropin; hCG, human chorionic gonadotropin; Man, D-mannose; Fuc, L-fucose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; g.l.c./m.s., gas liquid chromatography and mass spectrometry.

0006-291X/80/191192-08$01.00/0

oxidation and deamination techniques and enzymatic degradation with exo-glycosidases.

MATERIALS AND METHODS

Analytical Methods: Paper chromatography was performed on Whatman paper No. 1 in two solvent systems: A. 1-butanol:ethanol:water, 4:1:1 (v/v) and B. 1-butanol:pyridine:water, 4:3:4 (v/v). Thin layer chromatography was carried out on silica gel-G in solvent systems: C. 1-butanol:ethanol:water:ammonia, 50:70:40:1 (v/v) and D. 1-propanol:water, 7:1 (v/v). The neutral sugars, hexosamines, hexosaminitols and amino acid analyses were performed as described earlier (7). N-Acetylglucosamine was also determined by the method of Reissig et al. (10). 2,5-Anhydromannose and 2,5-anhydrotalose were prepared by the deamination of glucosamine hydrochloride and galactosamine hydrochloride. The anhydro sugars were reduced, acetylated and analyzed by g.l.c. on a column of 3% OV-225 at 170° isothermally for 5 min followed by an increase in temperature of 5°/min to 190° and maintained at that temperature using a Varian Gas Chromatograph Series 3700.

Preparation of Oligosaccharides from bLH and oLH: Bovine and ovine LH were isolated from pituitary glands by the procedures of Papkoff and Gan (11) and Sherwood et al. (12) respectively. The hormones (15 mg) were subjected to alkaline hydrolysis with 1.5 ml of 1 M NaOH containing 4 M $NaBH_4$ at 80° for 24 h. After cooling the reaction mixture in ice, an equal volume of water was added and the pH was adjusted to 5.0 with glacial acetic acid. The boric acid was removed by distillation with methanol and the hydrolysate was desalted on a column (1.5 x 95 cm) of Sephadex G-25 (fine). The column was eluted with 0.02 N acetic acid and the appropriate fractions were pooled and lyophilized. After N-acetylation (13) the oligosaccharides were purified by paper chromatography in Solvents A & B.

Partial Hydrolysis of Oligosaccharides: The bLH and oLH oligosaccharides (150 nmol) were heated in 0.5 N H_2SO_4 (200 µl) for 9 min in a boiling water bath. The hydrolysate was applied to a column (1.0 x 5 cm) of Bio-Rad AG-3 (CH_3COO^-) eluted with water and the eluate was lyophilized. Fucose released by this treatment was separated from the residual oligosaccharide by paper chromatography on Whatman paper No. 1 in Solvent B for 12 h.

Structural Analysis: Methylation (14) and periodate oxidation - Smith degradation studies were carried out as described (7). The deamination of the oligosaccharide and the isolation of the resulting products was done as follows: A 150 nmol aliquot of the oligosaccharide in 150 µl of water (prior to N-acetylation) was subjected to deamination with a mixture of 150 µl of sodium nitrite at 20° for 6 h. The reaction mixture was neutralized by passing through a column (1.0 x 10 cm) of Amberlite IRA-400 (HCO_3^-) and deionized by passing through a column (1.0 x 10 cm) of AG-50 (H^+). The column was eluted with water, the aqueous extract dried and the sample was reduced with $NaBH_4$. After the removal of the borate as above the resulting mixture was then passed through a column (1.0 x 5 cm) of AG-50 (H^+) eluted with water. The effluent was dried and fractionated by paper chromatography in Solvent B for 2 days. Enzymatic hydrolysis with the various Aspergillus niger exoglycosidases were carried out according to the procedures described earlier (7).

