THE CARBOHYDRATE-PROTEIN LINKAGE IN OVOMUCOID

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The amino acid residues involved in linkage with the carbohydrate of ovomucoid have been proposed as aspartic and glutamic acids (Tanaka, 1961) and serine or threonine (Hartley and Jevons, 1962). Since it was indicated earlier (Chatterjee and Montgomery, 1962) that ovomucoid contains several oligosaccharides and not a single polysaccharide, the possibility existed that each oligosaccharide could be linked to different types of amino acid. The present work shows that ovomucoid contains three similar oligosaccharide units, each of which is linked to aspartic acid.

Experimental and Results - The ovomucoid used in the present study was described in a previous paper (Chatterjee and Montgomery, 1962).

The analytical procedures for carbohydrates, amino acids and amino groups, as well as the fractionation procedures of gel filtration on Sephadex G-25 and zone electrophoresis,have been described earlier (Lee and Montgomery, 1961, 1962; Chatterjee and Montgomery, 1962).

In a typical preparation of ovomucoid glycopeptides, salt-free ovomucoid (4 g.) was dissolved in 0.1 M pyridine acetate buffer (pH 5.0, 400 ml.) and the solution was incubated at 60° with crystalline papain which had been activated with 0.001 M BAL and 0.001 M Na-Versenate. The enzyme-substrate ratio was increased during the course of the digestion from an initial 1% w/w to 2% by the addition of papain in 40 mg. portions. After about 6 hrs. of digestion

1/ This work will form part of a thesis to be presented by Y. C. Wu to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Ph.D.

the carbohydrate-containing material was isolated by gel-filtration; the recovery in terms of hexose was quantitative. The resulting glycopeptide mixture was submitted to the entire procedure three more times and the final material (1.08 g.) was fractionated in 250 mg. portions by zone electrophoresis in M acetic acid at 800 v. and 25 ma. for 24 hrs. Four fractions were obtained, representing a 90% recovery of hexose from the original ovomucoid. The composition of these glycopeptide fractions is summarized in Table I.

A mixture of ovomucoid glycopeptides from the papain digestions, containing fractions I (2%), II (76%) and III (22%), was treated with Pronase P (Kaken Chem Corp., Ltd., Tokyo) in 0.1 M borate buffer, pH 8.0, containing 0.01 M $CaCl_2$. After incubation at 40° for 3 days the mixture was fractionated as before into three fractions, the compositions of which indicated little further digestion by Pronase. The fraction corresponding to II was further fractioned on a column (84 x 0.9 cm.) of Dowex 50W x2 ion exchange resin, eluting by two procedures. Elution with 0.2 M Na citrate, pH 3.25, afforded a fraction (II-50W) (peak effluent volume, 30 ml.) containing 75% of the carbohydrate placed on the column. The analytical data for this fraction is summarized in Table II. The residual 25% of the carbohydrate could be gradually eluted by Na citrate (200 ml.) but the fractionation was better if carried out by an elution procedure that involved a gradient, with a constant volume mixing chamber, starting from 3 M acetic acid (200 ml.) and adding 0.2 M Na citrate (200 ml.). By this procedure five carbohydrate-containing fractions were obtained, the last of which showed the amino acid mole ratios, referred to one mole of the N-terminal phenylalanine (Table I), asp. 1.23, pro 0.93, ala 0.89, thr <0.01, ser <0.01. After two successive Edman degradations the amino acids in the glycopeptide are asp 1.01 mole and ala 0.89 mole, again referred to one mole of phenylalanine in the original.

Discussion - The digestion of ovomucoid with papain affords two major fractions designated II and III in Table I. The composition of III supported the linkage of the carbohydrate to an asparagine residue. The phenyliso-

TABLE 1 - Composition of Glycopeptide Fractions from the Papain Digestion of Ovomucoid

Component[1]	Glycopeptide Fraction			
	I (1%)[2]	II (66%)[2]	III (23%)[2]	IV (11%)[2]
	Moles of component per eight moles of N-acetyl D-glucosamine			
Aspartic acid	1.54	1.12 (0.39*)	1.05	1.12 (0.37*)
Threonine	1.02 (0.28**)	1.18 (0.33*, 0.26**)	0.02	0.90 (0.19*, 0.25**)
Serine	0.44 (0.09**)	0.38 (0.10*, 0.06**)	0.02	0.36 (0.03*, 0.06**)
Iso-leucine	0.20	0.30		0.26
Proline	0.25	0.26		0.39
Alanine	0.29	0.25 (0.08*)		0.29 (0.02*)
Phenylalanine	0.25	0.24 (0.22*)		0.35 (0.12*)
Glycine	0.26	0.06		0.08
Leucine	0.06	0.03		0.04
Lysine	0.54 (0.16**)			
Arginine	0.31 (0.31*)			
Glutamic acid	0.01			
Histidine	0.15			
Ammonia			1.24	
N-acetyl neuraminic acid	0.00	0.00	0.00	0.93
N-acetyl-D-glucosamine	8.00	8.00	8.00	8.00
D-Mannose	5.64	3.94	3.90	4.10
D-Galactose		0.88	1.05	1.86

* Moles of amino-terminal amino acid residue per eight moles of N-acetyl-D-glucosamine, determined by dinitrophenylation (Fraenkel-Conrat, Harris and Levy, 1955).

