THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 15, Issue of August 10, pp. 8755–8760, 1982
*Printed in U.S.A.*

# The Structures and Microheterogeneity of the Carbohydrate Chains of Human Plasma Ceruloplasmin

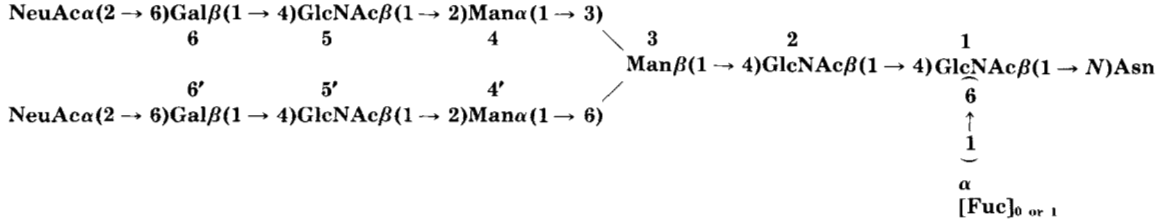
A STUDY EMPLOYING 500-MHz $^1$H-NMR SPECTROSCOPY*

(Received for publication, October 20, 1981)

**Masahiko Endo‡§, Koichi Suzuki‡¶, Karl Schmid‡, Bernard Fournet‖, Yannis Karamanos‖, Jean Montreuil‖, Lambertus Dorland**‡‡, Herman van Halbeek**, and Johannes F. G. Vliegenthart****

*From the ‡Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118, the ‖Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille I, Villeneuve d'Ascq, France, and the **Department of Bio-Organic Chemistry, University of Utrecht, NL-3522 AD Utrecht, The Netherlands*

**Sialo- and asialoglycopeptides were prepared from recrystallized monodisperse human plasma ceruloplasmin and analyzed by high resolution $^1$H-NMR spectroscopy and methylation analysis. This glycoprotein was found to possess only bi- and triantennary *N*-glycosidic glycans.**

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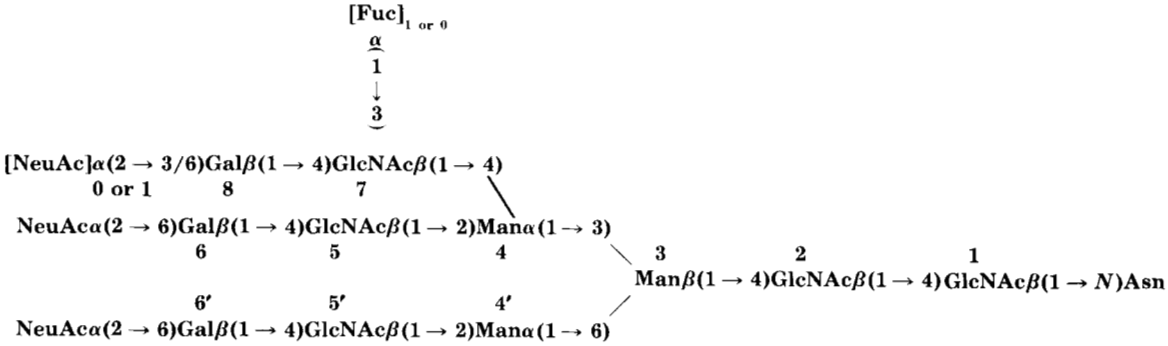


**As to the microheterogeneity of the carbohydrate units, it should be noted that the biantennary structure may be extended by a fucose residue in an α(1 → 6) bond to GlcNAc 1. In the triantennary glycan the 7-8 branch possesses either an α(2 → 3) or an α(2 → 6) linked NeuAc residue. If the NeuAc residue is absent in the 7-8 branch, this branch contains instead a fucose linked in an α(1 → 3) bond to GlcNAc 7.**

Ceruloplasmin, the copper-containing protein of human plasma, has been extensively characterized (1, 2). Recently, the amino acid sequence of this glycoprotein was established to a large extent (3, 4). Considerable progress has also been made in elucidating the carbohydrate units of this protein. Employing hydrazinolysis, exoglycosidase digestion, methylation analysis, and periodate oxidation, the presence of bi- and triantennary glycans were demonstrated (5). However, the position of fucose, a sugar present in relatively small

* This investigation was supported by grants from the National Institute of General Medical Sciences (GM-10374), United States Public Health Service, the Centre National de la Recherche Scientifique (Laboratoire Associé No. 217: Biologie physicochimique et moléculaire des glucides libres et conjugués), the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO), and the Netherlands Foundation for Cancer Research (KWF, grant UUKC-OC 79-13). 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, Hirosaki University School of Medicine, Hirosaki, Japan.

¶ Present address, Department of Biology, Saitama University, Urawa, Japan.

