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ISOLATION AND CHARACTERIZATION OF THE O-GLYCAN CHAIN OF THE HUMAN VITAMIN-D BINDING PROTEIN

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SUMMARY - On a highly purified preparation, the structure of the carbohydrate chain of the human vitamin D-binding protein was investigated and two genetic forms of this protein were considered (Gc 2 and Gc 1 proteins). It was found that only the Gc 1 protein (Gc1a isoform) was glycosylated, the glycan moiety representing about 1 % of the protein. The structure of this O-glycosidically linked glycan was determined to be : Neu Ac α(2→3) Gal ß(1→3) GalNAc α(1→O) Ser (or Thr). A tetrasaccharidic O-glycan with two N-acetylneuraminic residues was also characterized. The vitamin D-binding protein is a rare example of a serum protein O-glycosylated only on some genetic forms.

The serum protein (an α_2 globulin) called in 1960 by HIRSCHFIELD (1) group-specific component (Gc) was later identified as the vitamin D metabolic carrier protein or vitamin D-binding protein (DBP) (2, 3, 4) but its biological activity still remains incertain. Moreover, since 1977 (5), our knowledge of the genetic polymorphism of the molecule has largely improved. Today, 85 rare mutants are identified while three main alleles (Gc^{1F}, Gc^{1S}, Gc^{2}) are present in any human groups and correspond to 99 % of the polymorphism, based on an electrophoretic heterogeneity due to the existence of two kinds of protein bands : a one band pattern corresponding to the Gc2 protein (pI : 5.2) and a double band pattern for the Gc 1 proteins produced by the Gc^{1} allele (Gc 1a pI : 4.85 and Gc 1c pI : 4.95). The difference between Gc 1a (anodal mobility) and Gc 1c (cathodal band) is not due to an amino acid substitution but to the presence on Gc 1a of one sialic acid residue (6). The consequence is the probable existence of differences in the carbohydrate part of these two isoproteins.

The aim of this investigation was to determine the composition and structure of the glycan of the Gc 1 and Gc 2 proteins, highly purified (7).

MATERIALS AND METHODS

Purified proteins : Blood donor sera selected according to their DBP phenotype. Only samples belonging to Gc 1S and Gc 2 were considered. Details

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on the procedure used have been previously described (7). To put it briefly, the protein was purified by three successive chromatographies including Blue sepharose, immuno affinity (IgG anti DBP) and DEAE Tris Acryl columns. The purity of the proteins was controlled by SDS electrophoresis according to Laemmli's procedure, (8) ; determination of the isoelectric points (9) and ability to bind vitamin D derivatives (9). Protein concentration was estimated using the method described by Bradford (10) and by measuring the absorption at 280 nm ($E^{1\%}_{1cm}$: 5.5 ± 0.2).

Preparation and isolation of the glycopeptides : they were obtained from Gc 1 and Gc 2 proteins after incubation (15 mg of each protein) with 100 l of a pronase solution (10 mg/ml) at 37°C in 1M Tris buffer pH 7.8 containing 1mM $CaCl_2$ (11). After three successive additions of the pronase solution during seventy two hours, the solution was lyophilized. The dry product was solubilized in a 1 % (V/V) acetic acid solution. This solution was then chromatographed on a Biogel P6 column (200-400 mesh ; 100 x 1.6 cm) with a 9 ml/hr flow rate. Aliquots (15 µl) of each collected fraction (1. 5 ml) were directly applied on a silicagel plate (Kieselgel 60-Merck). After drying up, the plate was sprayed with a freshly prepared 0. 2 % orcinol solution in 20 % sulfuric acid. The fractions containing hexose were pooled and lyophilized.

Carbohydrate analysis : The molar ratio of monosaccharides was estimated by gas liquid chromatography (GLC) according to Zanetta et al. (12) after methanolysis with methanol 0. 5M HCl at 80°C for 20 hours.

Alkaline borohydride degradation of DBP : O-glycosidically linked oligosaccharides were released by treatment with 1M $NaBH_4$/0.05M NaOH for 20 hrs at 50°C in the dark under nitrogen (13). The solution was then cooled on ice, neutralized by the addition of Dowex 50 W x 8 (H+) resin according to Judson (14). Boric acid was removed as methylborate by five additions and evaporations of methanol 5 % (V/V) acetic acid and the reduced oligosaccharides were purified by chromatography on a Biogel P6 column as described above. Thin-larger chromatography (TLC) of purified oligosaccharides was performed on silicagel 60 plates (Merck) employing solvent n-butanol/acetic acid/water (2 : 1 : 1, V/V), and continuous flow for 16 h. The oligosaccharides were located after spraying the plate with the orcinol sulfuric reagent (15).

