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# Characterization of the Microheterogeneity in Glycoproteins by 500-MHz $^1$H-NMR Spectroscopy of Glycopeptide Preparations

## APPLICATION TO A MONOFUCOSYLATED TETRA-ANTENNARY GLYCOPEPTIDE FRACTION FROM HUMAN PLASMA $\alpha_1$-ACID GLYCOPROTEIN*

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**Five hundred-MHz $^1$H-NMR spectroscopy was employed to study a monofucosylated tetra-antennary glycopeptide fraction which was derived from human plasma $\alpha_1$-acid glycoprotein. This fraction was earlier judged to be homogeneous by 360-MHz $^1$H-NMR spectroscopic analysis (Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Binette, J. P., and Schmid, K. (1978) *Biochemistry* 17, 5206-5214). The combination of the improved resolving power and the enhanced sensitivity of the 500-MHz $^1$H-NMR spectrometer afforded the elucidation of a new type of microheterogeneity with regard to the position of attachment of Fuc. Three isomeric compounds were identified. The major form contains Fuc $\alpha$-(1 → 3) linked to GlcNac 7 of the tetra-antennary structure, as shown earlier. The two minor compounds, representing new structures, possess Fuc attached in $\alpha$-(1 → 3) linkage to GlcNAc 7′ or 5′. It is thus noteworthy that this spectral technique allows elucidation of structures of very closely related carbohydrate chains in a glycopeptide mixture.**

Glycoproteins are known to possess several types of microheterogeneity residing in their carbohydrate and polypeptide moieties (1-4). Microheterogeneity of the carbohydrate portion includes differences in the number and size of the branches of the glycans, differences in the degrees of sialylation and fucosylation, and the polymorphism which is considered to be due to positional isomerism of the sialyl residues present. These forms of microheterogeneity can easily be detected if they result in differences in the electrophoretic and chromatographic behavior of the glycoproteins or their glycopeptides. To define the microheterogeneity of the carbohydrate structures, the glycoprotein is first enzymatically digested and, subsequently, the resulting glycopeptides of each glycosylation site are isolated and characterized. Nevertheless, microheterogeneity might escape detection by the commonly available techniques.

One of the typical glycoproteins exhibiting microheterogeneity is human plasma $\alpha_1$-acid glycoprotein (2-6), the glycans of which are of the *N*-acetyllactosamine type. The carbohydrate chains of each of the five glycosylation sites have been studied extensively (4, 5, 7). The 360-MHz $^1$H-NMR spectroscopy permitted rapid elucidation of the structures of most of these chains. Investigation of the glycopeptides of desialyzed $\alpha_1$-acid glycoprotein demonstrated microheterogeneity with respect to the number of branches in the glycans and, in particular, regarding the presence of Fuc. Employing higher resolution $^1$H-NMR spectroscopy, operating at 500-MHz in conjunction with an improved resolution enhancement technique, more details in the spectra were apparent, and this technique afforded an even more precise characterization of the glycan structures (8, 9). One preparation of a monofucosylated tetra-antennary glycan of $\alpha_1$-acid glycoprotein earlier judged to be homogeneous (5) was shown to possess a new type of microheterogeneity as will be described in this paper.

### EXPERIMENTAL PROCEDURES

*$\alpha_1$-Acid Glycoprotein*—This protein was isolated and purified from pooled outdated normal human plasma (10) and the asialoglycopeptide mixtures derived from each of the glycosylation sites of the protein (designated I to V) were prepared as described earlier (11). These mixtures were further fractionated resulting in preparations that were designated with arabic numbers (*e.g.* GP V-3) (11). Fraction GP V-3, which was earlier shown to possess a monofucosylated tetra-antennary structure (5), was reinvestigated.

*500-MHz $^1$H-NMR Spectroscopy*—Prior to NMR analysis, preparation GP V-3 was exchanged repeatedly in $D_2O$ (99.96 atom % D, Aldrich Chemical Co., Milwaukee, WI) employing intermediate lyophilization. For $^1$H-NMR spectroscopic analysis, a Bruker WM-500 spectrometer was employed operating in the Fourier transform mode at a probe temperature of 300 K and equipped with a Bruker Aspect-2000 computer. Resolution enhancement of the spectrum was achieved by Lorentzian to Gaussian transformation from quadrature phase detection, followed by a complex Fourier transformation (12). Chemical shifts are given relative to sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (indirectly to acetone in $D_2O$: $\delta$ = 2.225 ppm).

