Primary structure of a new tetraantennary glycan of the *N*-acetyllactosaminic type isolated from human factor VIII/von Willebrand factor

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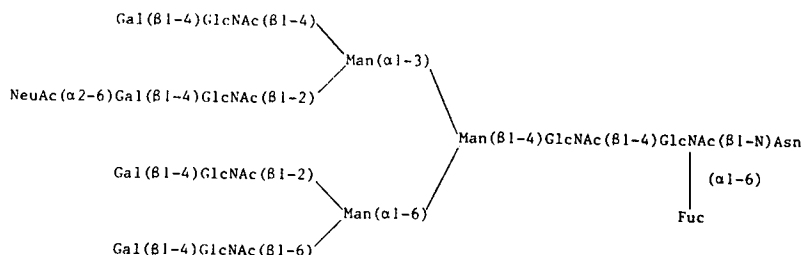
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N-Glycosidically linked glycopeptides released by mild alkaline treatment of human factor VIII/von Willebrand factor (FVIII/vWF) were fractionated by serial affinity chromatography on columns of Sepharose linked to concanavalin A (ConA) and *Lens culinaris* agglutinin (LCA). The fraction which is not retained on ConA-Sepharose, but eluted from LCA-Sepharose contains a pure minor glycopeptide which was structurally analysed. Based on the results of methylation analysis and 500-MHz ^{1}H-NMR spectroscopy, the following structure is proposed:

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Gal(β1-4)GlcNAc(β1-4)
                      \
                       Man(α1-3)
NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)      \
                                       Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn
Gal(β1-4)GlcNAc(β1-2)                 /                    |
                      \             /                    (α1-6)
                       Man(α1-6)                            |
Gal(β1-4)GlcNAc(β1-6)                                      Fuc
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Human plasma factor VIII/von Willebrand factor (FVIII/vWF), which plays an important role in hemostasis and coagulation, is a complex consisting of 90% von Willebrand factor (vWF) [1−3]. The physicochemical properties of this complex which have so far been studied are thus essentially ⟨tho⟩se of vWF. Human plasma vWF is a glycoprotein ⟨co⟩ntaining 15% carbohydrate and possessing *O*- and *N*-glycosidically linked glycans with a high degree of microheterogeneity [4−6]. Up to now, only the primary structure of the major biantennary asparagine-linked glycan has been described [7]. In the present study, *N*-glycosyl-peptides and oligosaccharide-alditols released by alkaline reductive treatment [8] have been fractionated using serial affinity chromatography on ConA-Sepharose and LCA-Sepharose. By this way, a minor glycopeptide not bound to ConA but retained by and eluted from LCA lectin has been purified. By associating data obtained by permethylation analysis and 500-MHz ^{1}H-NMR spectroscopy, we propose a monosialylated,

monofucosylated tetraantennary structure of the *N*-acetyl-lactosaminic type which has not previously been described in human glycoproteins.

MATERIALS AND METHODS

Reagents

Human FVIII/vWF was purified from therapeutic concentrates as in [9]. ConA-Sepharose was obtained from IBF (Villeneuve la Garenne, France). LCA purified by affinity chromatography [10] was insolubilized on Sepharose 4B (Pharmacia, Uppsala) according to [11] at a concentration of 2 mg lectin/ml gel. Methyl-α-D-glucopyranoside was from Koch-Light (Conbrook, England), Bio-Gel P-2 and P-4 (200−400 mesh) from Bio-Rad (Richmond, CA), D$_2$O from Aldrich (Milwaukee, WI) and anhydrous hydrazine from Pierce (Rockford, IL).

Preparation of N-glycosylpeptides and oligosaccharide-alditols

Human FVIII/vWF was submitted to mild alkaline reductive treatment according to [8] and oligosaccharide-alditols and *N*-glycosylpeptides were fractionated by affinity chromatography on lectin-Sepharose columns according to Debray et al. [12] and as described in Fig. 1. Hydrazinolysis,

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Abbreviations. ConA, concanavalin A; LCA, *Lens culinaris* agglutinin; NeuAc, *N*-acetyl-neuraminic acid; Gal, D-galactose; Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; Fuc, L-fucose; Asn, L-asparagine; FVIII/vWF, factor VIII/von Willebrand factor; MeGlc, methyl α-glucoside.

