Short Communication

The Peptide Moiety of Human-Blood-Group Active Glycoproteins Associated with the ABO and Lewis Groups

By A. S. R. DONALD, J. M. CREETH, W. T. J. MORGAN and WINIFRED M. WATKINS
The Lister Institute of Preventive Medicine, London S.W.1

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The carbohydrate components of the blood-group-specific glycoproteins that occur in the secretions and tissue fluids in man have been extensively investigated and it is established that the A, B, H, Le^a and Le^b immunological determinants are associated with the carbohydrate moiety (see Morgan & Watkins, 1969). However, because means were not available whereby the peptide moiety in the specific glycoprotein could be obtained intact and free from carbohydrate, little is known about this important structural component apart from its amino acid composition (see Pusztai & Morgan, 1963).

In this paper methods are described for the isolation from blood-group-active glycoproteins of a macromolecular glycopeptide that has a considerably increased content of peptide and an amino acid composition closely similar to that of the parent glycoprotein. Sugars other than *N*-acetyl-D-galactosamine are largely eliminated by chemical degradation and the resulting glycopeptide is enzymically degraded to remove the *N*-acetyl-amino sugar.

Blood-group B-active glycoprotein (1g.) isolated from a human ovarian-cyst fluid (see Morgan, 1967) was suspended in acetic acid (100ml.) containing 25mM-H₂SO₄ (A.R.). The reaction mixture, in a tightly stoppered flask, was held at 60 ± 2° for 24 hr. and cooled. The insoluble material, after repeated washing with acetic acid, was dissolved in water (5ml.) and dialysed at 3–4° until free from acid. The indiffusible material, freeze-dried (247mg.), was dissolved in 0.2M-acetic acid (5ml.), loaded on a column (2cm. × 60cm.) of Sephadex G-75 and eluted with 0.2M-acetic acid. The fractions emerging after the void volume were arbitrarily divided; the first 30ml. of eluent yielded 81mg. (fraction *a*), and the following 120ml. of eluent 169mg. (fraction *b*). The two materials were separately de-*O*-acetylated in 50mM-NaOH for 30 min. at 20°, and the solutions were neutralized with HCl, dialysed, concentrated and freeze-dried. The analytical values for the amino acid and carbo-

hydrate contents of the two fractions were closely similar. Each retained the characteristic amino acid pattern of the original glycoprotein (Table 1) and the content of amino acid had increased from 18 to 55%. Fucose was absent and the galactose and *N*-acetylglucosamine contents were less than 6 and 3% respectively. Both fractions contained about 25% of *N*-acetylglucosamine and were therefore essentially *N*-acetylgalactosaminyl-peptides. About 80% of the *N*-acetylglucosamine present in the starting material was recovered in the two fractions. The glycopeptide (fraction *b*) gave a single symmetrical boundary in a sedimentation-velocity experiment at 60000 rev./min., with *S*_{25,w} 1.38s. The molecular weight was determined from sedimentation-equilibrium experiments (Yphantis, 1964). Only slight curvature was visible in the plot of log(concentration) versus (radius squared), indicating substantial homogeneity of the solute. The results were *M*_n 12000, *M*_w 12100, based on a value for the partial specific volume, *v*, of 0.714ml./g. The optical rotation of the glycopeptide was [α]₅₄₆ or ₅₇₈ +8° (c 1.0 in water).

Alkaline reductive cleavage of intact blood-group-active glycoproteins implicated *N*-acetylglucosamine as the sugar linked by *O*-glycosidic bonds to serine and threonine (see Kabat *et al.* 1965). Direct proof of this linkage was obtained by degradation of the *N*-acetylgalactosaminyl-peptide (fraction *a*; 5mg.) in 0.2M-NaOH (1.0ml.) containing 20mg. of NaBH₄. The reaction mixture was kept in the dark for 9 days at 18–20°, neutralized with 0.5M-HCl and samples were hydrolysed and analysed for hexosamine, hexosaminitol and amino acids. The results (Table 1) revealed a loss of galactosamine, an increase in galactosaminitol, a loss of threonine (76%) and serine (69%) and an increase in alanine. The proportions of the other amino acids were not appreciably changed. The overall loss in the hydroxy amino acids (1.464 μmoles/mg.) was matched by a loss of 1.206 μmoles of galactosamine/mg. (96%). Similar changes were observed (Table 1) when the original

Table 1. *Analysis of the B-active glycoprotein and its degradation products*

Results are expressed as (1) g./100g. of substance, (2) μ moles/100 μ moles of total amino acid [determined by the method of Spackman, Stein & Moore (1958) in an amino acid analyser (Evans Electroselenium Ltd.)] and (3) as μ moles loss or gain/mg. of substance.

