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

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

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

NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)
6 5 4
NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)

and

[NeuAc]α(2→3/6)Galβ(1→4)GlcNAcβ(1→4)
8 or 1

NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)
6 5 4
NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)

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

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anomic protons

![Diagram of carbohydrate structures and NMR spectum]

Fig. 2. Structural reporter group regions of the resolution-enhanced 500-MHz $^1$H-NMR spectrum of sialo-glycopeptide fraction $d$, derived from ceruloplasmin. The numbers in the spectrum refer to the corresponding residues in the structures. Signals amounts in this protein (6), has not been reported.

In this paper, employing high resolution $^1$H-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.

The abbreviation used is: Fuc, fucose.

MATERIALS AND METHODS AND RESULTS$^2$

DISCUSSION

High Resolution $^1$H-NMR Spectroscopy—500-MHz $^1$H-

$^2$ 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.
Ceruloplasmin Carbohydrates Elucidated by 1H-NMR

NMR spectroscopy was performed on the sialo- and asialo-glycopeptide 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 sialylglycopeptides, Fuc as well as NeuAc could be clearly 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 δ = 4.257 (Man 3, biantenna), δ = 4.220 (Man 3 and 4, triantenna), and δ = 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 anomer protons of Man 4 and 4′ (8). In the biantennary glycopeptide (compound d1) this linkage proved to be α(2 → 6) in both branches based on the H-1 signals of Man 4 and 4′. In the triantennary structure (compound d2) another type of NeuAc linkage was observed viz. α(2 → 3) to Gal 8 (δ = 2.756 for H-3,δ) and δ = 1.801 for H-3,δ, in addition to the NeuAc residues α(2 → 6) linked to Gal 6 and 6′ (8). To a small extent, compound d3 is present in this fraction. Compound d3 contains a NeuAc residue in α(2 → 6) linkage to Gal 8 (δ(3,δ = 1.705; δ(NAc of GlcNAc 7 = 2.100) (8). In this mixture another triantennary structure (compound d4) is present, lacking a NeuAc residue linked to Gal 8 but instead possessing a Fuc α(1 → 3) linked to GlcNAc 7. This conclusion was derived from the structural reporter group signals of Fuc: δ(NAc of GlcNAc 7 = 5.104, δ(3,δ = 4.820, and δCH3 = 1.171, demonstrating the presence of a Fuc α(1 → 3) linked to a peripheral GlcNAc. The N-acetyl signal at δ = 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 (δ(NAc = 4.573 and δCH3 = 1.200) and from the chemical shift of the N-acetyl signal of GlcNAc 2 (δ = 2.094) (8). These data establish the presence of Fuc in α(1 → 6) linkage to GlcNAc 1. The intensity of the signals of the latter type of Fuc suggests that the α(1 → 6) linked Fuc occurs in the biantennary glycopeptide (d1).

Based on the intensity ratios of the various signals in the NMR spectra of the other sialylglycopeptide fractions (a, b, c, and e), it can be concluded that they consist primarily of bi-α(2 → 6)-sialo biantennary glycopeptides. Fractions b and e also contain small amounts of triantennary glycopeptide with NeuAc in (α2 → 6) linkage to Gal 6 and 6′, whereas a third NeuAc residue is α(2 → 3) linked to Gal 8.

The NMR spectral data of the investigated asialo-glycopeptide 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.

β Elimination—Exhaustive β-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 1H-NMR spectroscopy, the following glycan structures of ceruloplasmin could be established. Compound a was found to possess a biantennary structure without Fuc and compound d1 a biantennary structure with Fuc attached to GlcNAc 7 (for structures, see Summary), whereas compounds d2 and d3 have a triantennary structure without Fuc but differing in the type of their sialic acid-Gal linkage, and compound d4 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 α(2 → 3) or α(2 → 6) linkage. If NeuAc is absent at this Gal residue. Fuc occurs at GlcNAc 7 in α(1 → 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 α(1 → 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 1H-NMR spectroscopy.

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Ceruloplasmin Carbohydrates Elucidated by H-NMR

The structure and function of ceruloplasmin, a copper-containing protein, has been extensively studied. Ceruloplasmin is known to play a role in copper transport and metabolism. The elucidation of its carbohydrate structure is an important aspect of understanding its function.

The methods used for this study involved nuclear magnetic resonance (NMR) spectroscopy. Ceruloplasmin samples were dissolved in appropriate buffers and subjected to NMR analysis to determine the carbohydrate composition. The results were then compared with those obtained from other methods to validate the findings.

The data from the NMR analysis revealed that ceruloplasmin contains a complex carbohydrate moiety, which is essential for its biological function. The carbohydrate structure was further refined by interpreting the NMR spectra, yielding a detailed understanding of its composition.

The findings contribute to the broader understanding of protein-carbohydrate interactions and the role of carbohydrates in protein functionality. This knowledge is crucial for developing new therapeutic approaches and for understanding the intricate mechanisms of protein function in health and disease.

**Table 1**

| NMR ACID COMPOSITIONS OF DOMINANT FRAGMENTS OF CERULOPLASMIN |
|--------------------------|--------------------------|
| Glucopyranose fraction   | Glucopyranose fraction   |
| HN  | 2       | 3       | 4       | 5       | 6       | 7       | 8       | 9       | 10      | 11      | 12      |
| Glc  | 0.5     | 0.2     | 0.2     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |
| Gln  | 0.2     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |
| Gal  | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |
| GlcNAc| 0.1    | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |
| GlcNAc2| 0.1   | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |
| Man  | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |
| GlcNAc3| 0.1   | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |
| GlcNAc4| 0.1   | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     | 0.1     |

**Results**

The carbohydrate composition of ceruloplasmin was determined using NMR spectroscopy. The data showed a complex carbohydrate composition, with significant contributions from glucose (Glc), galactose (Gal), and N-acetylgalactosamine (GlcNAc). These sugars are essential for the protein's function, contributing to its stability and interactions with other molecules.

The detailed analysis revealed that the carbohydrate moiety includes a mix of linear and branched structures, characteristic of complex carbohydrates. The presence of N-acetylgalactosamine (GlcNAc) indicates that the carbohydrate is likely to be a complex type, which is common in many proteins.

**Discussion**

The elucidation of the carbohydrate structure of ceruloplasmin has important implications for understanding its function in copper transport and metabolism. The presence of specific sugars like glucose and galactose suggests interactions with other biological molecules, potentially affecting its biological activity.

Further studies using advanced spectroscopic techniques are needed to confirm these findings and to explore the full complexity of the carbohydrate structure, which may be crucial for developing targeted therapeutic approaches.
Ceruloplasmin Carbohydrates Elucidated by $^1$H-NMR

Fig. 1. Structural reporter group regimes of the resonances observed in the NMR spectrum of the carbohydrate moiety of ceruloplasmin. The structure of ceruloplasmin corresponds to the structure of compound 1, isolated from the urine of man. The peaks at $3.67$ ppm and $3.87$ ppm correspond to the anomeric protons of glucose and mannose, respectively. The peaks at $1.94$ ppm and $2.04$ ppm correspond to the methyl groups of the mannose and glucose, respectively. The peaks at $2.39$ ppm correspond to the protons of the non-carbohydrate moiety. The peaks at $2.49$ ppm correspond to the protons of the acetylated moiety.

[Ceruloplasmin structure diagram]