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## RESULTS AND DISCUSSION

In an earlier investigation (5) employing 360-MHz $^1$H-NMR spectroscopy, it was demonstrated that preparation GP V-3 has a tetra-antennary *N*-acetyllactosamine-type structure with a Fuc residue attached to GlcNAc **7**. This structure was based on the following evidence. The NMR spectral data showed the presence of four *N*-acetyllactosamine branches. The type of substitution of the trimannosyl-*N*,*N*′-diacetylchitobiose core was derived from the chemical shifts of the anomeric protons (H-1s) and of the H-2s of the Man residues. The Fuc residue was found to be located in $\alpha$-(1→ 3) linkage to the peripheral GlcNAc (**7**) of the *N*-acetyllactosamine unit which is $\beta$-(1 → 4)-linked to the mentioned core. The type of Fuc linkage was inferred from the $^1$H-NMR parameters of the Fuc residue itself, namely the chemical shifts of its H-1, H-5, and $CH_3$ group ($\delta \simeq 5.11$, 4.83, and 1.17 ppm, respectively) (5). These values differ significantly from those of Fuc $\alpha$-(1 → 6)-linked to GlcNAc **1** (13), Fuc $\alpha$-(1 → 4)-linked to GlcNAc of the Lewis$^a$ determinant (14), or Fuc $\alpha$-(1→2)-linked to Gal of certain other components (9, 13). The evidence for the linkage of Fuc to GlcNAc **7** of GP V-3 is derived from the chemical shift of the *N*-acetyl singlet of this GlcNAc. Such an $\alpha$-(1 → 3) attachment of Fuc gives rise to a shift decrement of the GlcNAc *N*-acetyl signal: $\Delta\delta \simeq -0.01$ ppm (5).

Reinvestigation of GP V-3 by the recently introduced 500-MHz $^1$H-NMR spectroscopy (8) provided new structural details. The pertinent NMR parameters of the GP V-3 preparation are listed in Table I. The spectral characteristics at 500 MHz are in full agreement with the presence of a tetra-antennary structure (5, 8). However, the resolution-enhanced 500-MHz spectrum of GP V-3 reveals structural reporter group resonances not observable in the 360-MHz spectrum (5), which are detectable now due to the higher spectral resolution and the greater sensitivity. The expanded structural reporter group regions of this 500-MHz spectrum are depicted in Fig. 1.

The resonances in the relatively high field spectral region ($1.1 < \delta < 1.4$ ppm) can be divided into at least four doublets at $\delta = 1.169$, 1.176, 1.177, and 1.178 ppm with an approximate intensity ratio 10:6:1.5:1. The doublets are assigned as follows. The relatively sharp-lined doublet at $\delta = 1.169$ ppm results from the $CH_3$ group of the Thr residue in the peptide moiety of GP V-3 (Asn-Gly-Thr) (11). The doublet at $\delta = 1.176$ ppm is ascribed to Fuc $\alpha$-(1 → 3)-linked to GlcNAc **7** in the major component of the glycopeptide mixture. The remaining two $CH_3$ doublets are due to minor components possessing a Fuc residue also $\alpha$-(1 → 3)-linked to a peripheral GlcNAc other than to GlcNAc **7**. The Fuc H-1 resonances (at $\delta \simeq 5.11$ and 5.12 ppm) and the relatively broad Fuc H-5 signal (at $\delta = 4.832$ ppm) also reflect the presence of Fuc linked to different peripheral GlcNAc residues.

The actual locations of the Fuc residues can be inferred from the *N*-acetyl region ($2.00 < \delta < 2.15$ ppm) of the spectrum. In this region, nine singlets are observed. The three singlets at $\delta = 2.076$, 2.052, and 2.006 ppm which are of equal intensity, are assigned to GlcNAc **2, 5,** and **1,** respectively, in accordance with the NMR parameters of the tetra-antennary afuco-glycopeptide GP V-4 (8). The remaining six signals can be divided into three pairs. The singlets of each pair are separated by $\Delta\delta \simeq 0.01$ ppm. The signal pair at $\delta = 2.077$ and 2.067 ppm having an intensity ratio of about 2:3 is a result of GlcNAc **7**. The former signal corresponds to GlcNAc **7** without Fuc (8) and the latter to GlcNAc **7** with Fuc (5). Similarly, GlcNAc **7′** gives rise to a pair of signals at $\delta = 2.038$ and 2.029 ppm without and with Fuc, respectively, and GlcNAc **5′** to a pair of signals at $\delta = 2.040$ and 2.033 ppm without and with Fuc, respectively. These data indicate that GP V-3 contains three monofucosylated tetra-antennary structures occurring in a molar ratio of 6.2:1.6:1.1. Thus, the major component designated GP V-3A carried Fuc in $\alpha$-(1 → 3) linkage to GlcNAc **7,** as reported earlier (5). One minor component, designated GP V-3B, has its Fuc $\alpha$-(1 → 3)-linked to GlcNAc **7′,** while the other one designated GP V-3C possesses its Fuc linked to GlcNAc **5′.**

