The Structures of the Carbohydrate Moieties of Bovine Blood Coagulation Factor IX (Christmas Factor)

OCCURRENCE OF PENTA- AND TETRASIALYL TRIANTENNARY SUGAR CHAINS IN THE ASPARAGINE-LINKED SUGAR CHAINS*

(Received for publication, November 15, 1982)

Tsuguo Mizuochi§, Takahiro Taniguchi, Kazuo Fujikawa¶, Koiti Taniai‖, and Akira Kobata‡

From the Department of Biochemistry, Kobe University School of Medicine, Chuo-ku, Kobe, Japan and the †Department of Biochemistry, University of Washington, Seattle, Washington 98195

Bovine blood coagulation factor IX (Christmas factor) contains four asparagine-linked sugar chains in one molecule. The sugar chains were quantitatively liberated as radioactive oligosaccharides from the polypeptide moiety by hydrazinolysis followed by N-acetylation and NaBH₄ reduction. The structures of these sugar chains were determined by sequential exoglycosidase digestion in combination with methylation analysis.

Bovine factor IX contained two unique penta- and tetrasialyl triantennary sugar chains with the structures shown below in addition to tetra-, tri-, and disialyl biantennary sugar chains of Siaα₂→3Galβ1→3(Siaα₂→6)GlcNAcβ1→2Manα1→6[Siaα₂→3Galβ1→3(Siaα₂→6)GlcNAcβ1 → 2Manα1 → 3]Manβ1 → 4GlcNAcβ1 → 4GlcNAc, Siaα₂ → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6[Siaα₂ → 3Galβ1→3(Siaα₂→6)GlcNAcβ1→2Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc, and Siaα₂ → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6[Siaα₂ → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3]Manβ1 → 4GlcNAcβ1 → 4GlcNAc and their partially desialized forms.

---

* This work has been supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, by research grants from the Yamanouchi Foundation for research on Metabolic Disorders, and from National Institutes of Health Grants HL-16919, GM-15731, and AM-7902. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Department of Biochemistry, The Institute of Medical Sciences, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108 Japan.

§ Recipient of a Grant-in-Aid for Encouragement of Young Scientists.

‖ Investigator of the Howard Hughes Medical Institute.
Blood coagulation factor IX (Christmas factor) is one of the plasma glycoproteins that requires vitamin K for its biosynthesis. It plays an important role in the intrinsic pathway of the blood coagulation process (1). Individuals with factor IX deficiency (Christmas disease or hemophilia B) show bleeding symptoms essentially identical with those of classic hemophilia or hemophilia A (2, 3). In the blood coagulation process, factor IX is converted to the active form factor IXa by the action of factor XIa in the presence of calcium ions, releasing an activation glycopeptide of 10,000 Da (4). Factor IXa then acts as the enzyme responsible for the proteolytic conversion of factor X to factor Xa in the presence of factor VIII, phospholipid, and calcium ions (1). Studies of the activation mechanism and amino acid sequence of bovine factor IX have shown that the protein contains four asparagine-linked sugar chains of which three contain up to as much as 75% of the total carbohydrate and are localized in the activation peptide of only 35 amino acid residues (4, 5). To elucidate the structures and physiological roles of the sugar moieties of blood coagulation factors, the structures of all sugar chains in bovine factor IX were investigated.

**EXPERIMENTAL PROCEDURES AND RESULTS**

Fig. 5. Proposed structures of the fully sialylated sugar chains of bovine blood coagulation factor IX.
DISCUSSION

Bovine factor IX is composed of a single polypeptide chain of 416 amino acid residues with \( M_r \approx 55,400 \). The protein contains as much as 15% carbohydrate which is linked to four asparagine residues (residues 158, 168, 173, and 261). Upon activation by factor Xa in the presence of calcium ions, a highly glycosylated peptide of 35 amino acid residues (residues 147–182), activation peptide, is released from the middle of the molecule to form the active factor IXa (4). Although the physiological role of sugar chains of factor IX has not been elucidated yet, it is obvious that this activation peptide contains three sialic acid-rich sugar chains at Asn-158, Asn-168, and Asn-173, as much as 75% of the total carbohydrate. Abundance of sialic acid residues in this region, as shown in the present study, may contribute to maintaining the conformation of the factor IX molecule by their negative charge, and their release with the activation peptide upon activation may cause a drastic change in the conformation to generate the active form factor IXa. Alternatively, it is conceivable that the carbohydrate moiety of the protein is almost quantitatively released under the conditions employed if it is linked to four asparagine residues (residues 158, 168, 173, and 261) (5). Upon activation by factor XIa in the presence of calcium ions, the active form factor IXa is generated and their release with the activation peptide upon activation by factor XIa in the presence of calcium ions, a highly glycosylated peptide of 35 amino acid residues (residues 147–182), activation peptide, is released from the middle of the molecule to form the active factor IXa (4).

