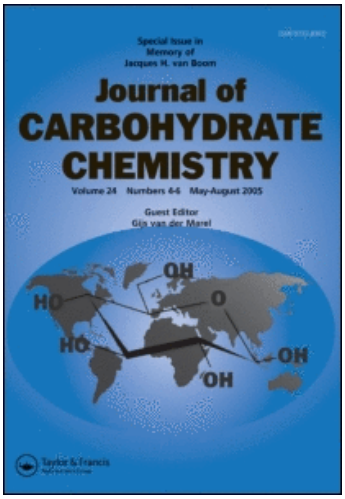

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TWO-DIMENSIONAL NMR SPECTRAL STUDY (AT 500 MHz) OF A GLYCOOCTAPEPTIDE DERIVED FROM GLYCOPHORIN A^M

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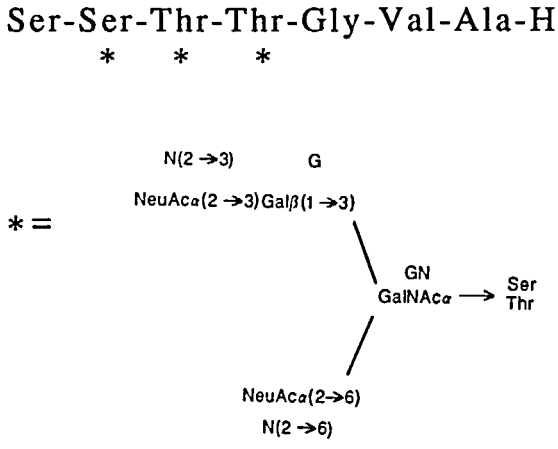
ABSTRACT

One-dimensional and two-dimensional spectral data are presented for the *N*-terminal glycooctapeptide derived from the transmembrane glycoprotein, glycophorin A^M. Two-dimensional homonuclear Hartmann-Hahn spectroscopy (2D-HOHAHA) and two-dimensional rotating frame nuclear Overhauser enhancement spectroscopy (2D-ROESY) were used to assign many of the resonances present in the spectra, with specific emphasis placed upon the oligosaccharide units. Not only were intra residue NOE's detected, but also inter residue NOE's involving two linked carbohydrates [β-Gal(1-->3)α-GalNAc] and also between the α-GalNac-->Thr linkage pair (between GalNAc H-1 and Thr H^β) indicating restricted motion about this glycosidic bond. Moreover, the H-1/H-2 cross-peaks in the 2D-ROESY spectra appear to be well resolved indicating some chemical shift differences for H-1 and H-2 of the three linkage GalNAc residues found in this glycooctapeptide.

INTRODUCTION

A biologically active portion of the *N*-terminal of the transmembrane glycoprotein, glycophorin A can be obtained by treatment of the glycoprotein with cyanogen bromide.[1-3] The reaction produces a glycooctapeptide whose amino acid sequence and carbohydrate structure are given below (1; structure of glycooctapeptide A^M).[1-7] This portion of the molecule is involved in the biological display of the MN blood group antigens.



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Previous studies dealing with this glycopeptide used the traditional one-dimensional ^{13}C-NMR spectroscopic technique.[2] From the authors' data, it was postulated that a unique structure existed about the *N*-terminus involving hydrogen bonding between the glycosidic oxygen atom of α-NeuAc(2-->3)β-Gal and the polypeptide backbone. Our recent multinuclear one- and two-dimensional spectroscopic study of intact glycophorin and tryptic fragments derived from glycophorin did not lead to a clarification concerning a

unique oligosaccharide structure;[8] however, it must be pointed out that this work was accomplished with a large glycopeptide fragment (M_r ~18,000) and it was virtually impossible to observe resonances specifically associated with the *N*-terminus of this molecule.

In this report, we present two-dimensional HOHAHA and ROESY spectral data for the glycooctapeptide derived from glycophorin A^M, which leads to additional information about the carbohydrate structure of the N-terminus of this molecule.

