Structure determination of the major asparagine-linked sugar chain of human factor VIII–von Willebrand factor

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N-glycosidically-linked glycans released by hydrazinolysis of human factor VIII/von Willebrand factor (FVIII/vWf) were separated by high-voltage electrophoresis. Five fractions were obtained, one of them representing 60% of the total amount of the *N*-glycosidically-linked glycans of FVIII/vWf. On the basis of the carbohydrate composition, methylation analysis and 500 MHz ^{1}H-NMR spectroscopy, we describe the primary structure of this major glycan which is of the monosialylated and monofucosylated biantennary *N*-acetyllactosaminic type.

Factor VIII *von Willebrand factor* N-*Glycosididic glycan*

1. INTRODUCTION

Human factor VIII–von Willebrand factor (F VIII–vWf) is a complex containing a coagulant activity (F VIII-C) and an activity required for normal platelet hemostatic function which is carried by a factor VIII-related antigen (F VIII R-Ag) and measured by its ability to agglutinate normal washed human platelets in the presence of ristocetin (F VIII R-RCo). Recent studies suggest that the carbohydrate moiety of F VIII–vWf is important for the binding of the latter to platelets in the presence of ristocetin and for the in vivo survival of the protein [1–7]. We have demonstrated that the carbohydrate moiety of F VIII–vWf contains *N*- and *O*-glycosidically-linked glycans. After hydrazinolysis, a major glycan of the *N*-acetyllactosaminic type has been characterized by thin-layer chromatography [8]. Here, on the basis of the carbohydrate composition and the results of methylation analysis, mass spectrometry and 500 MHz ^{1}H-NMR spectroscopy, we describe the primary structure of this major glycan.

2. MATERIALS AND METHODS

Human F VIII–vWf was purified from therapeutic concentrates as in [9]. A lyophilized and delipidated F VIII preparation (10 mg) was hydrazinolyzed as in [10] and the liberated, *N*-deacetylated glycans were *N*-reacetylated, first with [^{14}C]acetic anhydride (CEA, 5–10 mCi/mM) and then with non-labelled acetic anhydride [11]. The liberated glycans were separated by high-voltage electrophoresis on Whatman 3 MM paper in the buffer: pyridine–acetic acid–water (18:6:2320, by vol.; pH 5.4) [12], at 75 V/cm. The radioactive spots were cut and eluted with water. Following standards were used: desialylated (A), monosialylated (B) and disialylated (C) glycans of the *N*-acetyllactosaminic type prepared from human serum transferrin [13] by hydrazinolysis.

Molar ratios of neutral monosaccharides, *N*-acetylhexosamines and *N*-acetylneuraminic acid were determined after methanolysis and trifluoroacetylation by gas–liquid chromatography [14]. Permethylation of oligosaccharides (200 μg) was

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carried out as in [15]. Methylglycosides resulting from methanolysis of permethylated oligosaccharides were identified by gas–liquid chromatography and mass spectrometry as in [16].

For NMR analysis the oligosaccharide fraction 3 (200 μg) was repeatedly exchanged in D_2O (99.96 atom% D, Aldrich), after adjustment of the pH of the solution to 7. The 500 MHz ^{1}H-NMR spectrum was recorded on a Bruker WM-500 spectrometer F (SON-facility, Nijmegen University), operating in the Fourier transform mode at a probe temperature of 27°C (for further details, see [17]). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulphonate (indirectly to acetone in D_2O: δ = 2.225 ppm).

3. RESULTS AND DISCUSSION

3.1. *Separation of* N-*glycosidically-linked glycans by high-voltage electrophoresis*

By high-voltage electrophoresis of the *N*-reacetylated [^{14}C]glycans released from F VIII–vWf by hydrazinolysis, 5 fractions were obtained (fig.1), which are constituted of neutral (fraction 1), monosialylated (fractions 2 and 3) and disialylated (fractions 4 and 5) oligosaccharides. These fractions appear to be heterogeneous by paper electrophoresis and/or by thin-layer chromatography, except the major fraction 3.

3.2. *Carbohydrate composition of the* N-*glycosidic carbohydrate chains*

The molar carbohydrate composition of the 5 fractions is given in table 1. The sugar moiety of fraction 3 represents 60% of the total amount of the *N*-glycosidically-linked oligosaccharides of F VIII–vWf. The structural investigations were carried out on this major and homogeneous fraction.

