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Preliminary communication

The role of the *N*-acetyl group in determining the conformation of 2-acetamido-2-deoxy-D-galactopyranosyl-threonine-containing peptides

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The complex oligosaccharide moieties in glycoproteins may play an important role in biological systems[1,2], for example, in recognition signals in which conformation is thought be important[3,4]. The glycan moieties may be visualised[4] as antennae attached to the protein, but the conformations of the carbohydrate–peptide linkages have not been determined. Although early studies[5,6] suggested that glycosylation played only a minor role in the conformation of the protein backbone, recent data obtained using light-scattering[7], electron microscopy, and ultracentrifugation[8] on "mucin"-type O-glycoproteins indicated the importance of the conformation of the carbohydrate units near to the carbohydrate–peptide linkage. Removal of the GalNAc residue from ovine submaxillary mucin caused the extended filament to collapse to a globular form[8].

Recently, we presented evidence[9] that "mucin"-type, glycosylated threonine-containing dipeptides formed an intramolecular hydrogen bond between the Thr NH group and the GalNAc CO group (Fig. 1), the strength of which was influenced by neighbouring amino acids.

We now report ^{1}H-n.m.r. data for carbohydrate–peptide interactions in the glycosylated tripeptides[9,10], 3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-*N*-(benzyloxycarbonyl)-L-threonyl–L-alanyl–L-alanine methyl ester [ZThr(AcGalNAc)–Ala–Ala–OMe], *N*-acetyl-L-alanyl–L-alanyl-3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine methyl ester [AcAla–Ala–Thr(AcGalNAc)–OMe], *N*-acetyl-L-alanyl–3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonyl–L-alanine methyl ester [AcAla–Thr(AcGalNAc)–Ala–OMe], and 3-*O*-(2-acetamido-3,4,6-tri-*O*-

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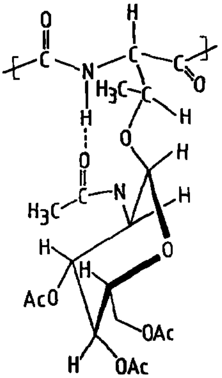

Fig. 1. Thr and AcGalNAc are oriented such that an intramolecular hydrogen bond can form between the Thr NH and the GalNAc CO.

acetyl-2-deoxy-α-D-galactopyranosyl)-*N*-acetyl-L-threonyl-L-alanyl-L-alanine methyl ester [AcThr(AcGalNAc)–Ala–Ala–OMe]. The last three glycopeptides differ only in the location of the glycosylated Thr residue.

The ^{1}H-n.m.r. spectra (270 MHz, JEOL GX 270 spectrometer) were recorded for 0.03M solutions in $(CD_3)_2SO$ (internal Me_4Si). Assignments were based on selective homo-spin decoupling and by comparison with intermediates and glycopeptides[9].

There were no observable concentration-dependent shifts in the range 0.01–0.04M and no significant change in the $^3J_{NH,C\alpha H}$ value in the temperature range 20–50°, so that the temperature coefficients ($d\delta/dT$) of the chemical shift of the amide proton resonance can be used to differentiate possible intramolecularly hydrogen-bonded amide protons. The $d\delta/dT$ data are presented in Table I.

The $d\delta/dT$ value for the Thr NH proton in ZThr(AcGalNAc)–Ala–Ala–OMe is -0.85×10^{-3} p.p.m. deg^{-1}, which confirms the presence of an intramolecular hydrogen bond between the Thr NH and the GalNAc CO group found previously[9]. Thus, the addition of an Ala residue to the Thr C-terminal side of ZThr(AcGalNAc)–Ala–OMe decreases the $d\delta/dT$ value of Thr NH from -2.83×10^{-3} to

TABLE I

TEMPERATURE COEFFICIENTS ($d\delta/dT$) OF THE CHEMICAL SHIFTS OF THE AMIDE PROTON RESONANCES FOR GLYCOSYLATED Thr-CONTAINING TRIPEPTIDES IN $(CD_3)_2SO$