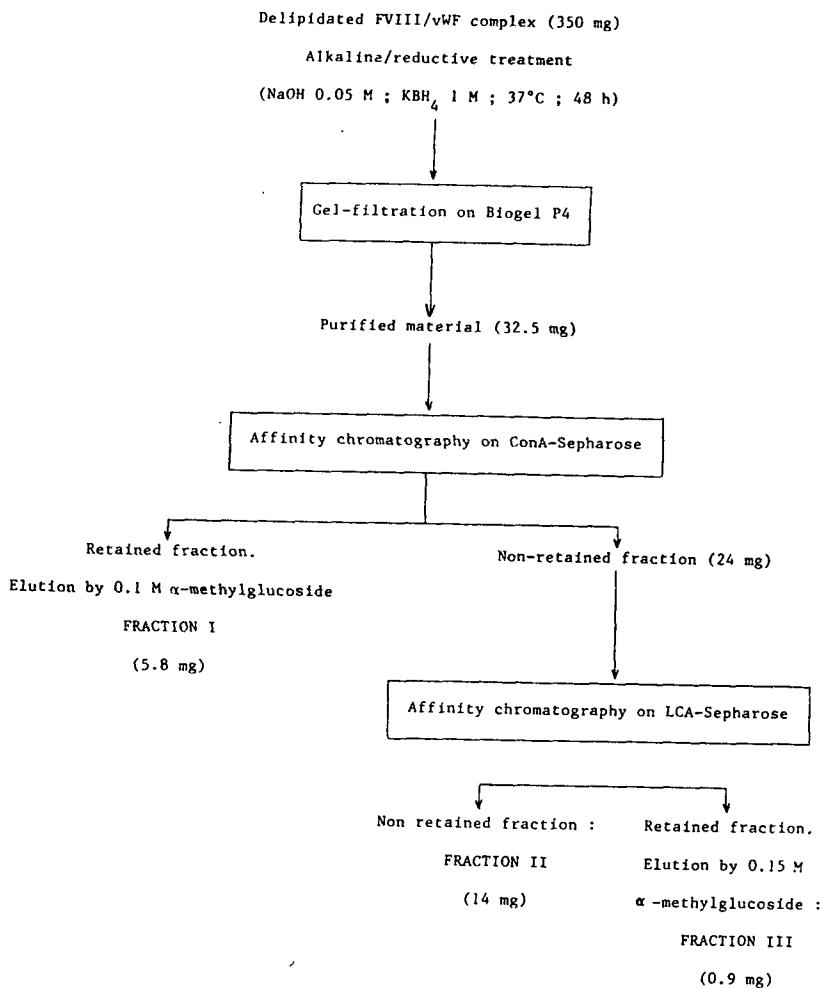Delipidated FVIII/vWF complex (350 mg)

Alkaline/reductive treatment

(NaOH 0.05 M ; KBH$_4$ 1 M ; 37°C ; 48 h)

Gel-filtration on Biogel P4

Purified material (32.5 mg)

Affinity chromatography on ConA-Sepharose

Retained fraction.

Elution by 0.1 M α-methylglucoside

FRACTION I

(5.8 mg)

Non-retained fraction (24 mg)

Affinity chromatography on LCA-Sepharose

Non retained fraction :

FRACTION II

(14 mg)

Retained fraction.

Elution by 0.15 M

α -methylglucoside :

FRACTION III

(0.9 mg)

Fig. 1. *Gel filtration and serial lectin fractionation of a mixture of* N-glycosylpeptides and O-oligosaccharide-alditols *obtained after alkaline/ reductive treatment of human factor VIII/von Willebrand factor*

N-reacetylation and reduction of glycan was realized according to [13].

Analytical methods

The carbohydrate content and the molar ratio of constituting monosaccharides of glycopeptide fractions were determined by gas-liquid chromatography after methanolysis and trifluoroacetylation [14]. Exhaustive micromethylation was carried out as in [15]. The methylated methylglycosides obtained by methanolysis of permethylated oligosaccharides were peracetylated and identified by gas-liquid chromatography coupled to mass fragmentometry (mass spectrometer Ribermag R10-10 coupled to the data system Sydar 121) as described in [16]. For ^{1}H-NMR analysis, the sample was repeatedly treated with D$_2$O at pD 6−7 at room temperature with intermediate lyophilization. 500-MHz ^{1}H-NMR spectroscopy was performed using a Bruker WM-500 spectrometer (SON-hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating in the pulsed Fourier-transform mode at a probe temperature of 27°C [17]. Chemical shifts (δ) are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm in D$_2$O at 27°C).