RESULTS AND DISCUSSION

Isolation and Characterization of Oligosaccharides from bLH and oLH:

The oligosaccharides were prepared by alkaline hydrolysis of the hormones in

Table I: Periodate oxidation of bLH, partially hydrolyzed bLH[a] and oLH oligosaccharides

Sugar	bLH		Partially hydrolyzed bLH	oLH	
	Native	IO_4^-	IO_4^-	Native	IO_4^-
Sialic Acid	undect	-	-	undect	-
Fucose	0.5	undect	undect	0.7	undect
Galactose	undect	undect	undect	0.4	undect
Mannose	2.9	0.7	n.d.[d]	2.8	0.9
N-Acetylglucosamine	2.0	1.5	1.9	2.7	2.1
N-Acetylglucosaminitol	0.5	0.4	undect	0.4	0.2
N-Acetylgalactosamine	1.0	1.0	1.0	1.0	1.0

a. Partial hydrolysis was carried out by incubating the oligosaccharide with 0.5N H_2SO_4 at 100° for 9 min.

b. Number of residues were calculated on the basis of N-acetylgalactosamine to be 1.

c. Undetectable; d, not determined.

the presence of $NaBH_4$ and contained fucose, mannose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylglucosaminitol. In addition, oLH contained galactose (Table I). Sialic acid content in both the hormones was negligible. With the exception of galactose and possibly N-acetylglucosamine the compositions of bLH and oLH oligosaccharides were essentially the same. Some of the differences in their compositions are probably due to microheterogeneity. The presence of N-acetylglucosaminitol at the reducing end of the oligosaccharides indicates that N-acetylglucosamine is involved in the linkage of the carbohydrate to the protein.

Methylation Studies of bLH and oLH Oligosaccharides Before and After Partial Acid Hydrolysis: The permethylated oligosaccharides from both bLH and oLH were found to consist of 2,3,4-tri-O-methyl fucose (0.3 and 0.4 residues), 2,3,4,6-tetra-O-methyl mannose (0.6 and 0.5 residues), 3,4,6-tri-O-methyl mannose (1.4 and 1.5 residues), 2,4,-di-O-methyl mannose (1.0 and 1.0 residue), 3,4,6-tri-O-methyl 2-N-methyl-N-acetylglucosamine (0.7 and 0.9 residues), 3,6-di-O-methy 2-N-methyl-N-acetylglucosamine (1.3 and 1.8 residues),

3,6-di-O-methyl 2-N-methyl-N-acetylgalactosamine (0.6 and 0.9 residues) and 1,3,5-tri-O-methyl 2-N-methyl-N-acetylglucosaminitol (0.2 and 0.4 residues), the latter being quantitated only approximately because of the diffused peak. The permethylated partially hydrolyzed oligosaccharides were found to lack 2,3,4-tri-O-methyl fucose as expected and also most of the 3,6-di-O-methyl 2-N-methyl-N-acetylgalactosamine but contained instead 3,4,6-tri-O-methyl 2-N-methyl-N-acetylgalactosamine. Based on these studies, it is clear that in the intact oligosaccharides, fucose, galactose (only in oLH) and about 1 residue of N-acetylglucosamine were terminally located. The remaining two N-acetylglucosamine residues were 1,4 and 1,4,6-linked while the mannose residues were terminal, 1,2 and 1,3,6-linked. The N-acetylgalactosamine residue was substituted at 1 and 4 positions. Since partial acid hydrolysis of the oligosaccharide resulted in the disappearance of 2,3,4-tri-O-methyl fucose, 3,6-di-O-methyl 2-N-methyl-N-acetylgalactosamine and 1,3,5,-tri-O-methyl 2-N-methyl N-acetylglucosaminitol and appearance of 3,4,6-tri-O-methyl 2-N-acetylgalactosamine, it indicates that the substitutents at C_4 and C_6 hydroxyl groups of N-acetylgalactosamine and N-acetylglucosamine respectively might be fucose or some other acid labile group.

Periodate Oxidation Studies of Oligosaccharides: Periodate oxidation studies of the oligosaccharides resulted in the destruction of galactose (in oLH only), fucose and mannose residues. The N-acetylglucosamine whether terminal in bLH or oLH oligosaccharide was not destroyed. Also, not destroyed was the N-acetylglucosaminitol residue. It can be concluded from the results of the periodate oxidation summarized in Table I that fucose and galactose (in oLH) residues were terminally located. Two of the three mannose residues were destroyed indicating that these were not linked by 1,3-linkage. Periodate oxidation of the partially hydrolyzed oligosaccharides resulted in the complete destruction of N-acetylglucosaminitol while neither N-acetylglucosamine nor N-acetylgalactosamine were affected indicating that fucose might be

linked to N-acetylglucosaminitol by 1,6-linkage. The data are thus consistent with those of the methylation studies.