** Moles of carboxyl-terminal amino acid residue per eight moles of N-acetyl-D-glucosamine, determined by hydrazinolysis (Akabori and co-workers, 1953).

1/ Not corrected for destruction during hydrolysis.

2/ Percentage of carbohydrate in the original ovomucoid corrected from a 90% experimental recovery to 100% recovery.

TABLE II - Data for Glycopeptide Fraction II-50W

Component	Moles of component per eight moles of N-acetyl-D-glucosamine			
	Composition[1]	Edman Degradation[2]		Periodate oxidized and BH_4-reduced product[3]
		Fraction A	Fraction B	
Aspartic Acid	1.20 (0.42*)	0.78	0.00	1.20
Threonine	0.87 (0.39*)	<0.01	0.46	0.46
Serine	<0.01			
Glycine	<0.01	< 0.01		
Alanine	<0.01	< 0.01		

* see Table I

1/ Hydrolyzed with 4 N HCl at 100° for 8 hrs. Not corrected for destruction during hydrolysis.

2/ Procedure of Konigsberg and Hill (1962) was used and the reaction product was fractionated by gel filtration on Sephadex G-25. Fraction A, containing all the carbohydrate, was excluded from the gel and was analyzed after hydrolysis with 4 N HCl at 100° for 8 hrs. Fraction B, containing no carbohydrate, was analyzed without hydrolysis.

3/ Fraction II-50W was treated with 0.1 M $NaIO_4$ at 25° in the dark for 24 hrs. The oxidized glycopeptide, isolated by gel filtration, was reduced with excess $NaBH_4$ at 5° overnight and the product, after separation by gel-filtration, was hydrolyzed with 4 N HCl at 100° for 8 hrs. The amino acids levels refer to the original II-50W since 57% of the hexosamine is oxidized in the reaction.

thiocarbamyl derivative of III cyclized in acid to a phenylthiohydantoin (Konigsberg and Hill, 1962). It is most probable therefore that this carbohydrate is present as a β-asparaginyl derivative.

Fraction II was further fractionated by ion exchange chromatography. The properties of one subfraction, II-50W (Table II), indicate that the main glycopeptide components have the dipeptide moieties thr-asp and asp-thr in approximately equimolar amounts. The carbohydrate linkage did not involve the hydroxyl group of threonine in either case bacause periodate oxidation of II-50W destroyed 50% of the threonine, which was the N-terminal residue on one component and the procedure of Edman (Konigsberg and Hill, 1962) on II-50W afforded a carbohydrate-containing material, isolated by gel filtration, that contained aspartic acid and its phenylthiohydantoin but no threonine or its phenylthiohydantoin.

The composition and end-group analyses of one other ion-exchange subfraction of II indicated a structure phe-pro-ala-asp(carbohydrate) with the carbohydrate recovery representing 10% of that in ovomucoid. Threonine and serine were present in this fraction at a contaminating level only. It is clear therefore that the amino acid sequence around the oligosaccharide units cannot be identical and whereas the presence of thr-asp(carbohydrate) and asp (carbohydrate)-thr in II-50W may support a peptide sequence -thr-asp-thr-, as in the case of α_1-glycoprotein (Kamiyama and Schmid, 1962), it is equally possible that these two glycopeptides derive from peptide segments with different amino acid sequences.

The carbohydrate composition of II and III were similar and the average molecular weights obtained from the absorbancy of the DNP-derivatives were 2700-2800, using a molar extinction coefficient of 15,000 in M-acetic acid (Lee and Montgomery). The procedure of partial dinitrophenylation (Silaev et al, 1960) showed that each component had one amino group per molecule. These results correspond to that required by the simplest oligosaccharide indicated for III (Table I; calculated molecular weight 2567)and show that ovomucoid contains three similar oligosaccharide units.

It will be seen that sialic acid was present only in IV, which also contained a higher level of D-galactose. However, since the recovery of sialic acid was low (50%) it is probable that the procedures of fractionation resulted in its hydrolysis from other fractions.

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