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Complete ^{1}H NMR assignments of synthetic glycopeptides from the carbohydrate-protein linkage region of serglycins*

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We present complete ^{1}H NMR assignments for two synthetic glycopeptides representative of the carbohydrate-protein linkage region of serglycin proteoglycans. The peptides are: Ser(Galp-Xylp)-Gly-Ser-Gly-Ser(Galp-Xylp)-Gly and, Ser(Galp-Xylp)-Gly-Ser(Galp-Xylp)-Gly-Ser(Galp-Xylp)-Gly. A number of 2D NMR spectra together with a 3D NOESY-TOCSY spectrum were acquired at 600 MHz to complete the assignments of the glycopeptides dissolved in water with 40% trifluoroethanol. Preliminary analysis of the NMR data suggests folded structures for the glycopeptides.

Keywords: glycopeptide, NMR, proteoglycan, serglycin, secondary structure

Introduction

Proteoglycans are macromolecules composed of a core-protein with a variable number of covalently attached glycosaminoglycan chains [1]. The biological roles of these molecules are quite diverse and their mechanisms are the subjects of intense investigations. Proteoglycans participate in cell adhesion, cell migration and growth, and have been implicated in the pathogenesis of atherosclerosis [1–5]. Generally, proteoglycan functions appear to be related to their overall physicochemical properties, especially their large size and the high density of negative charges on the attached glycosaminoglycan chains [6, 7]. However, specific interactions of proteoglycans with proteins have also been noted. For example, the anticoagulant action of heparin has been ascribed to the binding of lysine residues in antithrombin to a unique negatively charged pentasaccharide segment in the polysaccharide [8].

A detailed understanding of the molecular basis of proteoglycan function is aided by a knowledge of the tertiary structure of these molecules. Some progress has been made in this general area, and a number of recent investigations by NMR spectroscopy has provided valuable information about hyaluronan and the carbohydrate-protein linkage region of proteoglycans e.g. [9–15]. As part of our continuing effort to study the structural basis for biosynthesis of proteoglycans, we determined the complete ^{1}H NMR resonance assignments at 600 MHz for two glycopeptide fragments representative of the carbohydrate-protein linkages of serglycins [16]. The glycopeptides used in these investigations are: [*O*-β-D-Galp-(1 → 4)-*O*-β-D-Xylp-(1 → *O*)]-L-Ser-Gly-L-Ser-Gly-[*O*-β-D-Galp-(1 → 4)-*O*-β-D-Xylp-(1 → *O*)-L-Ser-Gly, and [*O*-β-D-Galp-(1 → 4)-*O*-β-D-Xylp-(1 → *O*)-L-Ser-Gly-[*O*-β-D-Galp-(1 → 4)-*O*-β-D-Xylp-(1 → *O*)-L-Ser-Gly-[*O*-β-D-Galp-(1 → 4)-*O*-β-D-Xylp-(1 → *O*)]-L-Ser-Gly, designated as bis-glycosylated-hexapeptide (BGH) and tris-glycosylated-hexapeptide (TGH), respectively (Fig. 1)

*A preliminary account of this work was presented at an International Symposium held at the University of Alabama at Birmingham in November, 1994 on the occasion of the 65th birthday of Professor Lennart Rodén.

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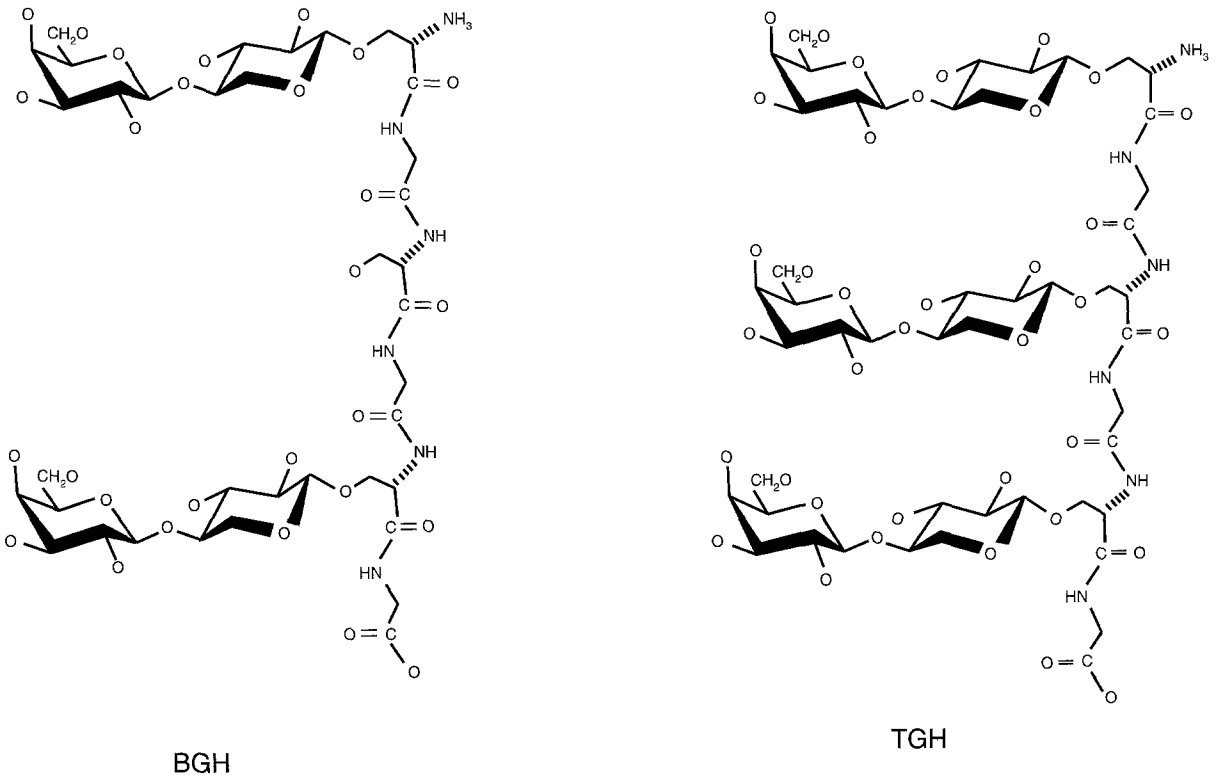

