A New Type of Carbohydrate-Protein Linkage in a Glycopeptide from Normal Human Urine*

(Received for publication, December 2, 1974)

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The most common linkages between carbohydrate and protein in glycoproteins and proteoglycans are the following: N-acetylglucosamine-asparagine, N-acetylgalactosamine-serine, N-acetylgalactosamine-threonine, galactose-hydroxylysine, and xylose-serine. The latter is as yet specific for proteoglycans (1, 2), the galactose-hydroxylysine linkage has only been found in the collagen type of glycoprotein (3), but the others are distributed more widely.

Human urine, which is a rich source of different products of degradation, contains some of these compounds. Tominaga et al. (4) isolated and identified O-β-D-xylonsyl-L-serine, and Cunningham et al. (5) isolated O-β-D-galactopyranosyl-β-hydroxylysine from normal urine. 2-Acetamido-β-D-glucopyranosylamine (GlcNAc-Gly) was first isolated and characterized from the urine of patients with a deficiency of 2-acetamido-β-D-glucosamine amidohydrolase (6) and was reported later to be present in normal urine in small amounts (7, 8).

A new type of carbohydrate-protein linkage was discovered by the isolation of digalactosyleysteinle from a urinary glycoprotein (9). An analogous compound containing trigalcosylcysteine was later found in human erythrocyte membranes (10). We now report the presence of another glycopeptide in normal human urine, containing the new sugar-amino acid linkage fucose-threonine.

**SUMMARY**

A glycopeptide, 3-O-β-D-glucopyranosyl-α-L-fucopyranosyl-α-L-threonine, has been isolated from normal human urine. The glycopeptide was isolated by gel chromatography, preparative zone electrophoresis, paper chromatography, and high voltage electrophoresis. The average yield of the glycopeptide was in the range of 0.2 to 0.3 mg/liter of urine. Sugar analysis and amino acid analysis gave equimolar amounts of glucose, fucose, and threonine. Linkages and sequential order were established by methylation analysis of the glycopeptide after degradation of the amino acid residue with ninhydrin. The permethylated product was analyzed on gas-liquid chromatography and mass spectrometry. Anomeric configuration was deduced from optical rotation.

**EXPERIMENTAL PROCEDURES**

**General Methods**—Four-hour urine specimens from 18 individuals, three of each of A, B, and H secretors and nonsecretors, were collected following a 24-hour period of fasting. For preparative purposes 30 liters of urine were collected from a healthy male (A secretor) without any restrictions on food intake. The urines were stored at −20º until used. Sephadex G-25 (fine) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Pevikon C 870 was obtained from Kema Nord AB (Stockholm, Sweden). Bio-Gel P-2, −400 mesh, was obtained from Bio-Rad Laboratories (Richmond, Calif.). Bacterial growth was prevented by addition of phenyl mercuric nitrate (30 ml of saturated solution/liter of urine). After filtering the urine samples, ultrafiltration was performed at +4º using Visking 3/4-inch dialysis tubing (11). Concentration of urine and chromatographic eluates was done by rotary evaporation under reduced pressure. Gel chromatography, preparative zone electrophoresis, high voltage electrophoresis, and preparative paper chromatography were performed as described elsewhere (12, 13). The following solutions and solvent systems were used: A, 2 M acetic acid; B, ethyl acetate/acetic acid/water (2/1/1, v/v); C, 1-propanol/ethyl acetate/water (6/1/3, v/v); D, ethyl acetate/pyridine/water (2/1/2, v/v, upper phase); E, ethyl acetate/pyridine/water (10/4/3, v/v).

**Analytical Methods**—Colorimetric methods were used for determination of total hexose (14) and 6-deoxyhexose (15). Determination of glucose was performed using glucose oxidase, Glox (Kabi AB, Stockholm, Sweden) (16).

Sugar analysis was performed by gas-liquid chromatography (17) and mass spectrometry (18). Methylation analysis was performed as previously described (19).

Gas-liquid chromatography was carried out on glass columns containing: (a) 3% w/w of ECNSS-M on Gas-chrom Q at 900º (for sugar analyses), (b) 3% w/w of OV-225 on Gas-chrom Q at 180º (for methylation analyses), and (c) 3% w/w of OV-1 on Gas-chrom Q at 240º (for permethylated oligosaccharide derivatives). A Perkin-Elmer 900 gas chromatograph with flame ionization detector was used.

For mass spectrometry a Varian MAT 311 gas-liquid chromatography-mass spectrometry instrument fitted with the appropriate column was used. Mass spectra were recorded at an ionization potential of 70 e.v., an ionization current of 1000 μA, and an ion source temperature of 120º. Optical rotations were measured on a Perkin-Elmer model 141 photoelectric polarimeter. Quantitative amino acid determinations were carried out on a Jel 6AH automatic amino acid analyzer (JEOL Co., Tokyo) with the two-column method (20). The glycopeptide was hydrolyzed in 6 N hydrochloric acid at 110º for 18 hours.