3.3. *Primary structure determination*

The primary structure of the major *N*-glycosidic glycan of F VIII–vWf was established by

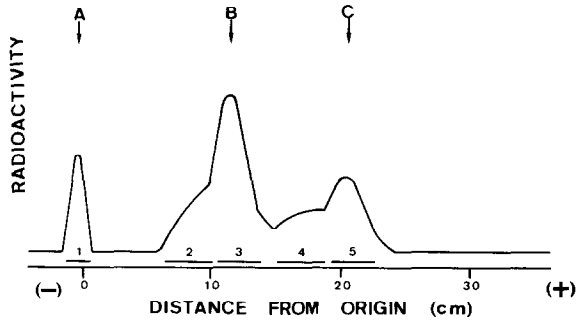

Fig.1. Radioelectropherogram of the oligosaccharides released from F VIII–vWf by hydrazinolysis (for details of the experimental procedure, see section 2).

Table 1

Molar carbohydrate composition[a] of the oligosaccharides released from F VIII–vWf by hydrazinolysis and separated by high-voltage electrophoresis

Monosaccharide	Combined oligosaccharides	Fraction				
		1	2	3	4	5
Man	3.0	3.0	3.0	3.0	3.0	3.0
Gal	2.6	3.0	3.4	2.0	3.4	3.0
Fuc	1.6	2.0	2.0	1.2	traces	1.2
GlcNAc	4.3	4.0	4.8	4.2	4.0	4.8
GalNAc	–	–	–	–	–	–
NeuAc	1.2	–	0.85	1.0	1.6	2.0
Sugar content for each fraction (%)	100	10	8	60	6	16

[a] Calculated on the basis of 3 mannose residues/molecule

500 MHz ^{1}H-NMR spectroscopy, in conjunction with permethylation analysis. The results of the latter are compiled in table 2.

The 500 MHz ^{1}H-NMR spectrum of F VIII–vWf fraction 3 shows the characteristic features of an oligosaccharide of the *N*-acetyllactosaminic type, derived from a carbohydrate unit *N*-glycosidically linked to asparagine of a glycoprotein. The presence of the usual mannotriose (4-3-4′) branching core is clearly deducible from the occurrence of the Man H-1 and H-2 signals. For Man-4, they are found at δ = 5.133 and 4.195 ppm; for Man-3, at $\delta \simeq$ 4.78 and 4.253 ppm; and for Man-4′, at δ = 4.926 and $\simeq$ 4.11 ppm, respectively. From these chemical shifts it can be concluded that a diantennary type of branching is concerned [17].

Table 2

Molar ratios[a] of monosaccharide methyl ethers present in the methanolysate of the permethylated major *N*-glycan isolated from F VIII–vWf

Monosaccharide methyl ethers	Fraction 3
(2,3,4)-Me_3-Fuc	0.8
(2,3,4,6)-Me_4-Gal	1.0
(2,3,4)-Me_3-Gal	1.2
(3,4,6)-Me_3-Man	2.0
(2,4)-Me_2-Man	1.2
(3,6)-Me_2-GlcNAc(Me)	2.6
(3)-Me_1-GlcNAc(Me)	0.8
(4,7,8,9)-Me_4-NeuAc(Me)	0.9

[a] The molar ratios were calculated on the basis of 2 residues of (3,4,6)-Me_3-Man

As to the extension of the core at the peripheral side, one of the two *N*-acetyllactosamine units that are $\beta(1\rightarrow2)$-linked to Man-4 and -4′, bears a NeuAc residue in $\alpha(2\rightarrow6)$-linkage to Gal. Evidence stems from the chemical shifts (δH-3ax = 1.718 ppm; δH-3eq = 2.669 ppm; δNAc = 2.030 ppm) and the relative intensities of the NeuAc structural-reporter groups (cf. [17]), as well as from the presence of two distinct sets of reporter-group signals for the *N*-acetyllactosamine units. The sialylated branch possesses the anomeric doublets of Gal and GlcNAc at δ = 4.445 and 4.606 ppm, respectively. The asialo counterpart shows H-1 doublets for Gal and GlcNAc at δ = 4.469 and 4.581 ppm, respectively. Localization of NeuAc in a certain branch could be readily achieved on the basis of the chemical shifts of the α-Man H-1 signals [17]. The chemical shift value for H-1 of Man-4 (δ = 5.133 ppm) points unambiguously to the presence of the $\alpha(2\rightarrow6)$-linked NeuAc in the upper (i.e., 4-5-6) branch, whereas δH-1 for Man-4′ (4.926 ppm) confirms the asialo character of the lower branch. This location of the NeuAc residue in the upper branch is corroborated by the chemical shifts of the *N*-acetyl signals of GlcNAc-5 (δ = 2.069 ppm, pointing to $(2\rightarrow6)$-sialylation of the 5-6 branch) and GlcNAc-5′ (δ = 2.048 ppm, indicating that Gal-6′ is the terminal residue in the lower branch) [17].