Finding both N-acetyl- and N-glycolyneuraminic acid in the sugar chains of factor IX casts doubt on our previous speculation that the asparagine-linked sugar chain contains only N-acetylmuramic acid (15). At the initial stage of developing the hydrazinolysis technique, we noticed that the N-glycolyl group is removed more slowly than the N-acetyl group. However, we later found that the glycolyl group can mostly be removed by 10-h hydrazinolysis. In our recent experiment, we also found that the glycopeptide which contains amino acid residues 34 to 41 of the heavy chain of factor X contains both N-acetyl- and N-glycolyneuraminic acid. Therefore, our previous speculation must be withdrawn.

Acknowledgments—We would like to express our gratitude to Dr. E. W. Davie for his continuous interest in this study. Thanks are also due to I. Ueda for her expert secretarial assistance.

REFERENCES

1. Davie, E. W., and Hanahan, D. J. (1977) in The Plasma Proteins (Putnam, F. W., ed) Vol. 3, pp. 421–544, Academic Press, New York
2. Aggeler, P. W., White, S. G., Glendening, M. B., Page, E. W., Leake, T. B., and Bates, G. (1952) Proc. Soc. Exp. Biol. Med. 79, 692–694
3. Biggs, R., Douglas, A. S., Macfarlane, R. G., Dacie, J. V., Pitney, W. R., Merkey, C., and O’Brien, J. R. (1962) Br. Med. J. 2, 1378–1382
4. Fujikawa, K., Legaz, M. E., Kato, H., and Davie, E. W. (1974) Biochemistry 13, 4508–4516
5. Katayama, K., Ericsson, L. H., Enfield, D. L., Walsh, K. A., Neubrath, H., Davie, E. W., and Kobata, A. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4900–4904
6. Fujikawa, K., Thompson, A. R., Legas, M. E., Meyer, R. G., and Davie, E. W. (1973) Biochemistry 12, 4938–4945
7. Mizuochi, T., Yamashita, K., Fujikawa, K., Kisiel, W., and Kobata, A. (1979) J. Biol. Chem. 254, 6419–6425
8. Takasaki, S., Mizuochi, T., and Kobata, A. (1982) Methods Enzymol. 83, 263–298
9. Ogata, S., Muramatsu, T., and Kobata, A. (1975) J. Biochem. (Tokyo) 78, 687–697
10. Mizuochi, T., Fujii, J., Kisiel, W., and Kobata, A. (1981) J. Biochem. (Tokyo) 90, 1023–1031
11. Yamashita, K., Mizuochi, T., and Kobata, A. (1982) Methods Enzymol. 83, 105–126
12. Takasaki, S., and Kobata, A. (1978) Methods Enzymol. 50, 216–220
13. Yamashita, K., Ohkura, T., Yoshima, H., and Kobata, A. (1981) Biochem. Biophys. Res. Commun. 100, 228–232
14. Paulson, J. C., Prieels, J.-P., Glasgow, L. R., and Hill, R. L. (1978) J. Biol. Chem. 253, 5617–5624
15. Mizuochi, T., Yamashita, K., Fujikawa, K., Titani, K., and Kobata, A. (1980) J. Biol. Chem. 255, 5026–5031

\(^2\) T. Mizuochi, K. Fujikawa, K. Titani, and A. Kobata, manuscript in preparation.
The specific activity of the radioactive oligosaccharides was calculated from the radioactivity incorporated into a definite amount of 6-sialyllactose added to the hydrazinolytic acid as an internal standard.
**Sugar Chains of Blood Coagulation Factor IX**