Component	Untreated B-active glycoprotein		Glycopeptide fraction b		Enzyme-treated glycopeptide fraction b	Alkali- borohydride- treated glycopeptide	Alkali- borohydride- treated B substance
	(1)	(2)	(1)	(2)	(2)	(3)	(3)
Asp	1.04	5.12	2.39	4.57	4.68	-0.008	+0.012
Thr	4.11	22.70	14.16	25.65	25.96	-0.945	-0.258
Ser	2.48	15.53	7.54	15.48	16.06	-0.519	-0.169
Glu	1.41	6.29	4.25	6.23	6.04	-0.008	+0.009
Pro	2.03	11.57	6.46	12.11	12.53	-0.038	+0.008
Gly	0.64	5.59	1.84	5.28	5.03	+0.017	+0.024
Ala	1.01	7.46	3.32	8.04	7.34	+0.141	+0.075
CyS	0.41	2.22	1.39¶	2.48¶	2.13¶	-0.004¶	+0.009
Val	0.94	5.29	2.67	4.91	5.06	-0.010	-0.001
Met	0.11	0.50	0.15	0.22	0.48	-0.001	-0.002
Ile	0.48	2.39	1.39	2.28	2.25	0	-0.001
Leu	0.80	4.03	1.96	3.23	3.16	-0.002	-0.001
Tyr	0.34	1.20	0.72	0.86	0.86	-0.003	+0.006
Phe	0.47	1.86	1.11	1.44	1.36	-0.004	-0.002
Lys	0.62	2.81	1.47	2.17	2.37	+0.007	+0.003
His	0.43	1.97	1.24	1.86	1.82	+0.002	+0.004
Arg	0.91	3.45	2.54	3.18	2.83	-0.008	+0.022
Total amino acids	18.23	99.98	54.60	99.99	99.96		
Fucose*	16.5		0			—	—
Galactose†	34.5		5.8			—	—
Glucosamine‡	18.5		2.3			-0.110	-0.146
Galactosamine‡	6.5		21.8			-1.206	-0.326
Glucosaminitol‡	—		—			+0.061	0
Galactosaminitol‡	—		—			+0.906	+0.241
Acetyl§	—		7.9			—	—

* Determined by the method of Gibbons (1955).

† Determined by the method of Dubois, Gilles, Hamilton, Rebers & Smith (1956).

‡ Determined by the method of Donald (1968).

§ Determined by the method of Radhakrishnamurthy, Dalferes & Berenson (1968).

|| Values corrected for amino acids in added enzyme.

¶ Values not entirely satisfactory because asymmetry of the peak indicated the presence of a contaminant.

B substance was treated with alkaline borohydride; threonine (75%), serine (71%) and *N*-acetyl-galactosamine (90%) were destroyed.

The anomeric linkage of the *N*-acetylgalactosamine to the hydroxyamino acids is inferred to be α from the results of serological-inhibition and enzymic-degradation experiments on the glycopeptide. The agglutinins from the albumin gland of the snail *Helix hortensis* are believed to react with human A₁ cells mainly by virtue of the α -*N*-acetylgalactosaminyl grouping that constitutes the terminal non-reducing unit in the A determinant (Prokop, Uhlenbruck & Köhler, 1968). Although, as expected, the *N*-acetylgalactosaminyl-peptide did not inhibit human anti-A agglutinins it nevertheless strongly inhibited the agglutination of A₁ cells by the *Helix hortensis* reagent; complete

inhibition was given by 7.5 μ g. of the glycopeptide, a value to be compared with 0.25 μ g. of purified A substance and 250 μ g. of α -methyl *N*-acetyl-galactosaminide. Two other anti-A reagents known to be directed primarily towards the α -*N*-acetylgalactosaminyl grouping, namely agglutinins from the seeds of *Dolichos biflorus* and *Vicia cracca* (see Bird, 1959), were also inhibited by the glycopeptide. The untreated B substance from which the glycopeptide was isolated gave no inhibition with these test reagents, suggesting that degradation of the substance had exposed terminal α -*N*-acetyl-galactosaminyl residues.