Figure 1. Structures of BGH (left) and TGH (right).

[17]. In addition to the chemical shifts, we also present vicinal coupling constants for the peptides.

Serglycins are a class of proteoglycans aptly named for their long repeating sequences of serine-glycine dipeptides. Both serine and glycine have a strong propensity to form β-turns [18] suggesting that serglycins may form some type of poly-β-turn structures, such as a β-spiral [19] or a 3_{10} helix. Currently there is no evidence to demonstrate this, and indirect evidence appears to contradict such models. Solubilized fragments of silk, which contain regular repeats of the sequence Gly-Ala-Gly-Ala-Gly-Ser are known to serve as efficient substrates for xylose transferase [20]; however, silk forms β-sheet structures, *not helices*. Based upon the silk structure, Schwartz and co-workers proposed a strand dimer model for the structure of some amphiphilic core proteins [21]. The effects of glycosylation on the secondary structure of core proteins are unknown. The current study serves as a basis for further investigations on the nature of interactions between the core protein and the serine-linked carbohydrates.

Materials and methods

Samples

Synthesis of BGH and TGH has been described previously [17]. All samples (~1 mM) were contained in 5 mm NMR tubes (Wilmad Glass Co., Buena, NJ). All solvents were purchased from Cambridge Isotope Laboratories. Spectra of the peptides were recorded in 60:40 (by vol) water and 2,2,2-trifluoroethanol-d_3 [99.94% D] (TFE-d_3). Samples were prepared by adding 300 µl of D_2O [99.97% D] or H_2O to weighed samples of each compound, followed by pH adjustment, before 200 µl of TFE-d_3 was added. The pH meter readings were not corrected for solvent or isotope effects. Aqueous samples were prepared by adding either D_2O or 90:10 (by vol) H_2O/D_2O to the dry compounds as noted, followed by pH adjustment.

NMR Spectroscopy

The ^1H NMR experiments were performed using a Bruker AM-600 spectrometer equipped with an Aspect 3000 computer. Proton chemical shifts were referenced to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Chemical shifts of the amide resonances of BGH and TGH were monitored as a function of pH in 60:40 (by vol) H_2O/TFE-d_3 at 273 K. The data from these titrations were fitted using a Marquardt least squares program to obtain pK_a's of the N and C terminal groups [22].

Two-dimensional NMR data were collected in the phase-sensitive mode using the time-proportional phase incrementation method [23]. Nuclear Overhauser effect spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) experiments on the peptides in 60:40 (by vol)

H_2O/TFE-d_3, at 273 K, were performed with a jump-return (JR) read sequence for water suppression [24]. We also acquired phase sensitive correlation spectroscopy (PH-COSY) spectra of the peptides with selective low-power presaturation of the water resonance. The decoupler and transmitter phases were synchronized to improve water suppression except in the TOCSY experiments, which were performed with the spectrometer in 'reverse mode'. The TOCSY experiments were performed with pulsing on the high-power decoupler channel using an MLEV-17 spin-locking sequence, without additional trim pulses [25]. Typically, a spin-lock power of 9.2 kHz was used (27 μs 90° pulse). A spin-lock time of 74 ms was used in order to observe various spin system connectivities. To minimize sample heating, the duty cycle was kept below 5% for all TOCSY experiments. The NOESY experiments were done with mixing times ranging from 100 to 600 ms. Both NOESY and TOCSY data were recorded with 512 t_1 experiments and 2 K complex t_2 data points. One-hundred twenty-eight and 256 scans were acquired for the TOCSY and NOESY experiments, respectively. In order to reduce the residual water signal on successive scans, the jump-return sequence also employed a 45° phase shift of the NOESY mixing pulse and the TOCSY preparation pulse [26]. Phase-sensitive COSY experiments were recorded with 1024 serial files of 4 K complex data points.