Fig. 2. Papier chromatography of oligosaccharides. Neutral oligosaccharides obtained by acid hydrolysis digestion of the radioactive A-IX were purified by paper electrophoresis as described in the legend of Fig. 1. The neutral oligosaccharides were applied to the origin of Whatman No. 3MM paper, and then developed for 10 days with ethanolic-pyridine-water (v/v/v) 9:1:2. Arrow indicates the position where authentic sugars migrated. A, GalGlcNAC(Man1→4)GlcNAcGlcNAc; B, GalGlcNAC(Man1→4)GlcNAcMan; C, GalGlcNAC(Man1→4)GlcNAcGlc; D, GalαGlcNAC(Man1→4)GlcNAcGlcNAc; E, GalαGlcNAC(Man1→4)GlcNAcMan; F, GalαGlcNAC(Man1→4)GlcNAcGlc; G, GalαGlcNAC(Man1→4)GlcNAcGlcNAc. A-F, the neutral fractions obtained from A-IX. G, A-IX-α; H, A-IX-β; I, A-IX-γ; J, A-IX-δ. From A-IX-α, B-IX, C-IX, D-IX, E-IX, F-IX, H-IX, I-IX, J-IX, from a mixture of A-IX.

![Fig. 2](image)

**Fig. 3.** Essential carboxylidase digestion of oligosaccharides H. The radioactive sugars were subjected to Sep-Pak P-4 column chromatography and the radioactivity in each tube (10 μl) was determined on an aliquot of sample by liquid scintillation spectrometry. The small arrows indicate the eluting positions of glucose oligomers numbers indicate the glucose units added are internal standards, and the big arrow indicates the elution position of authentic Man1→6Man1→6Man(α→1)GlcNAc-Gly-Gly. A, intact oligosaccharide H; B, a mixture of oligosaccharides after carboxylidase digestion; C, the radioactive peak in 10 after incubation with N-acetylglucosaminidase digestion; D, the radioactive peak in 10 after incubation with jack bean α-amylase; E, the radioactive peak in 10 after incubation with diphenyl-β-sulfonatofluorescein; F, the radioactive peak in 10 after incubation with N-acetylglucosaminidase digestion.

![Fig. 3](image)

**Table I.** Methylation analysis of oligosaccharides.

| Oligosaccharide | Molar ratio of methyl group to acetylation | Before digestion | After digestion |
|-----------------|------------------------------------------|-----------------|----------------|
| Sialosyl-1 | [1,5-di-O-Methyl][1,2-di-O-Methyl] | 3.0 0.5 | 2.3 0.1 0.13 0.8 0.2 0.3 1.0 |
| Sialosyl-2 | [1,4-di-O-Methyl][1,2-di-O-Methyl] | 0 1.0 | 0 0.4 0.2 0.6 0.13 1.5 1.0 2.0 2.0 |
| Sialosyl-3 | [1,5-di-O-Methyl][1,2-di-O-Methyl] | 0 0.7 | 0 0.4 0.7 0.8 0.9 0.6 0.4 0.7 0.9 |
| Sialosyl-4 | [1,4-di-O-Methyl][1,2-di-O-Methyl] | 0 1.0 | 0 0.4 0.7 0.8 |

| Sialomannosyl | [1,5-di-O-Methyl][1,2-di-O-Methyl] | 0 1.0 | 0 0.4 0.7 0.8 0.9 0.6 0.4 0.7 0.9 |
|---------------|---------------------------------|-----------------|----------------|
| Sialomannosyl-1 | [1,5-di-O-Methyl][1,2-di-O-Methyl] | 0 1.0 | 0 0.4 0.7 0.8 0.9 0.6 0.4 0.7 0.9 |
| Sialomannosyl-2 | [1,4-di-O-Methyl][1,2-di-O-Methyl] | 0 1.0 | 0 0.4 0.7 0.8 0.9 0.6 0.4 0.7 0.9 |
| Sialomannosyl-3 | [1,5-di-O-Methyl][1,2-di-O-Methyl] | 0 1.0 | 0 0.4 0.7 0.8 0.9 0.6 0.4 0.7 0.9 |
| Sialomannosyl-4 | [1,4-di-O-Methyl][1,2-di-O-Methyl] | 0 1.0 | 0 0.4 0.7 0.8 |

**a)** Numbers in the table were calculated by making the use of 2,4-di-O-Methylsialomannosyl-1.6.
**b)** The molar portion was the detection label in sample corresponding to the peak in Fig. 1.
**c)** Oligosaccharide mixture isolated from factor IX by hydrazinolysis.
**d)** Tr. is less than 0.1.