RESULTS

Glycopeptide fractionation

The mixture of *N*-glycosylpeptides and *O*-oligosaccharide-alditols obtained after alkaline/reductive treatment of human FVIII/vWF was fractionated by gel filtration and serial lectin fractionation according to Fig. 1. Fraction I retained by and eluted from the ConA-Sepharose column contained mostly the sialylated biantennary glycopeptides of the *N*-acetyllactosamine type previously described [7]. Fraction II, which was retained neither on immobilized ConA nor on LCA, was constituted mostly of oligosaccharide-alditols of the *O*-glycosidic type which will be described elsewhere. Fraction III, constituted of an *N*-glycosylpeptide, was eluted from the LCA-Sepharose column with 150 mM methyl α-glucoside, and desalted on a Bio-Gel P-2 column. The hydrazinolysis/ *N*-reacetylation/reduction of fraction III led to the formation of the free oligosaccharide-alditol with the following molar sugar composition: Man 3, Gal 3.7, GlcNAc 4.7, NeuAc 0.8, Fuc 0.9.

Methylation analysis

Methylation analysis (Table 1) demonstrated that all mannose residues were twice substituted suggesting a

Table 1. *Molar ratios of monosaccharide methyl ethers present in the methanolysate of the reduced and permethylated glycan from human factor VIII/von Willebrand factor*

Values are calculated on the basis of one residue of (2,4)-Me$_2$-Man

Monosaccharides methyl ethers	Molar ratio
(2,4)-Me$_2$-Man	1
(3,4)-Me$_2$-Man	0.85
(3,6)-Me$_2$-Man	0.95
(2,3,4)-Me$_3$-Gal	0.90
(2,3,4,6)-Me$_4$-Gal	2.75
(3,6)-Me$_2$-Glc(Me)Ac	3.45
(3)-Me$_1$-GlcN(Me)Ac	0.40
(2,3,4)-Me$_3$-Fuc	0.95
(4,7,8,9)-Me$_4$-NeuAc	0.80

Table 2. *^{1}H chemical shifts of structural reporter groups of constituent monosaccharides for the monosialylated oligosaccharide-alditol obtained from human factor VIII/von Willebrand factor*

For numbering of monosaccharides, see Fig. 2. Chemical shifts are given for neutral solutions at 27°C, downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in D$_2$O. n.d. means not detected, hidden under D$_2$O signal

Reporter group	Residue	Chemical shift
		ppm
H-1	GlcNAc-2	n.d.
	Man-3	n.d.
	Man-4	5.141
	Man-4'	4.850
	GlcNAc-5	4.609
	GlcNAc-5'	4.609
	GlcNAc-7	4.550
	GlcNAc-7'	4.550
	Gal-6	4.444
	Gal-6'	4.468
	Gal-8	4.468
	Gal-8'	4.468
	Fuc	1.896
H-2	GlcNAc-ol	4.22
	Man-3	4.208
	Man-4	4.208
	Man-4'	4.097
H-3ax	NeuAc	1.716
H-3eq	NeuAc	2.672
NAc	GlcNAc-ol	2.056
	GlcNAc-2	2.086
	GlcNAc-5	2.073
	GlcNAc-5'	2.045
	GlcNAc-7	2.073
	GlcNAc-7'	2.042
	NeuAc	2.031
CH$_3$	Fuc	1.225

tetraantennary structure and that three galactose residues were in terminal position, the fourth residue being substituted in the C-6 position as indicated by the presence of one 2,3,4-tri-O-methyl-galactose residue. The presence of 3-monomethyl N-acetylglucosaminitol may suggest the C-6 fucosylation of 1-N-acetylglucosamine residue. This structural feature is in line with the retardation of fraction III on immobilized

LCA [10, 12, 18]. High-resolution ^{1}H-NMR only was able to determine precisely and unambiguously the primary structure of such a complex structure.

NMR analysis

500-MHz ^{1}H-NMR spectra of the oligosaccharide-alditol were recorded in neutral D$_2$O solutions at a probe temperature of 27°C. Relevant NMR parameters are listed in Table 2.

