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Note

^{1}H NMR studies of hydroxy protons of the V[β-Gal(1 → 3)-α-GalNAc(1 → O)]THPGY glycopeptide

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Abstract

The hydroxy protons of the disaccharide moiety in the glycopeptide Val-[β-Gal(1 → 3)-α-GalNAc(1 → O)]-Thr-His-Pro-Gly-Tyr (**1**) have been investigated in aqueous solution using ^{1}H NMR spectroscopy. The chemical shifts (δ), coupling constants ($^3J_{CH,OH}$), temperature coefficients ($d\delta/dT$), exchange rates (k_{ex}), and NOEs have been measured. The data show that the O(2′)H of Gal has a reduced contact with water due to steric interference caused by the 2-acetamido group of GalNAc. No interaction, in terms of hydrogen bonding exists between the disaccharide and the peptide moieties, but the rotation around the sugar-peptide linkage is restricted.

Keywords: Conformation; Glycopeptide; Hydroxy protons; NMR; Fibronectin

1. Introduction

The carbohydrate moiety in glycopeptides has been found to influence the backbone conformation of peptides, but in what manner seems to depend on both the amino acid sequence and the structure of the carbohydrate moiety.[1–7] The high number of hydroxy groups render the carbohydrate moiety highly hydrophilic and adds a high number of possible hydrogen bonding sites to the glycoprotein that can not be found in the corresponding unglycosylated protein. One of the two main possible influences of the carbohydrate moiety upon the overall glycopeptide conformation is that it might function as a hydrophilic bulk group with no strong interaction with the peptide backbone. The other possibility is that the formation of strong hydrogen bonds with the peptide part could influence the overall conformation.

The observation of hydroxy proton signals by NMR requires that their rate of exchange with the solvent is sufficiently slow. This can be achieved by using an organic solvent such as DMSO, but in this case the influence of an aprotic solvent on the conformation must be considered.[8] Since it is desirable to study properties such as conformations in conditions that resemble the physiological conditions to as high extent as possible, an aqueous solution is preferred over organic solutions. This has so far been achieved by letting the sample solution be highly concentrated[9] or by adding a small amount of organic solvent.[8,10–20] Some of the hydroxy protons of an anti-

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freeze glycopeptide have also been observed by NMR at a temperature of 5 °C and at a concentration of 17 mg/mL in water, but the signals were not assigned and no attempt was made to use them as an aid in the interpretation of the glycopeptide conformation.[21]

As part of a continuing study[15–20] on the use of hydroxy protons in conformational analysis of saccharides, we have in this work investigated the conformation of the glycopeptide Val-[β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc]-Thr-His-Pro-Gly-Tyr (**1**), which is a partial structure of oncofetal fibronectin.[22] The study was performed in aqueous solution by ^{1}H NMR spectroscopy. The goal of this work was to show (i) that by careful preparation of the NMR sample, it is possible to observe the hydroxy proton signals in **1** and (ii) to obtain additional structural information in terms of chemical shifts, coupling constants, temperature coefficients, exchange rates and NOEs of the hydroxy protons.

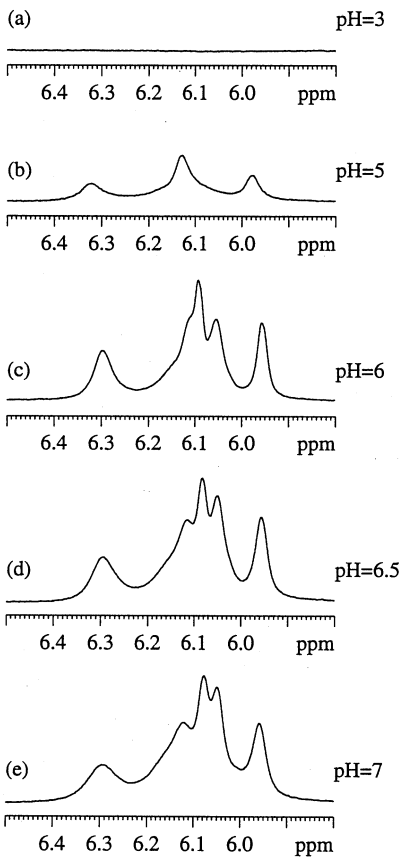

Fig. 1. One dimensional ^{1}H NMR spectra of **1** at −10 °C (a–b) or −8 °C (c–e) of the exchangeable OH protons in 85% water–15% acetone-d_6 at different pH.