‡‡ Present address, University Children's Hospital, Utrecht, The Netherlands.

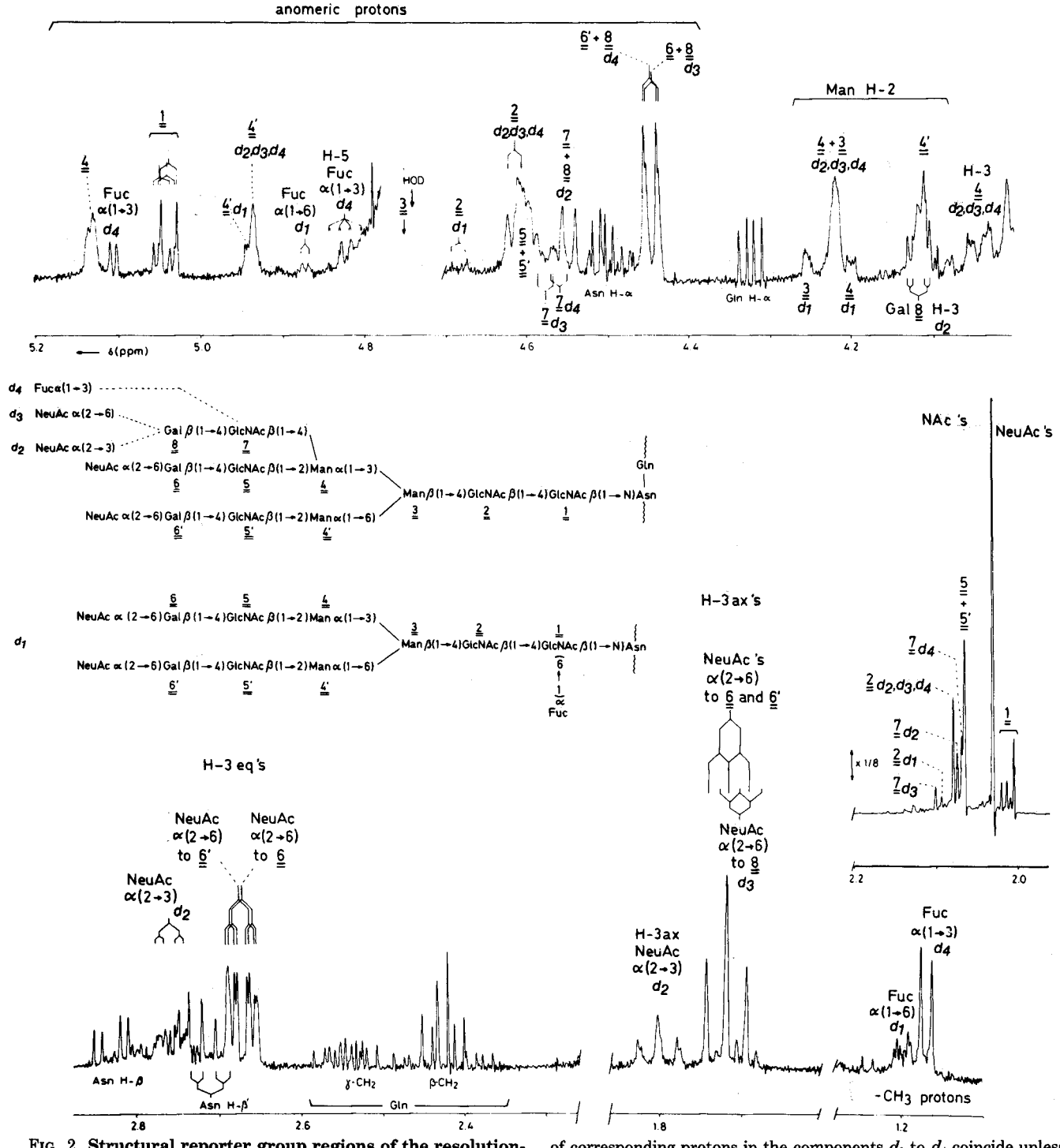


FIG. 2. **Structural reporter group regions of the resolution-enhanced 500-MHz ¹H-NMR spectrum of sialo-glycopeptide fraction *d*, derived from ceruloplasmin.** The *numbers* in the *spectrum* refer to the corresponding residues in the structures. Signals of corresponding protons in the components $d_1$ to $d_4$ coincide unless otherwise indicated. Further, the relative intensity scale of the *N*-acetyl proton region differs from that of the other parts of the spectrum, as indicated.

amounts in this protein (6), has not been reported.

In this paper, employing high resolution ¹H-NMR spectroscopy which has proved to be particularly advantageous in elucidating the complete primary structures of carbohydrate units of glycoproteins (7, 8), we have extended the structural studies of the glycans of ceruloplasmin and have established the precise linkages of Fuc[1] and NeuAc in the carbohydrate units of this protein. Moreover, it was also possible to assess the microheterogeneity in the carbohydrate branches of ceruloplasmin.