The presence of a tetraantennary type of structure can be deduced from the chemical shift values of the H-2 atoms of the three mannose residues (for Man-3 and Man-4 δ = 4.208 ppm, and for Man-4' δ = 4.097 ppm). In line with this interpretation, the chemical shift for H-1 of Man-4' is δ = 4.850 ppm [17]. A low-intensity signal at δ = 4.257 ppm, typical for H-2 of Man-3 in a biantennary structure, shows that some heterogeneity exists. The spectrum indicates the occurrence of one N-acetylneuraminic acid residue as is shown by the intensity of its N-acetyl signal. By the set of chemical shift values of its structural reporter groups (H-3ax δ = 1.716 ppm, H-3eq δ = 2.672 ppm and N-acetyl protons δ = 2.031 ppm), the linkage of N-acetylneuraminic acid is characterized as (2→6)Gal [7]. The chemical shift value of H-1 on Man-4 being δ = 5.141 ppm, demonstrate that N-acetylneuraminic acid is attached to Gal-6. The presence of a low signal at δ = 5.119 ppm indicates that a small amount of asialo Gal-6 occurs. The N-acetyl signals of GlcNAc-5', 7 and 7' resonating at δ = 2.045 ppm, δ = 2.073 ppm and $\overline{\delta}$ = 2.042 ppm respectively, correspond to asialoantennae. The structural reporter group signals for fucose (H-1 δ = 1.896 ppm and CH$_3$ δ = 1.225 ppm) are typical for fucose α-1,6-linked to N-acetylglucosaminitol [19]. The N-acetyl signals of N-acetylglucosaminitol and GlcNAc-2 are at δ = 2.056 ppm and at δ = 2.086 ppm respectively. In summary, the main component of this oligosaccharide-alditol fraction is a tetraantennary structure having N-acetylneuraminic acid α-2,6-linked to Gal-6 and fucose α-1,6-linked to N-acetylglucosaminitol.

DISCUSSION

Based on the results of NMR and methylation analysis, the primary structure of a glycopeptide released from human FVIII/vWF by alkaline treatment may be established as depicted in Fig. 2. From a technical point of view, it must be mentioned that the alkaline treatment of glycoproteins as proposed by [8] may replace the pronase digestion in obtaining N-glycosylpeptides [20]. Since the LCA lectin used in this study recognized glycopeptides (but not oligosaccharides) with a fucose residue linked at the C-6 position of GlcNAc-1, the β-elimination approach together with the serial affinity chromatography on immobilized lectin as proposed by [21] represents a powerful method for the separation of relatively pure families of glycans. Concerning the specificity of LCA as established by [10, 12, 18], it was demonstrated that only the triantennary glycopeptide with the 4'-mannose residue substituted in C-2 and C-6 positions and possessing a fucose residue α-1,6-linked to the N-acetylglucosamine conjugated to asparagine were recognized by the lectin. However, triantennary structures with the 4-mannose residue substituted in C-2 and C-4 position do not interact. In this connection, it was surprising to note that the tetraantennary glycopeptide described in this paper interacts with the lectin. This fact may be correlated with the presence of a single sialic
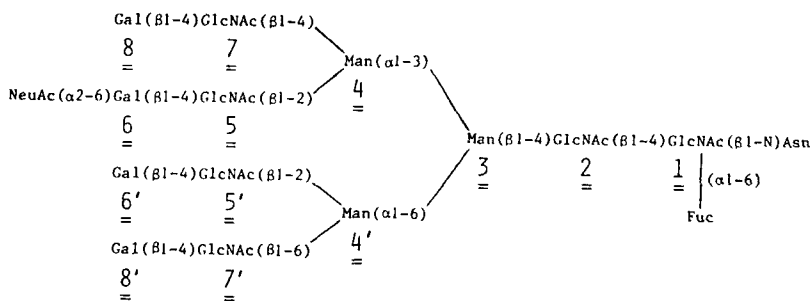
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          Gal(β1-4)GlcNAc(β1-4)
           8           7
           =           =          Man(α1-3)
  NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)        4
           6           5          =
           =           =                            Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn
          Gal(β1-4)GlcNAc(β1-2)                      3          2          1
           6'          5'                            =          =          = |(α1-6)
           =           =          Man(α1-6)                                  Fuc
          Gal(β1-4)GlcNAc(β1-6)     4'
           8'          7'          =
           =           =
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Fig. 2. *The primary structure of a glycopeptide released from factor VIII/von Willebrand factor by alkaline treatment, based on the results of* ^1H-*NMR and methylation analysis*

acid residue, giving the glycopeptide a conformation allowing the lectin to recognize its specific determinant. The glycan structure presented here has not yet been characterized in any biological fluid [22]. It is interesting to note that, like the previously described biantennary glycan of FVIII/vWF [7], the proposed structure possesses galactose residues in non-reducing terminal position and only one sialic acid and fucose residue. Such externally exposed galactose residues may play an important role in the FVIII/vWF binding to platelets [23]. However, the role of the carbohydrate moiety in its multimeric repartition and biological function, studied after enzymatic removal of carbohydrate by either exoglycosidases or endo-*N*-acetyl-*β*-D-glucosaminidase, remains controversial [23—25]. So, one must be cautious in interpreting the results obtained by enzymatic modifications of the FVIII/vWF complex glycans. Knowledge of the primary structure of FVIII/vWF carbohydrate chains seems essential to elucidate the relationship between structure and biological activities of this molecule.