2. Results and discussion

Hydroxy and amide protons of the disaccharide moiety. To be able to observe the hydroxy protons of the disaccharide moiety in aqueous solution, the pH was adjusted to 7 by addition of minute amounts of NaOH (Fig. 1). The rate of exchange of the hydroxy protons with the bulk water was further reduced by lowering the temperature to −13 °C. Acetone-d_6 (15%) was added to the solution to be able to reach this temperature without freezing. The exchangeable OH and NH proton signals (Table 1) were assigned on the basis of their scalar connectivities to the non-exchangeable protons (Tables 2 and 3) using COSY and TOCSY experiments.

Table 1 shows that all hydroxy proton signals have $\Delta\delta$ (chemical shift difference between hydroxy proton signals for the disaccharide and the corresponding signals for the monosaccharide methyl glycoside) less than 0.15 ppm with the exception of O(2′)H ($\Delta\delta = -0.45$ ppm) and O(6)H ($\Delta\delta = +0.28$ ppm). A large negative $\Delta\delta$ of 0.39 and 0.42 ppm was previously measured for O(2′)H in β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-OMe and β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-O-Ser, respectively.[20] This shielding was attributed to the steric interference caused by the 2-acetamido group which competes with solvation and leads to the upfield shift of the hydroxy proton resonances. A large positive $\Delta\delta$ has been previously observed for hydroxy protons which are close to hydroxy protons of neighbouring sugars.[16,19] The $\Delta\delta$ of 0.28 ppm measured for O(6)H can not however, be explained by the proximity to a hydroxy group of a neighbouring sugar and the reason for this shielding is still unclear. Due to broad resonance lines, only two $^3J_{OH,CH}$-values, for O(2′)H and O(4′)H could be measured from the one-dimensional NMR spectra. These values, around 4 Hz, indicate conformational averaging with free rotation of the hydroxy proton around the C(2′)–O(2′) and C(4′)–O(4′) bonds. The temperature coefficients have large values, in the range −11.2 to −17.2 ppb/°C, suggesting that the hydroxy protons are not involved in hydrogen bonding interaction. The rate of exchange of O(2′)H, O(4′)H and O(4)H

Table 1
^{1}H NMR chemical shifts, chemical shift differences [a], $^3J_{NH,CH}$-values, temperature coefficients, and exchange rates [b] measured at −13 °C in 85% water–15% $(CD_3)_2CO$ for the NH and OH protons of **1**

	δ (ppm)	$\Delta\delta$	$^3J_{NH,CH}$ (Hz)	dδ/dT (ppb/°C)	k_{ex} (s^{-1})
N(V)H	[c]				
N(T)H	9.19		7.9	−11.2	2
N(H)H	8.88		6.0	−9.4	1
N(G)H	8.95		10.5 [d]	−9.4	[e]
N(Y)H	7.91		7.7	−8.1	1
N(2)H	8.22	−0.13	9.7	−10.4	[e]
O(2′)H	6.14	−0.45	3.8 [f]	−13.7	25
O(3′)H	6.18	−0.07	[g]	−12.9	41
O(4′)H	6.02	0.06	4.8 [f]	−11.2	25
O(6′)H	6.23	0.05	[g]	−15.2	59
O(4)H	6.12	0.04	[g]	−14.9	19
O(6)H	6.37	0.28	[g]	−17.2	62

[a] Chemical shift of the hydroxy proton signal in the disaccharide minus that of the corresponding monosaccharide (methyl β-D-Gal*p* and D-Gal*p*NAc, respectively). A positive difference indicates a downfield shift.
[b] The exchange of amide protons was measured at +5 °C.
[c] The signal could not be detected in the spectra.
[d] $^3J_{NH,\alpha H}+{}^3J_{NH,\alpha' H}$.
[e] Could not be calculated since the rate of exchange of NH protons with water at +5 °C is too low.
[f] $^3J_{CH,OH}$.
[g] Broad signal.