[1] The abbreviation used is: Fuc, fucose.

## MATERIALS AND METHODS AND RESULTS[2]

## DISCUSSION

*High Resolution ¹H-NMR Spectroscopy*—500-MHz ¹H-

[2] Portions of this paper (including "Materials and Methods," the first part of "Results," Figs. 1 and 3, and Tables I-IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-1199, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

NMR spectroscopy was performed on the sialo- and asialoglycopeptide fractions derived from ceruloplasmin. The NMR spectral data are summarized in Table IV. The interpretation of the NMR spectra was carried out on the basis of our earlier pertinent studies (7, 8, 16, 21). The structural reporter group signals of the Man residues are consistent with the presence of bi- and triantennary structures.

Concerning the sialoglycopeptides, Fuc as well as NeuAc could clearly be observed in the NMR spectrum of glycopeptide fraction *d* (Fig. 2). This spectrum reveals that fraction *d* is a mixture of bi- and triantennary glycopeptides, occurring in a molar ratio of 2:5. This conclusion is based on the relative intensities of the Man H-2 signals at $\delta$ = 4.257 (Man **3**, biantenna), $\delta$ = 4.220 (Man **3** and **4**, triantenna), and $\delta$ = 4.200 (Man **4**, biantenna) being 1:5:1. The type of linkage of NeuAc to Gal is evident from the chemical shifts of the anomeric protons of Man **4** and **4'** (8). In the biantennary glycopeptide (compound $d_1$) this linkage proved to be $\alpha(2 \rightarrow 6)$ in both branches based on the H-1 signals of Man **4** and **4'**. In the triantennary structure (compound $d_2$) another type of NeuAc linkage was observed *viz.* $\alpha(2 \rightarrow 3)$ to Gal **8** ($\delta$ = 2.756 for H-$3_{eq}$ and $\delta$ = 1.801 for H-$3_{ax}$) in addition to the NeuAc residues $\alpha(2 \rightarrow 6)$ linked to Gal **6** and **6'** (8). To a small extent, compound $d_3$ is present in this fraction. Compound $d_3$ contains a NeuAc residue in $\alpha(2 \rightarrow 6)$ linkage to Gal **8** ($\delta$H-$3_{ax}$ = 1.705; $\delta$NAc of GlcNAc **7** = 2.100) (8). In this mixture another triantennary structure (compound $d_4$) is present, lacking a NeuAc residue linked to Gal **8** but instead possessing a Fuc $\alpha(1 \rightarrow 3)$ linked to GlcNAc **7**. This conclusion was derived from the structural reporter group signals of Fuc: $\delta$H-1 = 5.104, $\delta$H-5 = 4.820, and $\delta CH_3$ = 1.171, demonstrating the presence of a Fuc $\alpha(1 \rightarrow 3)$ linked to a peripheral GlcNAc. The *N*-acetyl signal at $\delta$ = 2.068 also shows that Fuc is attached to GlcNAc **7** (8, 16, 22). The occurrence of another Fuc, present to a small extent and in a different type of linkage, is evident from its structural reporter group signals ($\delta$H-1 = 4.873 and $\delta CH_3$ = 1.200) and from the chemical shift of the *N*-acetyl signal of GlcNAc **2** ($\delta$ = 2.094) (8). These data establish the presence of Fuc in $\alpha(1 \rightarrow 6)$ linkage to GlcNAc **1**. The intensity of the signals of the latter type of Fuc suggests that the $\alpha(1 \rightarrow 6)$ linked Fuc occurs in the biantennary glycopeptide ($d_1$).

Based on the intensity ratios of the various signals in the NMR spectra of the other sialoglycopeptide fractions (*a*, *b*, *c*, and *e*), it can be concluded that they consist primarily of bi-$\alpha(2 \rightarrow 6)$-sialo biantennary glycopeptides. Fractions *b* and *e* also contain small amounts of triantennary glycopeptide with NeuAc in $\alpha(2 \rightarrow 6)$ linkage to Gal **6** and **6'**, whereas a third NeuAc residue is $\alpha(2 \rightarrow 3)$ linked to Gal **8**.

The NMR spectral data of the investigated asialoglycopeptide fractions corroborate the identification of bi- and triantennary glycan structures. For the biantenna, the data are in full agreement with those previously published (8, 16, 21). The spectrum of the triantenna with Fuc attached to GlcNAc **7** (fraction *f*) is given in Fig. 3, while the spectral data of fraction *f* are presented in Table IV.