REFERENCES

1. Marchesi, S. L., Shuman, N. R. & Gralnick, H. R. (1972) *J. Clin. Invest. 51*, 2151—2161.
2. Shapiro, G. A., Andersen, J. C., Pizzo, S. V. & McKee, P. A. (1973) *J. Clin. Invest. 52*, 2198—2210.
3. Legaz, M. E., Schmer, G., Counts, R. B. & Davie, E. W. (1973) *J. Biol. Chem. 248*, 3946—3955.
4. Sodetz, J. M., Paulson, J. C. & McKee, P. A. (1979) *J. Biol. Chem. 254*, 10754—10760.
5. Mazurier, C., Samor, B., Parquet-Gernez, A., Fournet, B., Goudemand, M. & Montreuil, J. (1980) *Protides Biol. Fluids Proc. Colloq. 28*, 229—302.
6. Samor, B., Mazurier, C., Goudemand, M., Debeire, P., Fournet, B. & Montreuil, J. (1982) *Thromb. Res. 25*, 81—89.
7. Debeire, P., Montreuil, J., Samor, B., Mazurier, C., Goudemand, M., Van Halbeek, H. & Vliegenthart, J. F. G. (1983) *FEBS Lett. 151*, 22—26.
8. Aminoff, D., Baig, M. M. & Gathmann, W. D. (1979) *J. Biol. Chem. 254*, 1788—1793.
9. Mazurier, C., Parquet-Gernez, A., Samor, B., Goudemand, M. & Montreuil, J. (1979) *C. R. Acad. Sci. Paris 288*, 1431—1434.
10. Debray, H., Decout, D., Strecker, G., Spik, G. & Montreuil, J. (1981) *Eur. J. Biochem. 117*, 41—55.
11. March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal Biochem. 60*, 149—152.
12. Debray, H. & Montreuil, J. (1981) in *Lectins — Biology, biochemistry, clinical biochemisry* (Bog-Hansen, T. C., ed.) pp. 221—230, De Gruyter, Berlin.
13. Michalski, J. C., Peter-Katalinic, J., Egge, H., Paz-Parente, J., Montreuil, J. & Strecker, G. (1984) *Carbohydr. Res. 134*, 177—189.
14. Zanetta, J. P., Breckenridge, W. C. & Vincendon, G. (1972) *J. Chromatogr. 69*, 291—304.
15. Paz-Parente, J., Leroy, Y. & Fournet, B. (1985) *Carbohydr. Res. 141*, 41—47.
16. Fournet, B., Strecker, G., Leroy, Y. & Montreuil, J. (1981) *Anal. Biochem. 116*, 489—502.
17. Vliegenthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209—374.
18. Kornfeld, K., Reitman, M. L. & Kornfeld, R. (1981) *J. Biol. Chem. 256*, 6633—6640.
19. Cahour, A., Debeire, P., Hartmann, L., Montreuil, J., Van Halbeek, H. & Vliegenthart, J. F. G. (1984) *FEBS Lett. 170*, 343—349.
20. Debray, H., Strecker, G. & Montreuil, J. (1984) *Biochem. Soc. Trans. 12*, 611—612.
21. Montreuil, J., Debray, H., Debeire, P. & Delannoy, P. (1983) *Falk Symp. 34*, 239—258.
22. Montreuil, J. (1980) *Adv. Carbohyd. Chem. Biochem. 37*, 157—223.
23. Goudemand, J., Mazurier, C., Samor, B., Bouquelet, S. & Montreuil, J. (1985) *Thromb. Haemost. 53*, 390—395.
24. Gralnick, H. R., Williams, S. B. & Rick, M. E. (1983) *Proc. Natl Acad. Sci. USA 80*, 2771—2774.
25. Federici, A. B., Elder, J. H., Demarco, L., Ruggeri, Z. M. & Zimmerman, T. S. (1984) *J. Clin. Invest. 74*, 2049—2055.