Compound	*$d\delta/dT \times 10^{-3}$ (p.p.m. deg^{-1})*			
	GalNAc NH	*Thr NH*	*Ala^1 NH*	*Ala^2 NH*
ZThr(AcGalNAc)–Ala^1–Ala^2–OMe	−5.08	−0.85	−4.20	−5.13
AcAla^1–Ala^2–Thr(AcGalNAc)–OMe	−5.44	−2.96	(−5.51, −4.77)	
AcAla^1–Thr(AcGalNAc)–Ala^2–OMe	−2.91	−4.68	−5.39	−5.04
AcThr(AcGalNAc)–Ala^1–Ala^2–OMe	−0.07	−4.58	−4.20	−5.82

-0.85×10^{-3} p.p.m. deg^{-1} and suggests that the amino acid residues on the Thr C-terminal side may play an important conformational role. The dδ/dT value for AcAla–Ala–Thr(AcGalNAc)–OMe is -2.96×10^{-3} p.p.m. deg^{-1}, which is similar to that found for ZThr(AcGalNAc)–Ala–OMe[9] and suggests that the strength of the hydrogen bonding is not dependent on the amino acid residues on the Thr N-terminal side.

The $^3J_{NH,C\alpha H}$ value for Thr in ZThr(AcGalNAc)–Ala–Ala–OMe and AcAla–Ala–Thr(AcGalNAc)–OMe is 9.2 Hz, indicating the Thr NH to be almost *trans* to Thr Hα. Also, the small $^3J_{C\alpha H,C\beta H}$ value of 1.6 Hz suggests[11] restricted rotational averaging and/or quasi orthogonal orientation of the Thr Hα and Hβ. As previously reported[9], both coupling constants change to the above values only on glycosylation. Molecular models show that an orientation where an intramolecular hydrogen bond can form between Thr NH and GalNAc CO is consistent with the above data. The conformational preference of the *O*-glycosylic linkage should be determined by the exo-anomeric effect[12]. The formation of a hydrogen bond between Thr NH and GalNAc CO does not oppose this effect. Model studies indicate that H-1,2 of the sugar and Thr Hβ are in close proximity. Such an orientation was thought to be due to the exo-anomeric effect from the n.O.e. study of synthetic T_N glycopeptides[11].

On substitution of the benzyloxycarbonyl group in ZThr(AcGalNAc–Ala–Ala–OMe for acetyl, the dδ/dT value for Thr NH indicates no intramolecular hydrogen bonding. However, the dδ/dT value for the GalNAc NH decreases to almost zero, suggesting its involvement in a strong intramolecular hydrogen bond. Although not as distinct as in AcThr(AcGalNAc)–Ala–Ala–OMe, the dδ/dT values of AcAla–Thr(AcGalNAc)–Ala–OMe also suggest a change in the tendency to hydrogen bond away from Thr NH to GalNAc NH. If there is an intramolecular hydrogen bond with the GalNAc NH as the donor, there are two possible acceptors, namely, the Thr CO and the CO adjacent to the Thr NH. The latter requires an orientation of the CO towards the sugar moiety and rotation about the Thr N–Cα bond, the consequence of which is a change in the Thr $^3J_{NH,C\alpha H}$ value,

TABLE II

CHEMICAL SHIFT (δ) AND $^3J_{H,H}$ VALUES (Hz) FOR THE AMIDE PROTONS OF GLYCOSYLATED Thr-CONTAINING TRIPEPTIDES[a]

Compound	δ (J)			
	AcGalNAc	*Thr*	*Ala^1*	*Ala^2*
ZThr(AcGalNAc)–Ala^1–Ala^2–OMe	7.60 (9.5)	7.19 (9.2)	8.29 (6.7)	8.13 (7.3)
AcAla^1–Ala^2–Thr(AcGalNAc)–OMe	7.59 (9.5)	7.88 (9.2)	[8.06 (7.3), 8.04 (7.3)]	
AcAla^1.Thr(AcGalNAc)–Ala^2–OMe	7.09 (9.5)	8.01 (9.2)	8.26 (7.3)	8.53 (7.0)
AcThr(AcGalNAc)–Ala^1–Ala^2–OMe	7.05 (9.5)	7.98 (9.2)	8.30 (6.7)	8.19 (7.6)

[a] 0.03M Solutions in $(CD_3)_2SO$ at 23°.

but no such changes were observed for AcThr(AcGalNAc)–Ala–Ala–OMe and AcAla–Thr(AcGalNAc)–Ala–OMe. The value of 9.5 Hz for these glycopeptides is similar to those found in ZThr(AcGalNAc)–Ala–Ala–OMe and AcAla–Ala–Thr(AcGalNAc)–OMe (Table II).