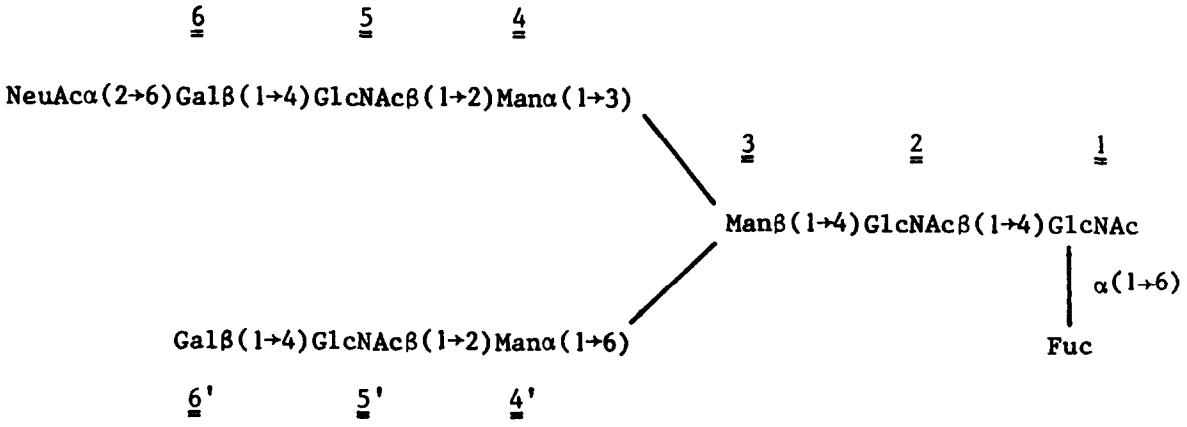

Fig.2. Primary structure of the major *N*-glycosidic glycan of F VIII–vWf.

Regarding the extension of the core at the reducing end, the Man-3 is $\beta(1\rightarrow4)$-linked to an *N,N'*-diacetylchitobiose unit which bears a Fuc residue in $\alpha(1\rightarrow6)$-linkage to GlcNAc-1. The presence of the GlcNAc $\beta(1\rightarrow4)$ [Fuc $\alpha(1\rightarrow6)$] GlcNAc structural element comes to expression in the occurrence of doublets for the anomeric protons of GlcNAc-2 (δH-1 $\simeq$ 4.68 ppm) and Fuc (δH-1 $\simeq$ 4.87 ppm). The H-5 and CH_3 signals of Fuc, at $\delta \simeq$ 4.11 and 1.208 ppm, respectively, point to its $\alpha(1\rightarrow6)$-linkage to GlcNAc-1. In addition to the chemical shift for H-1 of GlcNAc-2, the position of its *N*-acetyl signal is decisive for the presence of Fuc in $\alpha(1\rightarrow6)$-linkage to GlcNAc-1 [17]. The latter signal is, for the larger part ($\simeq$ 90%), observed at δ = 2.096 ppm, whereas only a minor part is found at δ = 2.080 ppm, indicating that most of the chains bear such a Fuc residue.

Based on the aforementioned NMR results, and those of the methylation analysis (table 2), the primary structure of F VIII–vWf fraction 3, representing the major *N*-glycosidic glycan of this glycoprotein, is established to be as depicted in fig.2. The structure is identical to that of human lactotransferrin glycopeptide D [18], and also to that of glycopeptide fraction B, derived from secretory immunoglobulins A from human milk [19]. Comparison of the 500 MHz ^{1}H-NMR data for the F VIII–vWf fraction 3 described here with those acquired at 360 MHz for the aforementioned glycopeptides reveals that the reducing character of the oligosaccharide released by hydrazinolysis and *N*-reacetylation does not markedly influence the chemical shifts of the structural-reporter groups of Fuc $\alpha(1\rightarrow6)$-linked to GlcNAc-1 nor those of GlcNAc-2, $\beta(1\rightarrow4)$-linked to GlcNAc-1. Obviously, the reporter-groups of GlcNAc-1 differ considerably when comparing oligosaccharide and glycopeptides (e.g., the NAc resonance of GlcNAc-1 is found here at δ = 2.039 ppm). Nevertheless, it should be emphasized that this study shows again the general applicability of the structural-reporter-group concept for the identification of glycopeptides, oligosaccharides and oligosaccharide-alditols [17,20].

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