*$\beta$ Elimination*—Exhaustive $\beta$-elimination afforded no evidence of the presence of *O*-glycosidically linked heteroglycans. This procedure yielded small quantities of *N*-glycosidic carbohydrate moieties, possessing bi- and triantennary structures.

*Conclusions*—Based on the data of methylation analysis and $^1$H-NMR spectroscopy, the following glycan structures of ceruloplasmin could be established. Compound a was found to possess a biantennary structure without Fuc and compound $d_1$ a biantennary structure with Fuc attached to GlcNAc **1** (for structures, see Summary), whereas compounds $d_2$ and $d_3$ have a triantennary structure without Fuc but differing in the type of their sialic acid-Gal linkage, and compound $d_4$ a triantennary structure with Fuc attached to GlcNAc **7** (Fig. 2).

The glycans exhibit several types of microheterogeneity. NeuAc may be attached to Gal **8** in $\alpha(2 \rightarrow 3)$ or $\alpha(2 \rightarrow 6)$ linkage. If NeuAc is absent at this Gal residue, Fuc occurs at GlcNAc **7** in $\alpha(1 \rightarrow 3)$ linkage. This observation is in support of Hill's exclusion principle regarding NeuAc or Fuc as terminal residues (23). In addition, as a third form of microheterogeneity, Fuc may be present in $\alpha(1 \rightarrow 6)$ linkage to GlcNAc **1**. This investigation further shows that it is now feasible to elucidate the complete structure of closely related glycans by high resolution $^1$H-NMR spectroscopy.

*Acknowledgments*—We are very much obliged to L. Larsen, Institute of Laboratories, Massachusetts Department of Health, Jamaica Plain, MA 02130, for the generous supply of Cohn fraction IV; Kathleen Hoar for isolating and crystallizing ceruloplasmin; G. Crombie for many amino acid analyses; and G. J. Gerwig for sugar analyses. Thanks are also due to Dr. W. E. Hull (Bruker Analytische Messtechnik, Rheinstetten, Federal Republic of Germany) for recording the 500-MHz $^1$H-NMR spectra.

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SUPPLEMENTARY MATERIAL TO

"The Structures and Microheterogeneity of the Carbohydrate Chains of Human Plasma Ceruloplasmin. A Study employing 500-MHz $^1$H-NMR Spectroscopy" by:

Masahiko Endo, Koichi Suzuki, Karl Schmid, Bernard Fournet, Yannis Karamanos, Jean Montreuil, Lambertus Dorland, Herman van Halbeek, and Johannes F.G. Vliegenthart

## MATERIALS AND METHODS

Crystallization of ceruloplasmin. This protein was isolated from freshly prepared Cohn fraction IV of pooled normal human plasma by a procedure based on the methods of Deutsch et al. (9), Heide and Haupt (10) and Van Leeuwen and Van Gelder (2). Briefly, ceruloplasmin was adsorbed from a suspension (10 l) of Cohn fraction IV (1.5 kg) on DEAE-cellulose (500 g) at pH 5.5. The resulting DEAE-cellulose was then thoroughly washed with sodium acetate buffer pH 5.5, 0.05 M and stepwise eluted at pH 7.3 with a sodium acetate buffer using an ionic strength gradient from 0.05 to 0.5. The eluate containing the crude ceruloplasmin ($OD_{280}/OD_{610}$ = 26-32) was fractionated with ammonium sulfate and a fraction between 50-60% saturation was collected. Subsequently, this precipitate was dissolved in a small volume of water and dialyzed overnight against sodium acetate buffer pH 5.25, 0.025 M. The formed crystals were centrifuged off, washed, dissolved in water, and dialyzed against the same buffer. The resulting crystals were washed, dissolved in water and lyophilized. To obtain ceruloplasmin in a homogeneous state it is important that a protease inhibitor (ε-amino caproic acid, 0.1 M) is present in all steps of the procedure and that the isolation is carried out at low temperature (4°C), except for the ammonium sulfate fractionation, and in a short period of time. Such preparations do not reveal the heterogeneity of the polypeptide chain described in earlier studies (1).

The N-terminal amino acid was determined by the dansyl procedure (11). Disc polyacrylamide gel electrophoresis was carried out according to Davis (12). Immuno-electrophoresis was carried out, using goat serum active against normal human serum as well as rabbit serum against human ceruloplasmin (Behring Diagnostics, Somerville, NJ).