RESULTS AND DISCUSSION

The glycooctapeptide in question contains ~60% carbohydrate by weight (M_r ~3,000) and the carbohydrate residues are present in three identical tetrasaccharides (1). Hence, the carbohydrate residues should be responsible for the majority of intense resonances found in the ^{1}H-NMR spectra. Fig. 1 shows the 2D-HOHAHA contour plot for the glyco-octapeptide. The top spectrum in Fig. 1 is the 1D spectrum obtained for this sample. There are clearly a large wealth of resonances present and many of theses have been assigned based on previous work dealing with related oligosaccharides,[9-11] glycooctapeptides,[2] and tryptic glycopeptides derived from glycophorin.[8] There are also a number of small resonances in our spectra indicating the presence of minor components such as modification by-products.

The 1D spectrum shows that the majority of carbohydrate resonances appear in the region 3.5-5.1 ppm, with some exceptions; the methyl protons of the acetyl moieties of the α-GalNAc and α-NeuAc residues, and H-3 (axial and equatorial) of α-NeuAc residues occur outside this region. Protein resonances occur throughout the spectral region and not all of the resonances overlap with the carbohydrate resonances. For instance, there are three sharp doublets in the region 0.8-1.4 ppm, which represent H^β of Ala and H^γ of the two Thr residues present. The resonance for H^γ of Valine occurs at ~1 ppm but its intensity indicates that it overlaps with other resonances (minor components,