Mild acid hydrolysis of periodate oxidized-reduced bLH gave a low molecular weight fraction (about 372 nmol from 10 mg based on N-acetylgalactosamine content) which migrated with N-acetylglucosaminyl glyceraldehyde fraction on TLC in Solvents C & D. Apparently, this low molecular weight fraction was derived from 1,2-β-linked mannose residues in the outer branches of the carbohydrate moiety and was made up of an equimolar mixture of N-acetylglucosamine and N-acetylgalactosamine and glyceraldehyde. It may be added that asialo hCG which has a total of 8 1,2-β-linked N-acetylglucosaminyl mannose linkages (7), also yielded N-acetylglucosaminyl glyceraldehyde on the first cycle of Smith degradation. Periodate oxidation of the fraction resulted in the destruction of most of the N-acetylglucosamine (81%) and a small fraction of N-acetylgalactosamine (21%). However, when the periodate oxidation was repeated after partial acid hydrolysis, an additional amount of N-acetylgalactosamine (56%) was destroyed indicating that the small molecular weight carbohydrate probably was made up of a mixture of N-acetylglucosaminyl glyceraldelhyde and X-N-acetylgalactosaminyl glyceraldehyde where X is an acid labile substituent. It is interesting to note that while the terminal N-acetylglucosamine in the intact bLH or oLH oligosaccharide was resistant to oxidation by periodate, it was, however, destroyed when the Smith degradation product, N-acetylglucosaminyl glyceraldehyde was subjected to periodate oxidation.

Deamination of bLH and oLH Oligosaccharides: The bLH and oLH oligosaccharides obtained by alkaline sodium borohydride hydrolysis were subjected to deamination by nitrous acid followed by paper chromatography which gave two major fractions R_{Glc} 0.4 (73 nmol) and R_{Glc} 1.3 (62 nmol) and one minor fraction R_{Glc} 1.0. All fractions were analyzed for carbohydrates. Fraction I was a tetrasaccharide made up of 2,5-anhydromannitol and mannose in a ratio of 1:3 (Table II). Fraction II was found to contain after mild acid hydrolysis 2,5-

Table II: Carbohydrate compositions[a] of the tetrasaccharide, (Man_3-) 2,5-anhydromannose before and after periodate oxidation.

Sugar	Native		After periodate oxidation		
			I cycle		II cycle
	bLH	oLH	bLH	oLH	oLH
2,5-Anhydromannose	1.0	1.0	1.0	1.0	1.0
Mannose	3.3	3.3	1.4	1.1	undect[b]

a. Number of sugar residues assuming 2,5-anhydromannose to be 1.
b. Undetectable.

anhydrotalitol indicating that it was probably some acid labile derivative of 2,5-anhydrotalitol. Fraction III was 2,5-anhydromannitol as determined by g.l.c. after acetylation.

The ratio of free 2,5-anhydromannose to 2,5-anhydrotalose generated during the deamination of the oligosaccharide was 1.0:0.25. However, when the deamination reaction was carried out on the partially hydrolyzed oligosaccharide, the free 2,5-anhydromannose and 2,5-anhydrotalose obtained were in almost equimolar proportion suggesting that an acid labile group was present on N-acetylgalactosamine rather than N-acetylglucosamine since there was no change in the 2,5-anhydromannose content before and after partial acid hydrolysis.

A detailed characterization of the tetrasaccharide was carried out by periodate oxidation and enzymatic hydrolysis with α and β-mannosidases. The tetrasaccharide after first cycle of Smith degradation yielded a disaccharide containing 2,5-anhydromannitol and mannose in a ratio of 1.0:1.4 in bLH and 1.0:1.1 in oLH. This disaccharide after another cycle of Smith degradation gave only 2,5-anhydromannitol (Table II). Hydrolysis with α-mannosidase also yielded a product identical to that obtained after the first cycle of Smith degradation thus indicating that two of the three mannose residues might be present at the nonreducing ends and were α-linked. When Fraction I was treated with a mixture of α and β-mannosidases, it gave 2,5-anhydromannitol indicating that the third mannose residue was β-linked. From the above data together