Permethylation and gas-liquid chromatography-mass spectrometry : the glycan liberated by ß elimination (5 µg) was methylated according to Hakomori's method (16) as modified by Bjorndal (17). After purification, the methylated compounds were treated with 0.5M methanol-HCl for 24 hrs at 80°C and the mixtures of methyl glycosides thus formed were peracetylated in pyridin-acetic anhydrid (1/1, 500 µl, 100°C for 30 min.). The methyl-glycoside methyl ethers were analyzed by GLC-MS as described (18).

RESULTS

In the determination of the glycan composition the purification of the serum DBP (4, 19, 20) and the procedures used account for the different results obtained in our investigation and in those previously published (21, 22, 23). The quality of the purified protein was controlled using different methods (figure 1). After loading as much as 6 µg of DBP, no additional bands were observed after SDS electrophoresis (MW = 56 000). After IEF, the measured isoelectric points were equivalent to those obtained for the native protein (9). The ability to bind the vitamin D deriva-

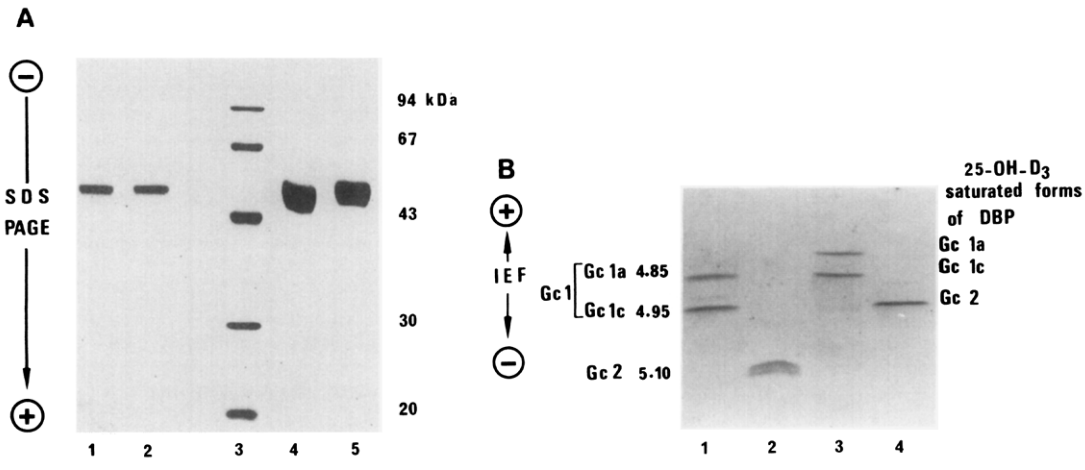

Figure 1 : A - SDS polyacrylamide gel electrophoresis of purified DBP
Lanes 1 and 2 = DBP 1.5 µg ;
Lane 3 = standard reference proteins of molecular weight ;
Lanes 4 and 5 = DBP 6 µ g.

B - Isoelectric focusing polyacrylamide gel electrophoresis prepared as described (9) using an ampholyte solution corresponding to the pH range 4 to 6.5 (pattern obtained after Coomasie Blue staining).
Lane 1 = purified DBP Gc 1S proteins (sample application 10 µ l of a 0.2 mg/ml solution ;
Lane 2 = purified DBP from Gc 2 phenotype (same concentration as above) ;
Lane 3 = Gc 1S proteins under saturated forms with 25-OH-D_3 solution (3 µg of 25-OH-D_3/ ml of serum ;
Lane 4 = Gc 2 purified under saturated form.

tives is also illustrated by the stability of the ligand bound forms and by their isoelectric points (9).

After pronase treatment of the purified DBP corresponding to the two genetic forms (Gc 1S and Gc 2), similar elution profiles were obtained by gel filtration on Biogel 6, (figure 2). We can also notice that all the protein used (15 mg for each experiments) was entirely digested by the pronase. The orcinol reaction on the collected aliquots showed a positive staining only with the glycopeptide isolated from the Gc 1S proteins and no reaction with the Gc 2 protein even after concentration of the corresponding aliquots.