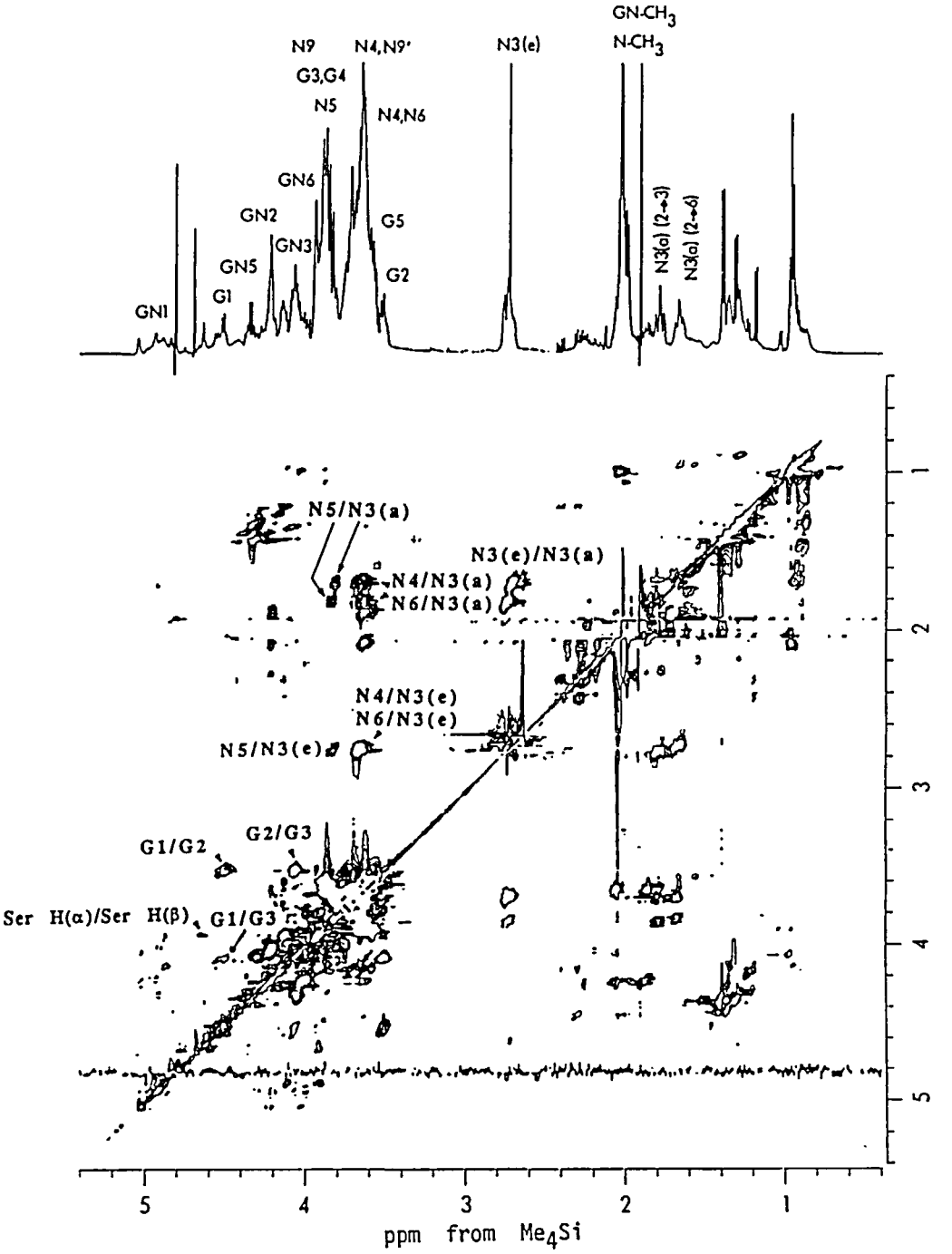

Fig. 1. 2D-HOHAHA contour plot of glycooctapeptide A^M. Sample was 10 mM in D_2O. Data were collected in a 1024 x 512 matrix (zero filled to 1024 x 1024) using a sweep width of 3,610 Hz, a τ value of 38 ms, and a recycle time of 2 s. Sixteen scans were collected per interval at 40 °C. The symbols for the carbohydrate residues given in the figure were taken from structure 1.

by-products). The resonances for H^{β} of Hse and H^{β} of Val overlap with the acetyl methyl resonances at ~2.0 ppm. The resonances for H^{α} and H^{β} of the remaining amino acids, and those not indicated above, all resonate in the region 3.6-4.5 ppm and, therefore, overlap with the carbohydrate resonances.

In order to obtain a clearer picture for the structure of the carbohydrates present, many of resonances need to be identified and the 2D-HOHAHA spectroscopic technique is a meaningful tool for accomplishing this because the method is used to determine total resonance connectivity via scalar coupling. Because of the known small $J_{4,5}$ values for α-GalNac and β-Gal,[12] the 2D-HOHAHA experiment will not show a total chemical shift connectivity for all the resonances of these residues. Likewise, this is also to be expected for α-NeuAc, because $J_{6,7}$ is very small.[11] Note in the region 3.4-4.0 ppm that there are a large number of resonances which overlap. This makes the total resonances assignments very difficult and in some cases the identification of the specific peak assignments in the 1D spectrum is based upon the chemical shifts for the respective model compounds. However, we have made many one-to-one cross-peak assignments in the 2D contour plots on the basis of the unequivocal identification of certain resonances (for instance, α-NeuAc H-3 (a,e)).

In the region between 3.4-5.05 ppm, the resonances for β-Gal and some of the cross-peaks associated with β-Gal can clearly be defined, as one might expect. Based upon model compound chemical shifts, α-GalNac H-1 should resonate at about 5.0 ppm. Only a few small cross-peaks are observed and this scattering of the cross-peaks may reflect a chemical shift non-equivalence for the α–GalNAc residues present; a clearer picture is given by the 2D-ROESY experiment (see later). This chemical shift non-equivalence for the α-GalNAc residues would not be unexpected because ^{13}C-NMR studies on related model compounds showed that chemical shift differences exist for α-GalNAc attached to Ser-2, Thr-3, and Thr-4.[13,14]