An enzyme isolated from *Trichomonas foetus* destroys the serological activity of blood-group A substance with the release of *N*-acetylgalactosamine (Harrap & Watkins, 1964). The enzyme prepara-

tion, which hydrolyses methyl α -*N*-acetyl-D-galactosaminide but not the β -anomer, released *N*-acetylgalactosamine from the *N*-acetylgalactosaminyl-peptide. These experiments indicated that up to two-thirds of the bound *N*-acetylgalactosamine could be removed; they do not exclude the possibility of the release of a larger percentage of this sugar by more intensive enzyme treatment. The molecular weight of the glycopeptide (fraction *b*) remaining after removal of 53% of the *N*-acetylgalactosamine, determined by sedimentation-equilibrium experiments, was M_n 10800, M_w 10400, based on a value for $\bar{v}$ of 0.740ml./g. A minor high-molecular-weight contaminant not present in the untreated glycopeptide was presumably derived from the added enzyme. The values of M are subject to revision if $\bar{v}$ is found to differ from its assumed value, but the results imply that the *T. foetus* enzyme is removing the *N*-acetylgalactosamine without appreciable fragmentation of the peptide moiety. After correction for the relatively small contribution of amino acids from the enzyme, the amino acid composition of the enzyme-treated glycopeptide was essentially the same as that of the untreated glycopeptide (Table 1).

The chemical degradation procedure applied to blood-group H- and Le^a-active glycoproteins yielded *N*-acetylgalactosaminyl-peptides essentially similar in chemical composition and behaviour to the fractions from B substance; thus giving further evidence (see Morgan & Watkins, 1969) for a common internal structural pattern in the specific glycoproteins. Consequently, a combination of the chemical and enzymic degradation methods can be expected to yield the peptide moiety from blood-group substances of differing specificity in a form

amenable to physical, chemical and immunological investigation by the standard techniques applied to proteins. Examination of the intermediate *N*-acetylgalactosaminyl-peptide described in this paper confirms that *N*-acetylgalactosamine is attached to serine and threonine in the peptide chain through *O*-glycosidic linkages and has demonstrated for the first time that the sugar is in the α -anomeric form.

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- Bird, G. W. G. (1959). *Brit. med. Bull.* **15**, 165.
Donald, A. S. R. (1968). *J. Chromat.* **35**, 106.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Gibbons, M. N. (1955). *Analyst*, **80**, 268.
Harrap, G. J. & Watkins, W. M. (1964). *Biochem. J.* **93**, 9P.
Kabat, E. A., Bassett, E. W., Pryzwansky, K., Lloyd, K. O., Kaplan, M. E. & Layug, E. J. (1965). *Biochemistry*, **4**, 1632.
Morgan, W. T. J. (1967). In *Methods in Immunology and Immunochemistry*, vol. 1, p. 75. Ed. by Williams, C. A. & Chase, M. W. New York: Academic Press Inc.
Morgan, W. T. J. & Watkins, W. M. (1969). *Brit. med. Bull.* **25**, 30.
Prokop, O., Uhlenbruck, G. & Köhler, W. (1968). *Vox sang., Basel*, **14**, 321.
Pusztai, A. & Morgan, W. T. J. (1963). *Biochem. J.* **88**, 546.
Radhakrishnamurthy, B., Dalferes, E. R. & Berenson, G. S. (1968). *Analyt. Biochem.* **26**, 61.
Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Biochem.* **30**, 1190.
Yphantis, D. A. (1964). *Biochemistry*, **3**, 297.