The formation of an intramolecular hydrogen bond between GalNAc NH and Thr CO does not greatly perturb the extended conformation of the peptide backbone found in glycopeptides having an intramolecular hydrogen bond between Thr NH and GalNAc CO. However, rotation about the Thr side-chain and the GalNAc C–N bond would be expected. The Thr $^3J_{C\alpha H,C\beta H}$ values (2.6 and 2.2 Hz, respectively) for AcThr(AcGalNAc)–Ala–Ala–OMe and AcAla–Thr(AcGalNAc)–Ala–OMe suggest less restricted rotational averaging or slight rotation away from the quasi orthogonal orientation of Hα and Hβ mentioned previously[9,11]. The fact that this coupling constant is larger for AcThr(AcGalNAc)–Ala–Ala–OMe, a compound having the stronger hydrogen bond, supports the latter explanation.

There is no significant change in the GalNAc $J_{2,NH}$ value, which is 9.5 Hz for each of glycopeptides, indicating a near-*trans* orientation. However, similar coupling constants do not necessarily imply no rotation about this bond. As shown in Fig. 2, two orientations about the GalNAc C-2–N bond may have similar coupling constants. Such a possibility is supported by the distinct change in the chemical shift of the GalNAc NH resonance (Table II), and the glycopeptides studied here and previously[9] can be divided into two groups according to this chemical shift. A value of ~7.6 p.p.m. is found with glycopeptides for which hydrogen bonding is suggested between Thr NH and GalNAc CO, whereas a value of ~7.1 p.p.m. is found with glycopeptides for which hydrogen bonding is suggested between GalNAc NH and Thr CO. Whether these shifts are characteristic of the two intramolecular hydrogen bonds needs to be determined, but this difference in chemical shift of GalNAc NH resonances clearly indicates two different magnetic environments, suggesting rotation about the C-2–N bond.

Model studies show that, in glycopeptides with an intramolecular hydrogen bond between GalNAc and Thr CO, there must be rotation about the *O*-glycosylic linkage away from the orientation found in glycopeptides having a hydrogen bond

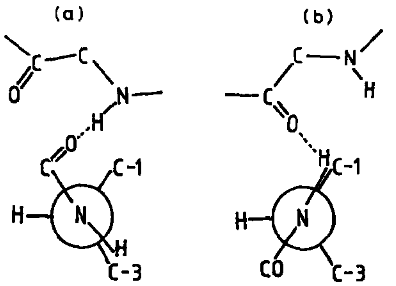

Fig. 2. Projection along the GalNAc C-2–N bond of AcGalNAc–Thr such that (a) the Thr NH can form an intramolecular hydrogen bond with GalNAc CO, and (b) the GalNAc NH can form an intramolecular hydrogen bond with Thr CO.

between Thr NH and GalNAc CO. This rotation need not be large to accommodate the new hydrogen bond, and the preferred orientation due to the exo-anomeric effect is not greatly perturbed. All available data suggest that switching between the hydrogen bonds is not sterically difficult (Fig. 2) and is determined by the amino acid residues on the Thr N-terminal side. The bulk of these residues is a factor which suggests that some presently unknown steric interactions decide the choice of hydrogen bonds.

An intramolecular hydrogen bond involving Thr NH and GalNAc CO implies an orientation where the plane of the sugar ring is roughly perpendicular to the peptide backbone[9]. Formation of the intramolecular hydrogen bond between GalNAc NH and Thr CO requires an orienation where the plane of the sugar ring is roughly in line with the peptide backbone. As these studies are on solutions in $(CD_3)_2SO$, the existence of such hydrogen bonds in biological systems is speculative. However, the idea of two intramolecular hydrogen bonds (which, in principle, can be formed regardless of peptide sequence) as the origin of some preferred carbohydrate orientation has implications in the conformational/biological roles of carbohydrates in glycoproteins.

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