Table 2
^{1}H NMR chemical shifts for the CH protons of the amino acids of **1** measured at −13 °C in 85% water–15% $(CD_3)_2CO$

	α_1	α_2	β_1	β_2	γ_1	γ_2	δ_1	δ_2	Ar-1	Ar-2
Val	4.02		2.26		1.07	1.01				
Thr	4.62		4.34		1.26					
	$J_{\alpha,\beta}=2.0$									
His	4.86		3.09	2.98					7.87	7.11
Pro	4.42		2.28	1.90	1.99	1.99	3.43	3.67		
G	3.99	3.86								
Tyr	4.41		3.13	2.91					7.10	6.77

Table 3
^{1}H NMR chemical shifts for the NH and CH protons of the disaccharide part of **1** measured at −13 °C in 85% water–15% $(CD_3)_2CO$

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	NH	Ac
β-D-Gal*p*	4.41	3.52	3.63	3.90	4.06	3.72	3.80		
	$^3J_{1,2}$ 8.9 Hz								
α-D-Gal*p*NAc	4.79	4.22	4.02	4.21	3.64	3.77	3.77	8.22	2.03
	$^3J_{1,2}$ 3.1 Hz								

with the bulk water is lower than that of the other hydroxy protons. Hydroxy protons whose contacts with the solvent are decreased should have lower exchange rates than hydroxy protons in full contact with the solvent. A lower rate of exchange was previously measured[20] for O(2′)H in the disaccharide β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-OMe and was attributed to a decreased accessibility of water because of the proximity to the 2-acetamido

group of the neighbouring sugar. The lower rate of exchange measured for O(4′)H and O(4)H in **1** was also observed for the monosaccharide methyl glycoside[15]. This might be due to the reduced solvation (hydration) of an axial hydroxy group relative to an equatorial group.[23,24] The data was not as conclusive in β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-OMe and β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→O)-Ser.[20]

The large $^{3}J_{\mathrm{NH\text{-}H2}}$-value of 9.7 Hz measured for the 2-acetamido group in GalNAc indicates a *trans* orientation of N(2)H and C(2)H (a preference for the *anti* conformation around the C(2)–N(2) bond). This coupling constant is very similar to the one measured in the free disaccharide, and indicates that the conformation of the 2-acetamido group is unaffected by the presence of the peptide.

Amide protons in the peptide moiety. The $^{3}J_{\mathrm{NH},\alpha\mathrm{H}}$-values measured from one-dimensional spectra are in the range of 6.0–7.9 Hz, which might indicate the absence of an ordered secondary structure. The temperature coefficients between −8.1 an −11.2 ppb/°C suggest that no hydrogen bond stabilises the structure.

Conformation at the site of glycosylation. The small value of the $^{3}J_{\alpha,\beta}$ coupling constant (2.0 Hz) measured for the Thr residue indicates a restricted rotation around the sugar-peptide linkage. This small coupling constant indicates a preference for an orthogonal orientation between the α- and β-protons of threonine. If the *N*-acetylgalactosamine residue was undergoing motion relative to the peptide backbone through rotation around the Cα–Cβ bond of Thr, a larger value of ~5 Hz would be expected.

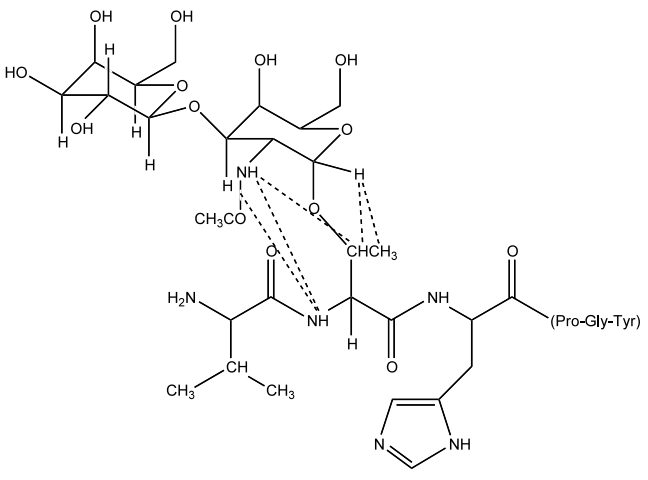

Fig. 2. Schematic representation of the inter-residue NOEs at the site of glycosylation between the disaccharide and the peptide.