This result was then confirmed by th GLC analysis. After methanolysis, no hexose or monosaccharide was detectable on the Gc 2 protein. The glycopeptide obtained from the Gc 1S proteins presents three residues : galactose, N-acetyl galactosamine and N-acetyl neuraminic acid These results show (table 1) that the same monosaccharide composition is found on native DBP and on its derived glycopeptide. The molar ratio of these three monosaccharides are in good agreement with a trisaccharidic

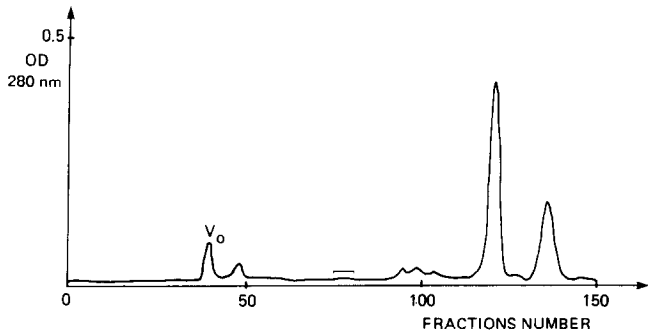

Figure 2 :Gel permeation chromatography of glycopeptides released after pronase digestion of the DBP (Gc 1S proteins). V_o is the void volume of the Bio-gel P-6 column. The fractions corresponding to a positive orcinol staining (fractions 75 to 81) are pooled for further characterization of the monosaccharides.

glycan O- glycosidically linked to serine or threonine. This structure was further confirmed, by TLC analysis of the released glycans after reductive ß elimination (figure 3). The purified glycan shows the same $R_{Lactose}$ than the O-glycan present in the fetuin (14) examined under the same conditions. In figure 3, we can notice the presence at a trace level of an additional glycan with the same mobility that the tetrasaccharidic O-glycan present in the fetuin (24).

The association of GLC and MS allows the identification of the methylated sugars obtained by methanolysis of the methylated glycan. On

TABLE 1.

MONOSACCHARIDE COMPOSITION OF THE DBP AND OF ITS GLYCOPEPTIDE

	I						II		
	Native protein			Glycopeptide fraction isolated from Gc1*					
Monosaccharides	Gc2	Gc1					Previous Studies	Mixture Gc2 + Gc1 %	Gc2 %
		M/M	relative amount (%)	R/M	M/M	R/M			
Galactose	-	0.46	0.14	1.11	0.28	0.95	Hexose	2.0	2.4
N-acetyl galactosamine	-	0.41	0.17	1	0.30	1	Hexosamine	2.0	0.7
N-acetyl neuraminidic acid	-	0.50	0.30	1.20	0.31	1.04	Fucose	0.2	0.2
							Sialic acid	0	0

* The values presented are an average of three determinations on three different DBP preparations corresponding to the Gc 1S and Gc 2 proteins respectively.

M/M ratio = moles of hexoses to mole of DBP

R/M ratio = mole of N-acetyl glycosamine to mole of DBP

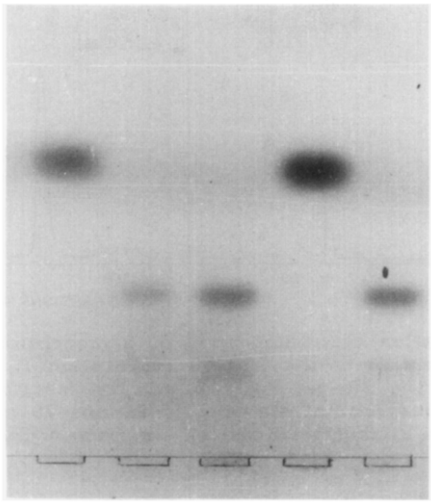

Figure 3 : Thin layer chromatography (5 to 10 µl applied volume) of glycans obtained from fetuin and DBP (Gc 1S proteins) after alkaline borohydride degradation. Pattern obtained after orcinol staining.
Lanes 1 and 4 = lactose as standard (5 µg)
Lane 3 = glycans released from DBP (5µg)
Lanes 2 and 5 = mixture of O-glycans released from fetuin (5 µg).

figure 4, the presence of fragments m/e 71 (K_2), 88 and 116 (H_1), 233 (E_1), 247 (A_1) associated with the absence of fragment B_1 m/e 176 or 204 and the low ratio J_1/K_2 (m/e 75/m/e 71) are characteristic of methyl 2,4,6-tri-O-methyl-3-mono acetyl galactose (17). On the basis of the methanolysis results and of these GLC/MS data, the structure for the carbohydrate unit of Gc 1S proteins can be given.

DISCUSSION

Two original results were obtained from this investigation :

1 - the absence of an N-glycan structure on the different genetic forms of the serum DBP.