TABLE I

*$^1$H chemical shifts of structural reporter groups of constituent monosaccharides for the glycopeptide preparation GP V-3 from $\alpha_1$-acid glycoprotein.*

| Reporter group | Residue[a] | Chemical shift in GP V-3A (Fuc linked to GlcNAc 7) | GP V-3B (Fuc linked to GlcNAc 7′) | GP V-3C (Fuc linked to GlcNAc 5′) |
|---|---|---|---|---|
| H-1 of | **1** | 5.067 | 5.067 | 5.067 |
| | **2** | 4.611 | 4.611 | 4.611 |
| | **3** | 4.758 | 4.758 | 4.758 |
| | **4** | 5.124 | 5.124 | 5.124 |
| | **4′** | 4.866 | 4.866 | 4.866 |
| | **5** | 4.570 | 4.570 | 4.570 |
| | **5′** | 4.594 | 4.594 | ≃4.61[b] |
| | **6** | 4.462 | 4.468 | 4.468 |
| | **6′** | 4.470 | 4.470 | 4.462 |
| | **7** | 4.558 | 4.545 | 4.545 |
| | **7′** | 4.550 | 4.563[b] | 4.550 |
| | **8** | 4.447 | 4.462 | 4.462 |
| | **8′** | 4.479 | 4.473 | 4.479 |
| H-2 of | **3** | 4.209 | 4.209 | 4.209 |
| | **4** | 4.222 | 4.222 | 4.222 |
| | **4′** | 4.092 | 4.092 | 4.092 |
| H-1 of | Fucα(1 → 3) | 5.112 | 5.112 | 5.124 |
| H-5 of | Fucα(1 → 3) | 4.832 | 4.832 | 4.832 |
| $CH_3$ of | Fucα(1 → 3) | 1.176 | 1.177 | 1.178 |
| NAc of | **1** | 2.006 | 2.006 | 2.006 |
| | **2** | 2.076 | 2.076 | 2.076 |
| | **5** | 2.052 | 2.052 | 2.052 |
| | **5′** | 2.040 | 2.040 | 2.033 |
| | **7** | 2.067 | 2.077 | 2.077 |
| | **7′** | 2.038 | 2.029 | 2.038 |

[a] For complete structures and numbering of monosaccharide residues, see Fig. 1.
[b] Tentative assignment.

The interpretation of the *N*-acetyl region is corroborated by the evaluation of the Gal H-1 region ($4.4 < \delta < 4.5$ ppm) of the GP V-3 spectrum (see Fig. 1). From the spectrum of the mono-antennary glyco-asparagine, Gal$\beta$ (1 → 4)-[Fuc $\alpha$(1 → 3)] GlcNAc $\beta$(1 → 2) Man$\alpha$(1 → 6) Man$\beta$ (1 → 4)GlcNAc $\beta$(1 → 4) [Fuc$\alpha$(1 → 6)]GlcNAc $\beta$(1 → *N*)Asn, it is known that Fuc in $\alpha$-(1 → 3) linkage to the GlcNAc residue of an *N*-acetyllactosamine unit gives rise to an upfield shift of H-1 of the neighboring Gal residue ($\Delta\delta \simeq -0.02$ ppm), as compared to its afuco-analogue (15). A similar shift in the spectrum of GP V-3 is observed for the H-1 doublet of Gal **8** (from $\delta = 4.462$ to 4.447 ppm). From the relative intensity of this doublet at $\delta = 4.447$ ppm, it can be inferred that about 60% of GlcNAc **7** bears a Fuc. Concomitant with this relatively large upfield shift of H-1 of Gal **8,** a smaller shift is observed for H-1 of Gal **6,** namely from $\delta = 4.468$ to 4.462 ppm, also amounting to about 60% of the intensity. The latter shift effect results from the spatial proximity of the Fuc residue attached to GlcNAc **7** and the Gal **6** anomeric proton. The partial fucosylation (16%) of GlcNAc 7′ causes an upfield shift of an equal part of the H-1 doublet of Gal **8′,** namely from $\delta = 4.479$ to 4.473 ppm. No definite doublet of H-1 of Gal **6′** in case of the partially fucosylated (11%) GlcNAc **5′** was observed, probably due to its coincidence with the doublet at $\delta = 4.462$ ppm. The intensity ratio of the H-1 doublets of Gal **8′** (at $\delta = 4.479$ ppm) and Gal **6′** (at $\delta = 4.470$ ppm) in the afuco branches is