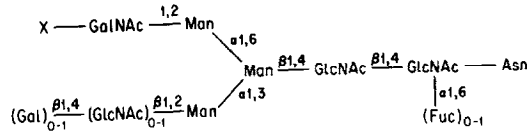

Figure 1. Proposed structure for the carbohydrates of bovine and ovine LH. Galactose is present only in oLH. X is an acid labile substituent whose precise identification remains to be established.

with the methylation data on the intact oligosaccharide, the structure of the tetrasaccharide can be derived as Man$\overset{\alpha 1,6}{\text{---}}$(Man$\overset{\alpha 1,3}{\text{---}}$)$\overset{\beta 1,4}{\text{---}}$Man-2,5-anhydromannitol.

Anomeric Linkages: The β-anomeric linkage of N-acetylglucosamine to mannose in bLH and oLH oligosaccharides was determined by treatment with β-N-acetylglucosaminidase. 0.8 and 0.7 residues of N-acetylglucosamine were released from bLH and oLH oligosaccharides, respectively. Treatment of bLH β-subunit with α-L-fucosidase resulted in the release of 0.3 residues of fucose indicating the anomeric linkage as α. The β-galactosidase was able to hydrolyze about 0.2 residues of galactose from oLH oligosaccharide indicating that the galactose was linked by a β-linkage. As shown above the two of three mannoses were α-linked and one was β-linked.

Our earlier studies had shown that N-acetylgalactosamine was an integral component of the α-subunits of LH and TSH (1) but its precise location in the structure was not known. Based on the above studies the following structure is proposed for the carbohydrate units of oLH and bLH (Fig. 1). This structure shares several common features, such as the presence of the pentasaccharide core with other 'complex' type carbohydrate units in various glycoproteins. However, the carbohydrate units of oLH and bLH have some striking differences with all other carbohydrate units so far known. Not only do these hormones contain N-acetylgalactosamine but also an undefined substituent 'X' is present at C_4 position of the aminosugar. The proposed structure is at variance with that reported for the α-subunits of bLH, hTSH and hFSH in which N-acetylgalactosamine was shown to be located in the core (16). The studies are in progress

to determine the chemical identity of 'X' and its role in the synthesis and function of the hormones.

ACKNOWLEDGMENTS: This work was supported by grants HD-08766 & HD-12581 from U.S.P.H.S.

REFERENCES

1. Bellisario, R., Carlsen, R.B., and Bahl, O.P. (1973) J. Biol. Chem. 248, 6796-6809.
2. Carlsen, R.B., Bahl, O.P., and Swaminathan, N. (1973) J. Biol. Chem. 248, 6810-6827.
3. Morgan, F.J., Birken, S., and Canfield, R.E. (1975) J. Biol. Chem. 250, 5247-5258.
4. Closset, J., Hennen, G., and Lequin, R.M. (1973) FEBS Lett. 29 (2), 97-100.
5. Saxena, B.B., and Rathnam, P. (1976) J. Biol. Chem. 251, 993-1005.
6. Sairam, M.R., and Li, C-H. (1977) Can. J. Biochem. 55, 755-760.
7. Kessler, M.J., Reddy, M.S., Shah, R.H., and Bahl, O.P. (1979) J. Biol. Chem. 254, 7901-7908.
8. Kessler, M.J., Mise, T., Ghai, R.D., and Bahl, O.P. (1979) J. Biol. Chem 254, 7909-7914.
9. Warren, L. (1959) J. Biol. Chem. 234, 1971-1975.
10. Reissig, J.L., Strominger, J.L., and Leloir, L.F. (1955) J. Biol. Chem. 217, 959-966.
11. Papkoff, H., and Gan, J. (1970) Arch. Biochem. Biophys. 136, 522-528.
12. Sherwood, O.D., Grimek, H.J., and McShan, W.H. (1970) Biochem. Biophys. Acta. 221, 87-106.
13. Wheat, R.W. (1960) Methods Enzymol. 8, 60.
14. Stellner, K., Saito, H., and Hakomori, S. (1973) Arch. Biochem. Biophys. 155, 464-472.
15. Bahl, O.P. (1969) J. Biol. Chem. 244, 575-583.
16. Hara, K., Rathnam, P., and Saxena, B.B. (1978) J. Biol. Chem. 253, 1582-1591.