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ISOLATION AND CHARACTERIZATION OF N- AND O- GLYCOSIDIC CARBOHYDRATE UNITS OF HUMAN CHORIONIC GONADOTROPIN*

Om P. Bahl, Leopold März and Michael J. Kessler

Department of Biological Sciences, Division of Cell and Molecular Biology, SUNY at Buffalo, Amherst Campus, Buffalo, New York 14260

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SUMMARY

The detailed structures of the carbohydrate moiety of hCG have been determined. The structural analyses were carried out on all four asparagine-linked glycopeptides as well as serine-linked carbohydrate chains. The glycopeptides were prepared from the tryptic hydrolysates of the reduced-S-carboxamidomethyl hCG-α and hCG-β and were purified by chromatography on Sephadex G-50 and preparative high voltage paper electrophoresis at pH 1.8. The serine-linked carbohydrate chains were cleaved by β-elimination with alkali in the presence of sodium borohydride and were purified by chromatography on Sephadex G-25. All glycopeptides and the oligosaccharide were examined for homogeneity by high voltage paper electrophoresis, paper chromatography, and chemical composition. The structural studies involved the determination of intersugar and anomeric linkages and monosaccharide sequences and were carried out by a combination of several techniques such as periodate oxidation, methylation and sequential enzymatic degradation.

Human chorionic gonadotropin (hCG) is made up of two subunits, α and β. The monosaccharide sequences and the tentative structures of the carbohydrate units based on the sequential degradation with exo-glycosidases have been proposed earlier (1,2). This communication describes the complete detailed structures, including monosaccharide sequences and intersugar and anomeric linkages, of all four asparagine-linked glycopeptides and the O-glycosidic carbohydrate chain. The structures were derived from enzymatic degradation, periodate oxidation and methylation studies.

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MATERIALS AND METHODS

Human chorionic gonadotropin was prepared from a commercial preparation (Organon, West Orange, N.J.), essentially according to Bahl (3), with a final potency of 12,000 i.u./mg. The α and β subunits were prepared by dissociation of purified hCG with 8 M urea followed by chromatography on DEAE-Sephadex, the separation of the subunits being accomplished by a stepwise salt gradient followed by chromatography on Sephadex G-100 (2,4). The enzymes β-galactosidase, β-N-acetylglucosaminidase, α-mannosidase and α-N-acetylgalactosaminidase were isolated in the laboratory from a commercial preparation of Aspergillus niger (5).

Preparation of N-Glycosidic Glycopeptides. The four N-glycopeptides, designated as αT-8, αT-11, βT-2, and βT-3, were prepared from the reduced S-carboxamidomethyl hCG-α and hCG-β (6,7). The subunits were desialized by treatment with 0.025 N HCl at 80° for 1 hr. After desalting on Sephadex G-25 (coarse), with asialo subunits were digested with TPCK-trypsin. The trypsin hydrolysates were subjected to chromatography on Sephadex G-50 (1.5 cm x 150 cm), the elution profile being followed by absorbance at 230 nm and by phenol-sulfuric acid test for sugars (8). The glycopeptide fractions were further purified by preparative high voltage paper electrophoresis.

Isolation of O-Glycosidic Carbohydrate Chain. hCG, 95 mg, was subjected to β-elimination with 0.1 N NaOH and 1.0 N $NaBH_4$ at 37°. The O-glycosidic chain was separated from hCG by chromatography on Dowex 50 (H^+) using H_2O as an eluant. The carbohydrate in the eluate was then purified by chromatography on a column of Sephadex G-25 (1.5 x 100 cm) previously equilibriated with 0.1 M pyridinium acetate, pH 5.0. The fractions containing N-acetylgalactosaminitol were pooled. Finally, the oligosaccharide in the pooled fraction was further purified by re-chromatography on Sephadex G-25 (1.5 x 200 cm).

Periodate Oxidation. The N-glycosidic glycopeptides and O-glycosidic carbohydrate (100 nmoles) were treated with 0.05 M $NaIO_4$ (5-fold excess) at 4° for 60 hrs in the dark. The reaction was then brought to pH 7.0 with 0.05 M NaOH and made 0.25 M in $NaBH_4$ by adding solid sodium borohydride. The reduction was allowed to proceed for 24 hrs at 4° after which the reaction was stopped by the addition of glacial acetic acid. The borate was removed either as methyl borate or by chromatography on a Sephadex G-25 (fine) column equilibrated in 0.1% NH_4HCO_3. The resulting periodate-oxidized and reduced material was then subjected to mild acid hydrolysis by incubation with 0.5 N HCl at room temperature for 24 hrs. The periodate-oxidized, reduced, and acid-hydrolyzed glycopeptide was isolated by chromatography on Sephadex G-25 (fine).