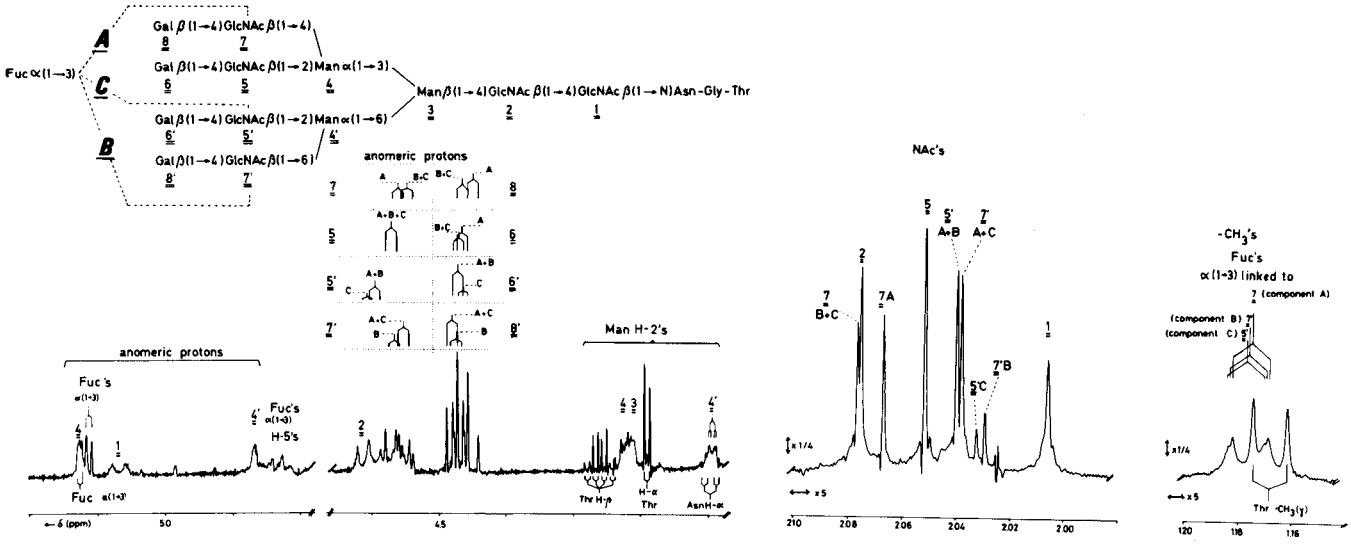


FIG. 1. **Structural reporter group regions of the resolution-enhanced 500-MHz $^1$H-NMR spectrum of glycopeptide preparation GP V-3 from $\alpha_1$-acid glycoprotein, in $D_2O$ at 300 K.** The numbers and letters in the spectrum refer to the corresponding residues in the structure and to the components in the mixture, respectively. The relative intensity scale as well as the chemical shift scale of the high-field spectral regions ($\delta < 2.10$ ppm) differ from those of the low field regions as indicated.

consistent with the relatively large amount of Fuc linked to GlcNAc **7'** (compound GP V-3B) in comparison to Fuc linked to GlcNAc **5'** (compound GP V-3C), as derived from the pattern of *N*-acetyl singlets.

In conclusion, a new type of microheterogeneity found in a glycopeptide fraction from $\alpha_1$-acid glycoprotein has been revealed by 500-MHz $^1$H-NMR spectroscopy. Three isomeric monofucosylated tetra-antennary glycans were demonstrated to be present. In addition the main component which possesses Fuc in $\alpha$-(1 → 3) linkage to GlcNAc **7**, the occurrence of two minor components, having Fuc $\alpha$-(1 → 3)-linked to either GlcNAc **7'** or **5'**, was established. The latter two components have new carbohydrate structures. This microheterogeneity probably reflects a multiplicity of the fucosyltransferase system in the biosynthesis of glycoproteins. Since it is known that a mutual exclusion exists in transferring fucosyl and sialyl residues to *N*-acetyllactosamine branches (16), this Fuc microheterogeneity might be associated with the well known phenomenon of sialic acid polymorphism of $\alpha_1$-acid glycoprotein (2, 6).

With regard to microheterogeneity, it should be emphasized that 500-MHz $^1$H-NMR spectroscopy, because of its high resolving power and sensitivity, is capable of detecting and identifying glycopeptides in mixtures, even if present in small amounts. This eliminates the necessity of separating from one another very closely related compounds such as the isomeric glycans investigated in this study. It can be anticipated that more glycopeptide preparations will be found to be microheterogeneous when investigated by this technique.

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