2 - the low amount of sugar (about 1 %) bound to the DBP.

These data differ significantly from those previously presented on this molecule (21, 22, 25) ; the reasons of these discrepancies are mostly technical ones. We know from our experience, that no procedure used to purify (4, 19, 20) the protein can remove the serum glyco proteins present as contaminants. Only affinity chromatography using purified specific IgG was able to give a highly purified material. Another reason is the methods used to study the glycopeptide and the sugar composition, GLC being more sensitive and specific than colorimetric procedures (21). We

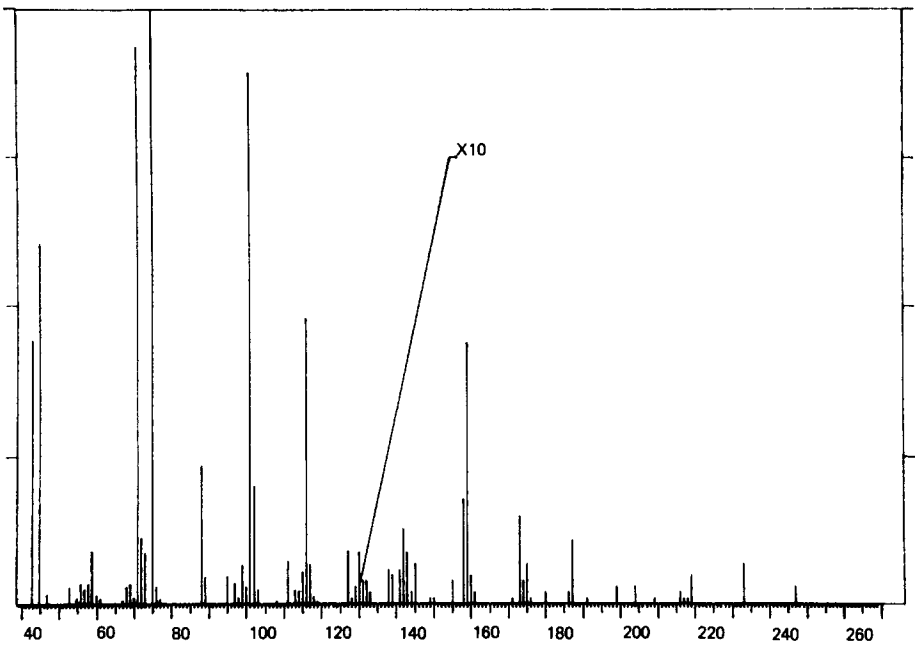

Figure 4 : Mass spectra of methyl glycoside of 0-acetyl-0-methyl substituted hexoses from the glycan liberated by ß elimination from the vitamin D-binding-protein

also found that some N-glycopeptides can be present in several pronase batches from different manufacturers and this accounts for the previous results published on the carbohydrate moiety of the vitamin D-Binding protein (6, 22, 23). The data presented demonstrate the absence of an N-glycan chain on the DBP. We were able to confirm these observations by hydrazinolysis on the native protein and on the purified glycopeptide (data not shown). Moreover, we verified that the DBP was not bound on a Sepharose Con A column (26) which is in agreement with the result published by Nilson and Bog-Hansen (27). The analysis of the saccharides shows the existence of only one residue of Neu. Ac. on the glycopeptide obtained from the Gc 1S proteins. In addition, we know that this residue, removed by neuraminidase treatment, is located on the Gc 1a isoprotein (28). In a previous study (29) we demonstrated that the mean level of the Gc 1a protein was about 40 % of the total serum DBP produced by the Gc 1 allele. We can then conclude that the studied glycan is only present on the Gc 1a protein.

From the sugar composition, the ß elimination and the TLC analysis and from the results obtained after the GLC/MS examination, we can conclude that Gc 1a isoprotein is a glycoprotein with an 0-glycosidically linked glycan whose structure is : Neu Ac α (2 → 3)Gal ß (1→3)GalNac α (1 → 0)Ser (or Thr). This very simple structure is identical with

that of the trisaccharide present on fetuin. The tetrasaccharide also identified on the Gc 1S proteins might be explained by the presence of a second N-acetyl neuraminidic acid residue attached to N-acetyl galactosamine in position (2 → 6). This additional glycan is probably present on a third Gc 1S isoprotein that we recently identified (29). We can deduce that Serine or threonine present on the Gc 1 protein (6) and to which O-glycans are usually bound (30), constitute an important site on the DBP during its evolution and for the metabolism of this protein.