Sialo-glycopeptides. For the preparation of these peptides, ceruloplasmin was treated as follows: papain digestion (Sigma, # 3125. 60°C, 5 h, pH 7.8; enzyme-substrate ratio 1:30), pronase digestion (Seikagaku Kogyo Co. Ltd., Tokyo, 1,000,000 tyrosine units per g; 48 h, 45°C pH 8.4; enzyme-substrate ratio 1:30), followed by gel filtration through Sephadex G-25, reduction and alkylation (14), redigestion with papain and pronase as mentioned and fractionation of the resulting glycopeptide mixture by chromatography on DEAE-cellulose. Subsequently, each glycopeptide fraction was desalted by gel filtration through a Sephadex G-25 column (1.7 x 45 cm) and lyophilized. It should be noted that Pronase E used in this investigation contains approximately 0.5% phenol-sulfuric acid positive sugars, mostly pentose while papain is devoid of carbohydrates.

Asialo-glycopeptides. Asialo-glycopeptides were prepared from the sialo-glycopeptide mixture by treatment of the latter with insoluble neuraminidase (C. perfringens, Sigma, St. Louis, MO, 2382) followed by fractionation of the resulting asialo-glycopeptide mixture on Dowex-50, X2 (200-400 mesh) (14).

β-Elimination. This procedure (0.05 M KOH, 1.0 M $NaBH_4$, 15 h, at 45°C) was carried out according to Carlson (15).

Carbohydrate and amino acid analysis. For the determination of their sugar compositions the glycopeptides were subjected to methanolysis (0.5 M HCl in methanol, 24 h, 80°C) followed by gas-liquid chromatography of the methyl glycosides of the trimethylsilyl- or acetyl-derivatives (16). To determine its hexosamines ceruloplasmin was hydrolyzed (4 M HCl, 6 h, 110°C, $N_2$) and analyzed on a Jeol amino acid analyzer. The amino acid compositions of these peptides were determined in the conventional manner (redist. HCl, 110°C, 24 h, $N_2$) using the above mentioned amino acid analyzer.

Methylation analysis. The glycopeptides were methylated according to Hakomori (17) as modified by Björndal et al. (18). After extraction with chloroform, the methylated products were purified on a Sephadex LH-20 column (1 x 20 cm, chloroform:ethanol = 1:1; 2 ml fractions; detection of carbohydrate by the phenol-sulfuric acid reagent of Dubois et al.) (19). The methylated compounds were then treated with 0.5 M HCl-methanol for 24 h at 80°C and formed mixtures of methylglycosides were analyzed by gas-liquid chromatography-mass spectroscopy after peracetylation in pyridine-acetic anhydride (1:1, 500 µl, 100°C, 30 min). The obtained products were analyzed by gas-liquid chromatography using a Girdel model 30 apparatus (Suresnes, France) equipped with a glass capillary column (60 cm x 0.35 mm), wall-coated with OV 101, and helium as a carrier gas. The temperature was programmed from 110°C to 240°C with a gradient of 3° per min. Mass spectra were recorded on a Riber-Mag 10-10 mass spectrometer (Rueil-Malmaison, France) using an electron energy of 70 eV and an ionizing current of 0.2 mA (16).

500-MHz $^1$H-NMR Spectroscopy. The 500-MHz $^1$H-NMR spectra were recorded on a Bruker WM-500 spectrometer (Bruker GmbH, Rheinstetten, FRG), operating in the Fourier transform mode, and equipped with a Bruker Aspect 2000 computer with 32k memory capacity. The D-resonance of the solvent $D_2O$ was used as field frequency lock signal. The spectra were taken up in 16k memory with an acquisition time of 3.28 sec and a spectral width of 2.5 kHz. Resolution enhancement was achieved by Lorentzian to Gaussian transformation from quadrature phase detection followed by employment of a 32k complex Fourier transformation (20). In general hundred acquisitions for each sample were accumulated. The indicated probe temperature was 300 K and was kept constant within 0.1 K. At this temperature the HOD-resonance is found at δ = 4.752 ppm. The chemical shifts (δ) are expressed in ppm downfield from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), but were actually measured by reference to internal acetone (δ = 2.225) with accuracy of 0.001 ppm.

## RESULTS

Homogeneity of crystallized ceruloplasmin. Two times crystallized ceruloplasmin appeared essentially homogeneous on disc polyacrylamide gel and immuno-electrophoresis, and possessed lysine as the single N-terminal amino acid. The ratio of the absorbances at 280 and 610 nm was found to be 22. As to the carbohydrate composition of this protein (1,6) (Table I) it is noteworthy that the sum of NeuAc (2.3 moles/mole protein) and Fuc (0.4) equals the amount of Gal (2.7). No amino sugars other than GlcNAc could be detected agreeing with earlier reports (6).