Sugar-peptide NOEs. No NOEs were observed between the hydroxy protons of the sugar residues and the protons of the peptide chain. This is not surprising since the hydroxy groups are facing away from the peptide chain (oriented towards the water). NOEs (Fig. 2) were observed between NH(α-D-Gal*p*NAc)-NH(Thr), NH(α-D-Gal*p*NAc)-Hβ(Thr), H1(α-D-Gal*p*NAc)-Hβ(Thr), H1(α-D-Gal*p*NAc)-Hγ(Thr), and between H3(α-D-Gal*p*NAc) and NH(Thr). Since the NOEs from the peptide are only to one face of the sugar, they indicate a relatively persistent interaction between the peptide and the sugar. These NOEs also confirm the restricted rotation around the sugar-peptide linkage.

3. Conclusion

NOEs in the peptide chain. No $d_{\mathrm{NN}}(i,i+1)$NOEs representative of folded conformations in terms of turn or helical structures were found, but the $d_{\alpha\mathrm{N}}(i,i+1)$ NOEs representative of extended conformations were present.

The large negative $\Delta\delta$ measured for O(2′)H together with its lower rate of exchange with water indicate a reduced solvation due to steric interference caused by the 2-acetamido group. The small $^{3}J_{\alpha,\beta}$-value of 2 Hz measured for the threonine residue suggests a restricted rotation of the sugar-peptide linkage around the Thr Cα–Cβ bond with a preference for an orthogonal orientation between the α and β protons of threonine.

4. Experimental

The synthesis of glycopeptide **1** has been previously reported.[25] All NMR experiments were performed on a Bruker DRX-600 spectrometer operating at 600.13 MHz for proton observation. Compound **1** was dissolved in a mixture of 85% water–15% $(CD_3)_2CO$. The pH was adjusted from 3 to 7 by adding a 1 mM NaOH solution to the sample in appropriate amounts. A $^{1}H-^{13}C$ dual microprobe operating in the inverse mode was used. A volume of 100

μL was used giving a sample concentration of 28 mM. $(CD_3)_2CO$ was added to the samples to allow a lowering of the sample temperature to −13 °C without freezing. All spectra unless specified were recorded at −13 °C, except for the temperature coefficients, which were measured by a variation from −10 to 25 °C in steps of 5 °C. The ^1H NMR spectra were referenced by setting the residual acetone-d_5 signal to δ 2.204 ppm. One and two dimensional ^1H NMR spectra were acquired using the WATERGATE pulse sequence for water suppression.[26] The 2D TOCSY and NOESY spectra were recorded in the phase sensitive mode using the TPPI method.[27] The 2D DQF-COSY and ROESY spectra were recorded in the phase sensitive mode using the state-TPPI method.[28] NOESY spectra were recorded with a mixing time (τ_m) of 100, 200 and 300 ms. ROESY spectra were recorded with a mixing time of 150 and 300 ms. Repetition delays of 1.5 s and 256 or 512 spectra of 2K data points were used. The data were zero-filled to 2K × 1K before applying a $\pi/4$ shifted sine-square bell window function in both dimensions. The rates of exchange of the hydroxy protons with water were calculated from 2D phase-sensitive chemical exchange experiments.[29] Mixing times of 3–24 ms in steps of 3 ms were used. 128 FIDs of 2K data points were acquired and a recycle delay of 1.5 s was used. A polynomial baseline correction was applied in both dimensions. The volumes of the NOE cross-peaks and diagonal peaks were measured using the program AURELIA (Bruker, Germany). The initial build-up rates of exchange cross peak volumes were determined from the spectra, and the volumes of the hydroxyl proton diagonal peaks at zero mixing time were obtained by extrapolation from the volumes of the diagonal peaks in the spectra. The exchange rate constants were calculated as the ratio of the initial build-up rates of the exchange peaks over the volume of the diagonal peaks at zero mixing time. The 3D structures of **1** were visualised using the CS CHEM 3D PRO program for Windows.

Acknowledgements

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