The DBP is a rare example of a serum protein poorly glycosylated, besides this O-glycan is only present on some genetic forms of the molecule. From this investigation, we can suppose that the turn-over of the Gc 1a, 1c and 2 proteins is different (31). This observation reinforces our hypothesis of a different biological activity of the DBP in relation to its genetic isoforms (9).

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REFERENCES

1 - HIRSCHFELD J., JONSSON B. and RASMUSON H. (1960) Nature 185, 931-932

2 - DAIGER S., SCHANFIELD M.S. and CAVALLI-SFORZA L.L. (1975) Proc. Natl. Acad. Sci. USA 72, 2076-2080

3 - BOUILLON R., VAN BAELEN H., ROMBAUTS W. and De MOOR P. (1976) Eur. J. Biochem. 66, 285-291

4 - HADDAD J.G., Jr. and WALGATE J. (1976) J. Biol. Chem. 251, 4803-4809

5 - CONSTANS J. and VIAU M. (1977) Science 198, 1070-1071

6 - SVASTI J., KUROSKY A., BENNETT A. and BOWMAN B.H. (1979) Biochem. 18, 1611-1617

7 - COUÉ M., CONSTANS J., VIAU M. and OLOMUCKI A. (1983) Biochem. Biophys. Acta under publication

8 - LAEMMLI U.K. (1970) Nature (London) 227, 680-682

9 - CONSTANS J., VIAU M. and BOUISSOU C. (1980) F.E.B.S. Lett. 111, 107-111

10 - BRADFORD M.M. (1976) Anal. Biochem. 72, 248-254

11 - SPIRO R.G. and BHOYROO V.D. (1974) J. Biol. Chem. 249, 5704-5717

12 - ZANETTA J.P., BRECKEURIDGE W.C. and VINCENDON G. (1972) J. Chromatogr. 69, 291-304

13 - WINZLER R.J., HARRIS E.D., PEKAS E.D., JOHNSON C.A. and WEBER P. (1967) Biochemistry 6, 2195-2202

14 - JUDSON P.A., ANSTEE D.J. and CLAMP J.R. (1982) Biochem. J. 205, 81-90

15 - HUMBEL R. and COLLART M. (1975) Clin. Chim. Acta 60, 143-145

16 - HAKOMORI S. (1964) J. Biochem. (Tokyo) 55, 205-208

17 - BJORNDAL H., HELLERQUIST C.G., LINDBERG B. and SVENSSON S. (1970) Angew. Chem. 82, 643-651

18 - FOURNET B., STRECKER G., LEROY Y. and MONTREUIL J. (1981) Anal. Biochem. 116, 489-502

19 - IMAWARI M., KIDA K. and GOODMAN De WITT S. (1976) J. Clin. Invest. 58, 514-523

20 - CHAPUIS-CELLIER C., GIANAZZA E. and ARNAUD Ph. (1982)) Biochem. Biophys. Acta 709, 353-357

21 - SCHULTZE H.E., BIEL H., HAUPT H. and HEIDE K. (1962) Naturwissenschfatten 48, 108-114

22 - CLEVE H., PRUNIER J.H. and BEARN A.G. (1963) J. Exp.. Med. 118, 711-726

23 - BOWMAN B.H. (1969) Biochem. 11, 4327-4335

24 - NILSSON B., NORDEN N.E. and SVENSSON S. (1979) J. Biol. Chem. 254, 4545-4553

25 - COPPENHAVEN D.H. and BOWMAN B.H. (1981) Am. J. Hum. Genet. 33, 6, 39 A Abstract n 121

26 - BAYARD B. and KERCKAERT J.P. (1981) Eur. J. Biochem. 113, 405-414

27 - NILSON M. and BOG-HANSEN T.C. (1979) in Protides of Biological Flinds Proceedings ed. by Peeters H. Pergamon Press. Oxford and New York 27, 599-602

28 - SVASTI J. and BOWMAN B.H. (1978) J. Biol. Chem. 253, 4188-4194

29 - CONSTANS J., ARLET Ph., VIAU M., and BOUISSOU C. (1983) Clin. Chim. Acta 130, 219-230

30 - CLAMP J.R., BHATTI T. and CHAMBERS R.E. (1972) in "Glycoproteins" (Gottschalk A. ed.) part A pp. 300-319 Elsevier-Amsterdam

31 - MORELL A.G., IRVINE R.A., STERNLIEB I., SCHEINBERG I.H. and ASHWELL G. (1968) J. Biol. Chem. 243, 155-159