Methylation. The samples (100-200 nmoles) were carefully dried over P_2O_5 *in vacuo* before dissolving in dimethylsulfoxide. Freshly prepared dimethylsulfinyl carbanion (9) was then added and the reaction mixture was kept for 2 hrs. Subsequently, after the addition of methyl iodide, the reaction was allowed to proceed for an hour. The methylated sample was extracted with chloroform and purified by chromatography on Sephadex LH-20 (1 x 100 cm) using chloroform:methanol (2:1, v/v). The fractions containing the methylated carbohydrate was subjected to acetolysis at 80-100° with 0.5 N H_2SO_4 in 95% glacial acetic acid for 16 hrs, followed by hydrolysis at 80° for 5 hrs after the addition of an equal volume of water. The hydrolysate was neutralized with AG-3 (acetate), and the methylated sugars were reduced with 10 mg sodium of borohydride at room temperature for 3 hrs. Following the removal of borate as methyl borate by the repeated additions of methanolic acetic acid, the methylated alditols were acetylated with pyridine-acetic anhydride at 100° for 1 hr. The alditol acetates in the sample were identified on a gas chromatograph equipped with a column of 3% OV-17 or 3% ECNSS-M using a linear temperature program, 120-190° at 1°/min.

Digestion with Glycosidases. The N- and O-glycopeptides and the subunits were digested with specific enzymes isolated from A. niger as described earlier (5).

Analytical Methods. The sialic acid was determined by the method of Warren (10). The neutral sugars were quantitated by a modification of the method of Lehnhardt and Winzler (11) for alditol acetates. The amino acid composition and hexosamine content was determined by an Amino Acid Analyzer as described earlier (1).

RESULTS AND DISCUSSION

Isolation and Chemical Compositions of N-glycopeptides and O-oligosaccharide. The N-glycosidically-linked carbohydrate units are located at asparaginyl residues 52 and 78 in the α-subunit and 13 and 30 in the β-subunit (6,7). The O-glycosidic carbohydrates are present in the carboxy terminus of hCG-β. The structural studies were performed on the tryptic N-glycopeptides, αT-8, αT-11, βT-2, and βT-3, prepared as described earlier (6,7) from the reduced-S-carboxamidomethyl-asialo hCG-α and hCG-β and the O-oligosaccharide. The glycopeptides were purified from the tryptic hydrolysates by chromatography on Sephadex G-50 and by preparative high voltage paper electrophoresis. The serine-linked carbohydrate chains were isolated by the β-elimination of hCG with NaOH and $NaBH_4$ followed by chromatography on Sephadex G-25. The N-glycopeptides and O-glycosidic oligosaccharide thus obtained were homogeneous by high-voltage paper electrophoresis, and paper chromatography. The amino acid and carbohydrate compositions of the N-glycopeptides (Table I) and of O-glycosidic carbohydrates further confirmed the purity of the preparations. Furthermore, the amino acid compositions of the N-glycopeptides agreed with those reported earlier (6,7).

The carbohydrate compositions of αT-8 and αT-11 showed the presence of about 4 N-acetylglucosamine, 3 mannose and 1 to 2 galactose residues. βT-2 and βT-3 also had similar compositions with the exception of the presence of 0 to 1 residues of L-fucose in each (Table I). The variation in the values of L-fucose residues in βT-2 and βT-3 and of galactose residues in all N-glycopeptides is due to the commonly known phenomenon of micro-heterogeneity in glycoproteins. The serine-linked carbohydrate chains were made up of about 2 residues of sialic acid, present as N-acetylneuraminic acid, 1 of galactose and 1 of N-acetylgalactosaminitol. The

