THE CARBOHYDRATE-PROTEIN LINKAGE IN THE α_1-GLYCOPROTEIN OF HUMAN PLASMA

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Evidence has been presented for ovalbumin (Johansen et al., 1961; Nuenke and Cunningham, 1961) and γ-globulin (Rosevear and Smith, 1961) that aspartic acid is linked to a single carbohydrate moity. On the other hand, there is little information about the carbohydrate-protein bond in glycoproteins like the α_1-glycoprotein where the carbohydrate is distributed in several large units (Eylar and Jeanloz, 1962). In the present work it has been found that aspartic acid is joined to N-acetylglucosamine in what appears to be a β-aspartylglycosylamine structure.

Experimental and Results: Sialic acid was removed from highly purified α_1-glycoprotein with pneumococcal neuraminidase. To 500 mg. of sialic acid-free α_1-glycoprotein were added 25 ml. of 0.2M tris buffer, pH 8.0, 0.2 ml. 0.4M $CaCl_2$, 20 mg. of Pronase (Cal. Corp. for Biochemical Research), and 1 ml. ethanol. At the end of 1 week incubation at 37°, during which time 5 mg. of Pronase were added daily, the solution was heated at 100° for 10 min., filtered, and passed through Sephadex G-25. The anthrone-positive fraction was collected, evaporated and submitted to the entire procedure (including incubation with Pronase) 4 more times. Finally, the anthrone-positive fraction was applied to a charcoal-celite column (Eylar and Jeanloz, 1962) and the fractions collected as shown in Table I. Fractions 5 and 6

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totaled 127 mg. and represented a yield of about 53% of the original carbohydrate.

Table I

Chromatography on Charcoal-Celite (30 g.)

Fraction	%Ethanol in Effluent	Volume(ml)	Weight(mg)
1	H_2O	500	-
2	5%	250	-
3	15%	250	-
4	20%	150	-
5	30%	30	23
6	30%	50	104
7	30%	100	5

Analysis by the method of sedimentation equilibrium in 0.1N NaCl (Eylar and Jeanloz, 1962) gave a molecular weight of 1800 for the fraction 6 glycopeptide. A partial specific volume of 0.65 was assumed for calculation.

On high voltage electrophoresis at pH 4.5 in pyridine, acetic acid, butanol buffer at pH 4.5 (Dintzis, 1961) two bands were detected for the fraction 5 glycopeptide. The slowest band was near the origin.

On paper chromatography in 3 systems, (1) butanol, acetic acid, water, 4:1:5, (2) pyridine, ethylacetate, water, acetic acid, 5:5:3:1, and (3) isopropanol, borate buffer, 4:1, (McMenamy et. al., 1957), the glycopeptides of fraction 5 or 6 did not move from the origin in 30 hrs. No other ninhydrin-positive spots were detected. Likewise, the DNP-glycopeptides, prepared by reaction of fractions 5 and 6 with fluorodinitrobenzene at pH 9.0 in a pH stat, showed only a single yellow spot at the origin in these systems.

Chemical analysis of both fractions gave approximately 46% galactose and mannose (anthrone), 43% N-acetylglucosamine (Elson-

Morgan), and 2-3% fucose. The amino acid and NH_3 analysis is shown in Table II.

Table II

Amino Acid Composition* of the Glycopeptides

Residue	Fraction 5 $\left[\frac{\text{mole}}{1800g}\right]$	Fraction 6 $\left[\frac{\text{mole}}{1800g}\right]$
Aspartic acid	0.55	0.73
Threonine	0.02	0.21
Glycine	0.0	0.06
Glutamic acid	0.0	0.02
Serine	0.0	0.02
Ammonia±	0.62	0.80

* (Spackman, Stein and Moore, 1958)
± Determined after hydrolysis in 1N HCl, 3 hrs., 100°.

Fraction 5 glycopeptide contained only aspartic acid in significant quantity while fraction 6 glycopeptide revealed some threonine and glycine as well. Both fractions yielded approximately 1 mole of NH_3 per mole of aspartic acid and suggest that an aspartylglycosylamine linkage is present. Further evidence for the proposed structure was obtained from hydrolysis of the DNP-glycopeptides which yielded DNP-aspartic acid from fraction 5 and 6 and in addition DNP-threonine in the latter case. The DNP-amino acids were identified by paper chromatography (Biserte and Osteux, 1951) after hydrolysis in 2N HCl for 3 hrs. or 6N HCl for 12 hrs. at 100°. No DNP-glucosamine was detected.

It was found that leucine aminopeptidase released threonine slowly from fraction 6. Threonine was also released by hydrolysis in 1N NaOH at 100° for 5 hrs. In neither case was aspartic acid released. Carboxypeptidase showed no activity toward the DNP-glycopeptides.

The low content of aspartic acid in the glycopeptides suggests that Pronase exhibits some activity toward the aspartic acid-carbohydrate bond. When fraction 5 glycopeptide was reduced with

$NaBH_4$, deionized on Amberlite MB-3 resin, hydrolyzed in 2N HCl at 100° for 8 hrs., and chromatographed in system (2), glucosaminitol was found. This finding is compatible with the structure of the octasaccharide (Eylar and Jeanloz, 1962) in which N-acetylglucosamine occupies the reducing position. It appears therefore that a β-aspartyl N-acetylglucosaminylamine linkage is present in the α_1-glycoprotein. It is probable that the β-COOH rather than the α-COOH of aspartic acid is involved (Eylar, E. H., unpublished observations). The type of linkage suggested for γ-globulin (Rothfus, 1961) is not likely in this case since the glucosamine residues are acetylated in the octasaccharide (Eylar and Jeanloz, 1962).

Summary: After prolonged digestion with Pronase, over 50% of the carbohydrate of the α_1-glycoprotein was isolated as glycopeptide fractions with molecular weight near 1800. One of the fractions yielded asparagine (aspartic acid plus NH_3) as the sole amino acid residue. Some of the aspargine had been cleaved from the carbohydrate unit (octasaccharide) by Pronase leaving N-acetylglucosamine in the reducing position. It was proposed that aspargine is joined through its amide group to form a β-aspartyl N-acetylglucosaminylamine linkage. Some of the asparagine residues of the other fraction appear to be in peptide linkage with threonine which is N-terminal.

This report does not concur with the proposal (Winzler and Inoue, 1961) that glutamic acid is joined to the amino group of glucosamine in the α_1-glycoprotein. Recently glycopeptides in which aspartic acid predominated were isolated from the α_1-glycoprotein (Kamiyama and Schmid, 1962).

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