The 2D-ROESY experiment gives a more concise picture of some of the resonance connectivities as well as provides

information concerning the three dimensional structure, if any, of the oligosaccharide. The 2D-ROESY contour plot (3.4-5.1 ppm) for this molecule is shown in Fig. 2. Although the 2D-ROESY contour plot may exhibit some of the same cross-peaks observed in the 2D-HOHAHA contour plot, additional peaks should also be present because of the spatial proximity of certain nuclei, even though no scalar coupling was previously observed. Such cross-peaks might result from the spanning of the glycosidic bond (between two attached sugar molecules or between peptide and the attached carbohydrate residue).[8,15] Because of 1,3 axial interactions, cross-peaks should now be observed between nuclei such as β-Gal H-1 and β-Gal H-5, which were not previously observed because of the small $J_{4,5}$ value.[12] The α-GalNac region in the 2D-ROESY contour plot has numerous cross-peaks associated with H-1 of this sugar molecule. The cross-peaks that should be present, based upon other studies, are those for α-GalNAc H-1/α-GalNAc H-2 and α-GalNAc H-1/Thr H^{β}.[8,15,16] The two cross-peaks furthest upfield have been assigned to α-GalNAc H-1/Thr H^{β}. This is based upon the cross-peak chemical shift (~4.4 ppm) as well as the fact that one of the components appears to be coupled to a resonance at 4.6 ppm which is Thr H^{α}. No cross-peak is observed for Ser H^{β} and α-GalNAc H-1, as is expected from our previous work.[8] The three remaining cross-peaks at around 4.2 ppm probably result from the dipolar interaction between α-GalNAc H-1/α-GalNAc H-2. This must indicate that we have unique chemical shifts for both the α-GalNAc H-1 and α-GalNAc H-2 nuclei.

The cross-peaks associated with β-Gal also show some unusual features. The expected cross-peaks are present as indicated in the figure: β–Gal H-1/β-Gal H-3 , β-Gal Gal H-1/β-Gal H-5, and β-Gal H-3/β-Gal H-5. However, an additional peak appears to be present that almost overlaps with the cross-peak associated with β-Gal H-1/β-Gal H-3; note the distended shape of this cross-peak. A 1D spectrum of the slice of this contour shows two peaks are clearly present. Based upon chemical shift and other resonance connectivities, this cross-peak must represent the