TABLE I

Periodate Oxidation[a] of N-Glycosidic Glycopeptides of hCG

Component	αT-8 Residues[b]			αT-11 Residues[b]			βT-2 Residues[b]			βT-3 Residues[b]		
	Before oxid.	After oxid. & red.	After oxid. red. & hyd.	Before oxid.	After oxid. & red.	After oxid. red. & hyd.	Before oxid.	After oxid. & red.	After oxid. red. & hyd.	Before oxid.	After oxid. & red.	After oxid. red. & hyd.
Aspartic acid	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
N-Acetylglucosamine	3.9	3.7	2.1	4.0	3.8	2.0	3.6	3.8	2.2	4.0	4.2	1.9
Fucose	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0
Galactose	0.7	0.0	0.0	1.8	0.0	0.0	1.9	0.0	0.0	2.1	0.0	0.0
Mannose	3.0	1.5	1.2	3.3	1.3	1.0	3.1	1.0	1.1	2.9	1.0	0.9
Erythritol	None			None			None			None		
Threitol	None			None			None			None		

a. See 'Materials and Methods' for details.
b. Calculated assuming aspartic acid as 1.

precise composition of the O-oligosaccharide chain is shown in Table II.

Periodate Oxidation Studies. Periodate oxidation and reduction of αT-8 and αT-11 resulted in the destruction of almost all of galactose, two mannose; and 0 to 1 residues of N-acetylglucosamine depending upon the amount of the terminal galactose present. Similar results were obtained with βT-2 and βT-3 since the fucose content of these preparations was quite low. The mild acid hydrolysis of the oxidized-reduced glycopeptide resulted in a trisaccharide containing one mannose and two N-acetylglucosamine residues, (Table I) and N-acetylglucosaminyl-glycerol. The data indicate that all galactose residues, with the exception of those attached to L-fucose, are terminal, and two of the mannose are linked by 1,2 or 1,6 linkages. All of the N-acetylglucosamine are linked by 1,3 or 1,4 linkages except those which are exposed as a result of microheterogeneity. The formation of N-acetylglucosaminyl glycerol would rule out the presence of 1,4-linked mannose. The periodate oxidation of O-glycosidic carbohydrate unit resulted in the complete destruction of sialic acid, indicating their terminal positions. This is also supported by their susceptibility to hydrolysis by V. cholerae neuraminidase. Only 20% of galactose residues were terminal whereas the others were linked to sialic acid by 2,3-linkages. The N-acetylgalactosaminitol was quantitively converted to threosaminitol, indicating that the N-acetylgalactosaminitol was substituted at positions 3 and 6, or 3.

Methylation Studies. The carbohydrate structures of the N-glycosidic and O-glycosidic carbohydrates were further investigated by methylation. These studies were performed individually on all N-glycopeptides, and also on the O-oligosaccharide. The results in Table III indicate that each of the N-glycopeptide contains about 2 residues of terminal galactose, 2 residues of 1,2-linked mannose, 1 residue of 1,3,6-substituted mannose and 4 residues of 1,4-linked N-acetylglucosamine. The βT-2 and βT-3 also showed some terminal fucose. The methylation data were consistent with those of the periodate oxidation. The O-oligosaccharide on methylation showed 0.2 residues of

TABLE II

Periodate Oxidation of Serine-Linked Carbohydrate of hCG

Carbohydrate	Before Oxidation	After 60 hrs of oxidation
	nmole (residues)[a]	nmoles (residues)[a]
Sialic acid	280 (1.92)	Nil
N-Acetylgalactosaminitol[b]	152 (1.04)	Nil
N-Acetylglucosamine	6 (0.1)	6 (0.1)
Galactose[c]	150 (1.03)	120 (0.82)
Thresoaminitol[b]	Nil	166 (1.14)
Moles of HCOOH/mole[d]	Nil	(2.0)

a. Values in parentheses represent the number of residues.

b. Amino sugars were quantitated by amino acid analysis at pH=5.28 using a buffer containing borate, after hydrolysis in 4N HCl for 4 hr at 100°.

c. Neutral sugars were determined as alditol acetates.

d. Determined by titration with NaOH under nitrogen.