**Isolation of Glycopeptide**—Urine ultrafiltrates were concentrated 10 times and fractionated on a Sephadex G-25 (fine) column. Eluted fractions were analyzed for total hexose and 6-deoxyhexose. Fig. 1 shows a typical pattern of the distribution of 6-deoxyhexose-containing material from a nonsecreted A secretor.
paper chromatography in the following solvents: B, -4BH blood groups and different secretor status. The yields of homogeneity of the isolated glycopeptide was investigated by the isolated compound were 0.2 to 0.3 mg/liter of urine. The
An insoluble residue was formed and removed by filtration.
Eluted fractions were assayed for 6-deoxyhexose to give fractions V, and Vb.
The eluted material was pooled into seven fractions (I to VII) as indicated Fraction V was further fractionated by preparative zone electrophoresis in 2 M acetic acid. Voltage, 3.2 volt/cm. Time, 40 hours. Arrow points to origin. Eluted fractions were assayed for 6-deoxyhexose to give fractions Va and Vb.

The eluted material was pooled into seven fractions (I to VII) as indicated Fraction V was further fractionated by preparative zone electrophoresis in 2 M acetic acid (Fig. 2). The bulk of the 6-deoxyhexose-containing material (Vs) remained at the origin and has previously been shown to contain neutral oligosaccharides (21, 22). Approximately 5% of the 6-deoxyhexose-containing material in region V had a slight mobility towards the cathode (Vb). Fraction Vb was concentrated and fractionated further by preparative paper chromatography in Solvent B. At least five different compounds stained with both silver nitrate and ninhydrin, but only one, R, 0.79; R, 0.62; C, R, 0.79; D, R, 0.62; and E, R, 0.28. The compound R, 1.15 showed [al], -111° (c 0.96, water).

**RESULTS**

Compound R, 1.15 was isolated from individuals of different ABH blood groups and different secretor status. The yields of the isolated compound were 0.2 to 0.3 mg/liter of urine. The homogeneity of the isolated glycopeptide was investigated by paper chromatography in the following solvents: B, R, 1.15; C, R, 0.79; D, R, 0.62; and E, R, 0.28.

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**DISCUSSION**

The glycopeptide was homogeneous in four solvent systems in paper chromatography, on high voltage electrophoresis at pH 1.9, and was eluted as a single peak on Bio-Gel P-2. Component analysis showed that the glycopeptide was composed of 1 mol of glucose, 1 mol of fucose, and 1 mol of threonine. The absolute configurations of the components were not determined but in the following it is assumed that glucose has the L, fucose the L, and threonine the L, configuration in analogy to previously known configurations of these compounds in human materials.

On ninhydrin degradation (23) the L-threonine residue was converted to a propionaldehyde derivative which on reduction with sodium borodeuteride yielded a 2-hydroxy-propionaldehyde residue. Permethylation of the ninhydrin-degraded glycopeptide gave a product which on gas-liquid chromatography showed one peak having the mass spectrum shown in Fig. 4. This mass spectrum is compatible with the structure given in Fig. 5. The A series of fragments (24-26) shows aA (m/e 219), aA2 (m/e 187), aA3 (m/e 155), and abA2 (m/e 361), abA3 (m/e 329). From those fragments it can be concluded that a hexose unit (a) is linked to a 6-deoxyhexose residue (b). The J series of fragments in the spectrum is ab4 (m/e 208), abb4 (m/e 248), and bbb4 (m/e 74). From the J series the structural element of a permethylated 6-deoxyhexose-OCH(CH3)2CHDOH is evident. By a combination of the A and J series of fragments the structural unit, permethylated hexose-6-deoxyhexose-OCH(CH3)2CHDOH, is shown.
Hydrolysis of the permethylated, ninhydrin-degraded, and reduced glycopeptide yielded equimolar proportions of 2,3,4,6-tetra-O-methyl-D-glucose and 2,4-di-O-methyl-L-fucose. This finding, in correlation with the mass spectrum of the intact permethylated derivative, demonstrates the following structural features: 3-glucopyranosyl-(1→3)-L-fucopyranosyl → OCH\_2(CH\_3)CHDOH. The optical rotation of the glycopeptide, $\varphi$-111°, is only compatible with a $\beta$-linkage for the $\alpha$-D-glucopyranosyl residue and an $\alpha$-linked L-fucopyranosyl residue using calculations based upon Hudson’s isorotation rules. From the evidence presented we postulate the structure shown in Fig. 3 for the glycopeptide, i.e. $\beta$-D-glucopyranosyl-(1→3)-$\alpha$-L-fucopyranosyl-(1→O)-L-threonine. The excretion of this compound is independent of blood group and secretor status and the origin is as yet unknown.

Acknowledgments—The help of Dr. Jörgen Eriksson, Institute of Medical Chemistry, Uppsala University, Uppsala, Sweden is acknowledged in kindly performing the amino acid analyses.
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