TABLE I

CARBOHYDRATE COMPOSITIONS OF THE SIALOGLYCOPEPTIDE FRACTIONS OF CERULOPLASMIN

| Monosaccharide | Sialo-glycopeptide - fraction a | b | c | d | e | Ceruloplasmin |
|---|---|---|---|---|---|---|
| Fuc | 0.2* | 0.4 | 0.4 | 0.5 | 0.3 | 0.4 |
| Gal | 2.0 | 2.2 | 2.1 | 2.7 | 2.3 | 2.7 |
| Man | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| GlcNAc | 3.4 | 3.5 | 3.3 | 4.0 | 3.4 | 4.4 |
| NeuAc | 1.8 | 1.9 | 1.8 | 3.1 | 2.0 | 2.3 |
| Total Carbohydrate Content | 74** | 76 | 82 | 86 | 74 | 41 |

* These numbers indicate the number of monosaccharide per three mannose residues.
** These numbers indicate the percentage of carbohydrate of the glycopeptides and of ceruloplasmin.

Sialo-glycopeptides. Chromatography of the sialo-glycopeptides on DEAE-cellulose resulted in fractions a-e (Fig. 1). The carbohydrate compositions of these fractions (Table I) suggest that they probably consist of mixtures of closely related glycans. The amino acid compositions and the N-terminal amino acid residues of the fractions a-e are given in Table II.

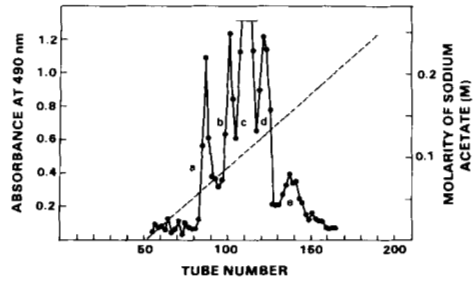


FIG. 1. Chromatography of the sialo-glycopeptides derived from human plasma ceruloplasmin. The glycopeptide mixture (32 mg) isolated from a papain-pronase digest of ceruloplasmin was fractionated on a DEAE-cellulose column (0.8 x 2.8 cm) at pH 5.8 in sodium acetate. The glycopeptides were eluted using an ionic strength gradient from 0.01 to 0.25 M. The flow rate was 0.85 ml per min. and 1.7 ml of effluent was collected per test tube. The effluent was monitored at 490 nm using the orcinol procedure (13). Five fractions designated a to e were obtained.

TABLE II

AMINO ACID COMPOSITIONS OF GLYCOPEPTIDE FRACTIONS OF CERULOPLASMIN

| Amino acid* | Glycopeptide - fraction a | b | c | d | e | f |
|---|---|---|---|---|---|---|
| Asx | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Thr | - | 0.6** | 0.2 | 0.2 | 0.2 | 0.1 |
| Glx | - | - | 0.8 | 0.7 | 0.5 | - |
| Ser | - | - | - | 0.1 | 0.1 | - |
| Gly | - | - | - | 0.1 | 0.1 | 0.2 |
| Ala | - | - | - | 0.1 | 0.1 | - |
| N-terminal amino acid | asx | asx | asx, glx | asx, glx | asx, glx | asx |

* Amino acids present in amounts smaller than 0.1 mole per mole of Asx are not included in this table.
** These numbers are expressed in moles per mole of Asx.

Asialo-glycopeptides. Chromatography of the asialo-glycopeptide mixture on Dowex-50 yielded seven major fractions which were further purified by gel filtration through a Sephadex G-50 column (1.2 x 85 cm).

Methylation analysis. The data of the methylation studies (Table III) indicate the presence of the type of linkages that usually occur in bi- and triantennary, N-acetyllactosamine type glycan structures (21).

TABLE III

COMPOSITION OF METHYLETHERS OF SIALOGLYCOPEPTIDES DERIVED FROM CERULOPLASMIN

| Glycopeptide fraction | 3, 4, 6-tri-OMe*-Man | 2, 3, 4-tri-OMe-Gal | 2, 4, 6-tri-OMe-Gal | 3, 6-di-OMe-Man | 2, 4-di-OMe-Man | 3, 6-di-OMe-GlcNAc NMe** | 6-mono-OMe-GlcNAc NMe | 4, 7, 8, 9-tetra-OMe NeuAcNMe | 2, 3, 4-tri-OMe-Fuc |
|---|---|---|---|---|---|---|---|---|---|
| a | 2.2 | 2.2 | - | - | 1 | 3.3 | - | 1.6 | - |
| b | 1.9 | 2.1 | - | - | 1 | 3.3 | - | 2.0 | - |
| c | 2.2 | 2.3 | - | - | 1 | 3.3 | - | 2.0 | - |
| d | 1.0 | 2.1 | 1.1 | 0.8 | 1 | 3.5 | 0.6 | 3.2 | 0.5 |
| e | 1.5 | 1.9 | 0.5 | 0.3 | 1 | 3.6 | 0.3 | 2.6 | 0.4 |

* O-Methyl is abbreviated as OMe.
** N-Acetyl-N-methyl is abbreviated as NAcNMe.