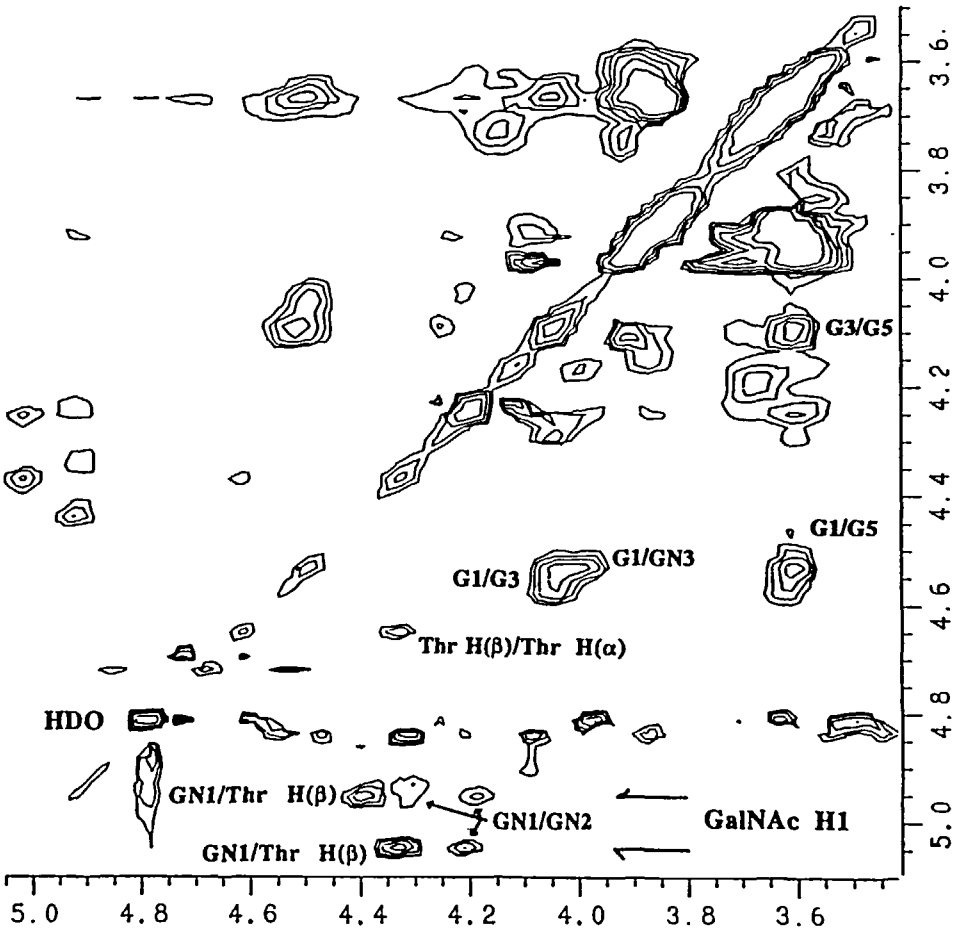

Fig. 2. 2D-ROESY contour plot of glycooctapeptide A^M. Sample was 10 mM in D_2O. Data were collected in a 1024 x 256 matrix (zero filled to 1024 x 512) using a sweep width of 6,024 Hz, a mixing time of 100 ms, and a recycle time of 1.75 s. Nine scans were collected per interval at 40 °C. The symbols for the carbohydrate residues given in the figure were taken from structure 1.

dipolar interaction between β-Gal H-1/α-GalNAc H-3, which is also expected since β-Gal is glycosylated to O-3 of α-GalNAc.

Our data clearly shows that the structure of the oligosaccharide present in Glycophorin A is consistent with 1. Our results clearly indicate that small but noticeable structural differences exist about the *N*-terminus of glycophorin A^M and and these are manifested in the carbohydrate-protein glycosidic linkages. Furthermore, a more extensive study using an elaborate number of mixing times may provide information about atomic distances and molecular interactions that might not have been observed using our single (and shortened) mixing time for the 2D-ROESY experiment.

MATERIALS AND METHODS

Cyanogen bromide and Sephadex G 150-50 were purchased from Sigma. The P-4 resin was obtained from Bio-Rad. Deuterium oxide (99.996% isotopically enriched) was a product of Merck, Sharpe & Dohme. All other chemicals were of reagent grade quality.

Glycophorin A^M and the tryptic glycopeptides obtained from glycophorin A^M were prepared as described previously.[8] Two hundred mg tryptic glycophorin glycopeptide was dissolved in 20 mL of 70% formic acid, and nitrogen was bubbled through the solution for 1 min. To this, 6 g of cyanogen bromide was added and the vessel was closed and the solution was stirred at room temperature (in the dark) for 35 min. The reaction was stopped by a 30-fold dilution with water and the solvent removed under vacuum; the sample vessel was kept in an ice bath during this process. The lyophilized sample was dissolved in 1 mL of 0.1 M acetate buffer, pH 6.8, and loaded onto a Sephadex G 150-50 column (1.5 x 145 cm) equilibrated with the acetate buffer. The effluent was monitored at 230 nm and 5 mL fractions collected. The glycooctapeptide was freeze-dried and chromatographed on a P-4 column (in distilled water containing a trace of sodium azide). Every 2.5 mL of the effluent was monitored at 230 nm and the glycooctapeptide was

collected and lyophilized. The glycooctapeptide sample was prepared for NMR studies by repeated exchange with D_2O and all spectra were recorded with the samples in D_2O.

The ^{1}H-NMR spectral studies on glycophorin glyco-octapeptides were carried out on a GE 500 MHz spectrometer at the National Institute of Environmental Health Sciences facilities located in the Research Triangle Park, NC. 2D-HOHAHA (two-dimensional homonuclear Hartmann-Hahn spectroscopy) and 2D-ROESY (two-dimensional rotating-frame nuclear Overhauser enhancement spectroscopy) spectra were obtained as previously described.[17,18] Further details are given in the respective figure captions.

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