TABLE III

Methylation of N-Glycosidic Glycopeptides and O-oligosaccharide of hCG-α and hCG-β

Methylated Sugar[a]	Asparagine-linked glycopeptides				Serine-linked Carbohydrate
	αT-8 Residues[b]	αT-11 Residues[b]	βT-2 Residues[b]	βT-3 Residues[b]	O-oligosaccharide Residues
2,3,4,6-Tetra-O-methylgalactose	2.2	1.9	2.0	1.8	0.2
3,4,6-Tri-O-methylmannose	2.2	1.8	2.0	1.8	-
2,4-Di-O-methylmannose	1.0	1.0	1.0	1.0	-
3,6-Di-O-methyl N-acetylglucosamine	3.8	3.2	3.5	2.8	-
2,4,6-Tri-O-methylgalactose	-	-	-	-	0.8
1,4,5-Tri-O-methyl N-acetylgalactosaminitol	-	-	-	-	1.0

a. Identified as alditol acetates.

b. Calculated assuming 2,4-di-O-methylmannose as 1 residue.

terminal galactose, 1.8 residues of 1,3-linked galactose and 1 residue of 3,6-linked N-acetylgalactosaminitol (Table III).

Degradation with Glycosidases. Our earlier studies had shown the sequences and anomeric linkages in the asparagine-linked carbohydrate units, based on the sequential degradation with glycosidases, to be NANA (Fuc)$\overset{\alpha}{-}$ Gal$\overset{\beta}{-}$ Glu NAc$\overset{\beta}{-}$ Man$\overset{\alpha}{-}$. Similar studies have been repeated on the individual N-glycopeptides and will be described elsewhere. Enzymatic studies have also been carried out on the carboxy-terminal peptide, BC-19 (7) using V. cholerae neuraminidase, and A. niger β-galactosidase and α-N-acetylgalactosaminidase. The neuraminidase hydrolyzed all of sialic acid, 7 to 8 residues, indicating their terminal positions. The β-galactosidase removed 3 to 4 galactose residues, and the α-N-acetylgalactosaminidase cleaved 3 to 4 N-acetylgalactosamine residues.

Based on these studies, the following structures have been assigned to the N- and O-carbohydrates of hCG-α and hCG-β (Fig. 1). Fig. 1 represents the complete structures which do not reflect the microheterogeneity present in the carbohydrates. For example, in αT-8 and αT-11 the galactose values have always been found to be lower and range between 1 and 2 residues. It may be noted that fucose residues are present only in the N-glycosidic carbohydrates in the β, but not in the α-subunit.

The structure of the asparagine-linked carbohydrate units presented in Fig. 1 differs considerably from those proposed for the carbohydrate in hCG-α by Kennedy and Chaplin (12). This structure was arrived at after several independent experiments and not only differs in monosaccharide sequence but also in intersugar linkages from those reported by the above authors. The monosaccharide sequences of peripheral sugar residues proposed in the present investigation are essentially identical to the tentative structures reported earlier (1,2). However, the major difference in the two proposals lies in the fine details. In addition, the size of the N-glycosidic carbohydrate units is found to be somewhat smaller than that proposed earlier, since the

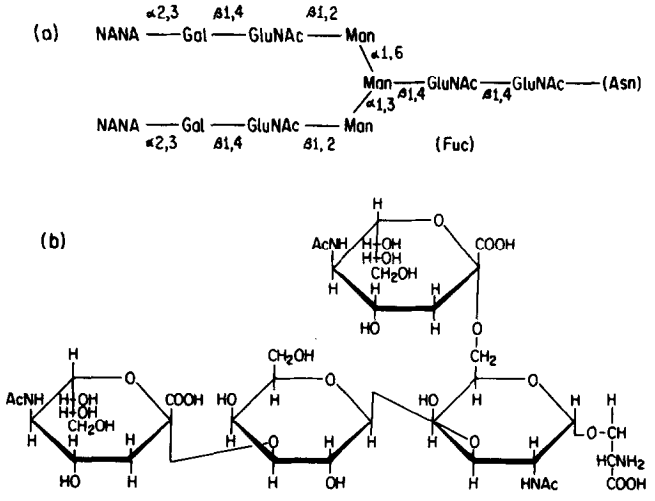

Fig. 1. The structures of the asparagine-and serine-linked carbohydrate units of hCG. (a). The structure of the asparagine linked units in hCG-α and hCG-β. Each of the hCG-β carbohydrate unit has one residue of L-fucose which may be linked to galactose or N-acetylglucosamine residues.(b). The structure of the four serine-linked units in hCG-β.

initial structures were based on a much higher molecular weight of hCG. Finally, it is interesting to note that similar structures for the asparagine- and serine-linked carbohydrate units have been found in other animal glycoproteins (13).

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