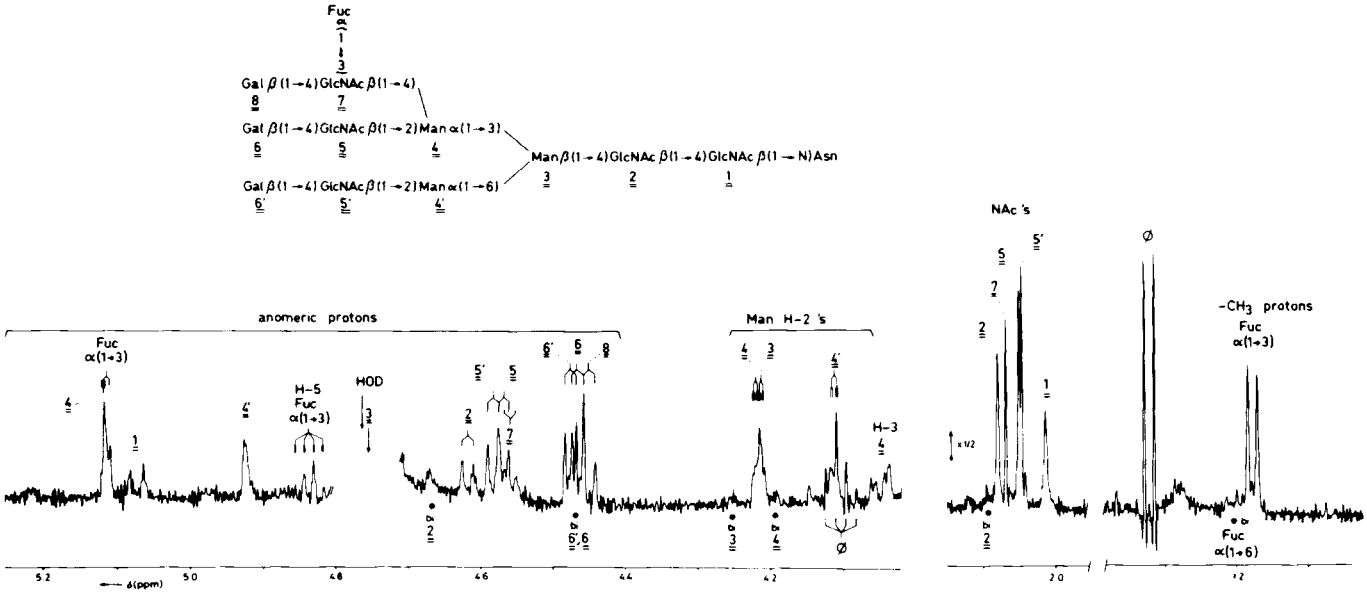


FIG. 3. Structural reporter group regions of the resolution-enhanced 500-MHz $^{1}$H-NMR spectrum of asialo-glycopeptide fraction 1, derived from ceruloplasmin. The structure of this compound corresponds to the asialoform of compound 4a (see text). From the very small signals at $\delta$ = 4.249 (H-2 of Man 3), $\delta$ = 4.194 (H-2 of Man 4), $\delta$ = 2.094 (NAc of GlcNAc 7) and at $\delta$ = 1.197 ($CH_3$ of Fuc) it is evident that the sample contains about 5% of a bi-antennary glycopeptide possessing a Fuc α(1→6) linked to GlcNAc 1. The relative intensity scale of the N-acetyl proton region differs from that of the other parts of the spectrum, as indicated. The signals marked by ↓ stem from a non-protein, non-carbohydrate contaminant.

TABLE IV

$^{1}$H-NMR chemical shifts of structural reporter groups of constituent monosaccharides for glycopeptide fractions derived from ceruloplasmin

| Structural reporter | Glycopeptide - fraction | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | a | b | c | d 1 | d 2 | d 3 | d 4 | e | f |
| **H-1 of** | | | | | | | | | |
| GlcNAc 1 | 5.075 | 5.066 | 5.041 | 5.045 | 5.040 | 5.037 | 5.037 | n.d. | 5.071 |
| GlcNAc 2 | 4.612 | 4.615 | 4.622 | 4.684 | 4.614 | 4.614 | 4.614 | 4.620 | 4.616 |
| Man 3 | 4.780 | 4.774 | 4.779 | ~4.77 | ~4.77 | ~4.77 | ~4.77 | 4.778 | 4.766 |
| Man 4 | 5.133 | 5.135 | 5.134 | 5.131 | 5.131 | 5.131 | 5.131 | 5.135 | 5.116 |
| Man 4' | 4.950 | 4.946 | 4.948 | 4.946 | 4.934 | 4.934 | 4.934 | 4.945 | 4.925 |
| GlcNAc 5 | 4.599 | 4.598 | 4.601 | ~4.60 | ~4.60 | ~4.60 | ~4.60 | 4.600 | 4.568 |
| GlcNAc 5' | 4.599 | 4.598 | 4.601 | ~4.60 | ~4.60 | ~4.60 | ~4.60 | 4.600 | 4.580 |
| Gal 6 | 4.443 | 4.444 | 4.444 | 4.444 | 4.444 | 4.444 | 4.444 | 4.443 | 4.464 |
| Gal 6' | 4.443 | 4.444 | 4.444 | 4.446 | 4.446 | 4.446 | 4.446 | 4.443 | 4.474 |
| GlcNAc 7 | - | (4.551)* | - | - | 4.546 | 4.572 | 4.557 | (4.549)* | 4.557 |
| Gal 8 | - | (4.551)* | - | - | 4.546 | 4.444 | 4.446 | (4.549)* | 4.448 |
| Fuc α(1→3)-GlcNAc 7 | - | - | - | - | - | - | 5.104 | - | 5.112 |
| Fuc α(1→6)-GlcNAc 1 | - | - | - | 4.873 | - | - | - | - | - |
| **H-2 of** | | | | | | | | | |
| Man 3 | 4.260 | 4.260 (4.220)* | 4.257 | 4.257 | 4.220 | 4.220 | 4.220 | 4.257 (4.219)* | 4.213 |
| Man 4 | 4.201 | 4.199 (4.220)* | 4.201 | 4.200 | 4.220 | 4.220 | 4.220 | 4.203 (4.219)* | 4.213 |
| Man 4' | 4.120 | 4.114 | 4.112 | 4.112 | 4.112 | 4.112 | 4.112 | 4.115 | 4.112 |
| **H-3eq of NeuAc** | | | | | | | | | |
| α(2→6)-Gal 6 | 2.669 | 2.668 | 2.670 | 2.670 | 2.670 | 2.670 | 2.670 | 2.670 | - |
| α(2→6)-Gal 6' | 2.669 | 2.668 | 2.670 | 2.673 | 2.673 | 2.673 | 2.673 | 2.670 | - |
| α(2→6)-Gal 8 | - | - | - | - | - | 2.670 | - | - | - |
| α(2→3)-Gal 8 | - | (2.758)* | - | - | 2.756 | - | - | (2.756)* | - |
| **H-3ax of NeuAc** | | | | | | | | | |
| α(2→6)-Gal 6 | 1.724 | 1.724 | 1.723 | 1.717 | 1.717 | 1.717 | 1.717 | 1.723 | - |
| α(2→6)-Gal 6' | 1.724 | 1.724 | 1.723 | 1.717 | 1.717 | 1.717 | 1.717 | 1.723 | - |
| α(2→6)-Gal 8 | - | - | - | - | - | 1.705 | - | - | - |
| α(2→3)-Gal 8 | - | (1.804)* | - | - | 1.801 | - | - | (1.804)* | - |
| **H-5 of Fuc** | | | | | | | | | |
| α(1→3)-GlcNAc 7 | - | - | - | - | - | - | 4.820 | - | 4.835 |
| α(1→6)-GlcNAc 1 | - | - | - | ~4.12 | - | - | - | - | - |
| **H-6 of Fuc** | | | | | | | | | |
| α(1→3)-GlcNAc 7 | - | - | - | - | - | - | 1.171 | - | 1.176 |
| α(1→6)-GlcNAc 1 | - | - | - | 1.200 | - | - | - | - | - |
| **N-acetyl protons of** | | | | | | | | | |
| GlcNAc 1 | 2.011 | 2.008 | 2.006 | 2.020 | 2.004 | 2.004 | 2.004 | 2.006 2.013 | 2.013 |
| GlcNAc 2 | 2.083 | 2.082 | 2.080 | 2.094 | 2.079 | 2.079 | 2.079 | 2.080 | 2.078 |
| GlcNAc 5 | 2.068 | 2.069 | 2.069 | 2.065 | 2.065 | 2.065 | 2.065 | 2.069 | 2.049 |
| GlcNAc 5' | 2.064 | 2.065 | 2.066 | 2.065 | 2.065 | 2.065 | 2.065 | 2.069 | 2.049 |
| GlcNAc 7 | - | - | - | - | 2.073 | 2.100 | 2.068 | - | 2.067 |
| NeuAcα(2→6)-Gal 6 | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | - |
| NeuAcα(2→6)-Gal 6' | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | 2.030 | - |
| NeuAcα(2→6)-Gal 8 | - | - | - | - | - | 2.030 | - | - | - |
| NeuAcα(2→3)-Gal 8 | - | - | - | - | 2.030 | - | - | - | - |

* Signals of relatively low intensity stemming from small amounts of triantennary glycopeptide present in the fractions b and e