For chemical-shift assignment purposes, we collected a 3D NOESY-TOCSY spectrum of BGH in 100% D_2O, pH 4.2, and 278 K, according to the phase cycling method of Vuister *et al.*, [27]. Incrementation in the second evolution period was accomplished with the use of an external timing accessory (Tschundin Associates). This also allowed for the storage of data in such a way that dummy scans were required only at the beginning of each new t_1 period. Mixing times of 70 ms and 800 ms were used, respectively, for the TOCSY and NOESY segments of the 3D experiment. In both increment dimensions 128 serial files were acquired.

Data were processed on a Silicon Graphics Indigo work-station using FELIX (Biosym Technologies, San Diego, CA). The NOESY and TOCSY data were zero-filled in the t_1 dimension to obtain square matrices of 1 K (real) data points. The COSY data were resolution-enhanced using unshifted sinebell window functions and zero-filled to 4 K (real) data points in both dimensions. This resulted in 0.6 Hz per pt resolution for the spectra of the peptides in D_2O and 1.3 Hz per pt resolution in 60:40 (by vol) H_2O/TFE-d_3. The 3D NOESY-TOCSY data were zero-filled to 256 (real) points and baseline flattened in all three dimensions using a third order polynomial function. The $^3J_{\alpha\beta}$ coupling constants of BGH were obtained from the PH-COSY experiments [28]. The $^3J_{NH\alpha}$ couplings of both glycopeptides were obtained from 1D spectra of samples in 60:40 (by vol) H_2O/TFE-d_3.

Results and discussion

600 MHz ^1H NMR resonance assignments

Our best spectra of the peptides were obtained at lower temperatures, 273 K in 60:40 (by vol) H_2O/TFE-d_3, and 278 K in aqueous solution. Adding TFE-d_3 to the peptides in water helped resolve much of the resonance overlap present in aqueous solution alone, and also increased the number of cross-peaks obtained in NOESY spectra of the peptides (only trivial cross-peaks were obtained in rotating frame Overhauser effect spectra (ROESY) of the peptides in water at 298 K). Figure 2 shows the peptide assignments for BGH. We assigned the ^1H NMR resonances of the glycopeptides in two-steps. First, we identified the peptide ^1H resonances in TOCSY spectra, and sequentially assigned them using standard NOESY methods [29]. The results for TGH are analogous. Second, we identified the carbohydrate resonances in TOCSY spectra, and further confirmed our identifications in COSY spectra. We

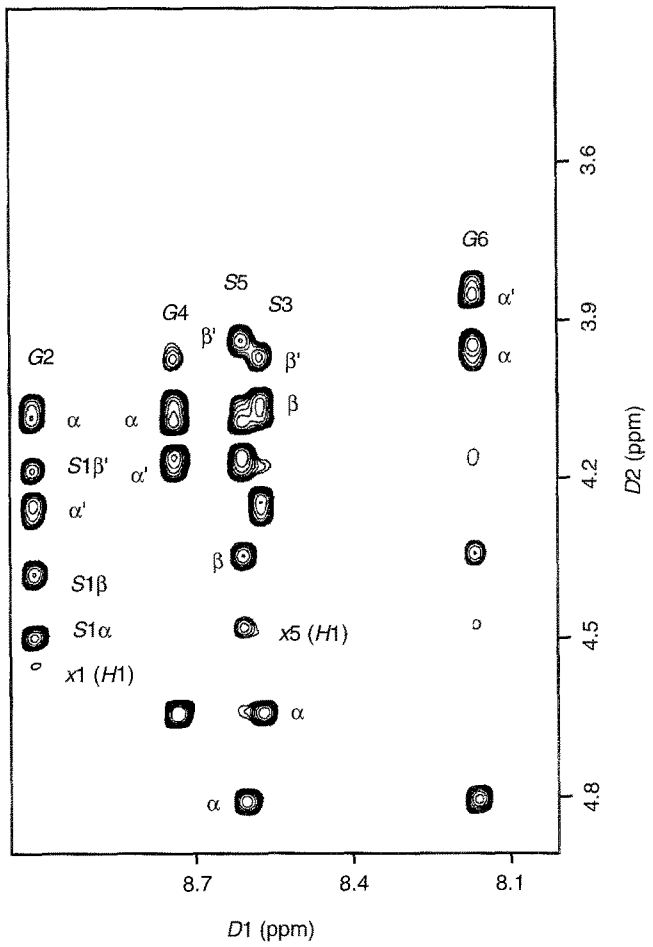

Figure 2. Fingerprint region of a 400 ms NOESY spectrum of BGH, indicating resonance assignments. We denote serine residues 1, 3 and 5 by S1, S3, and S5, glycines 2, 4 and 6 by G2, G4 and G6, and the xylose moities attached to S1 and S5 by x1 and x5 respectively. The sample was 1.0 mM, BGH at 273 K, pH 4.2, 60:40 (by vol) H_2O/TFE-d_3.

sequentially assigned the xylose residues by locating NOESY cross-peaks (NOEs) between their H1 resonances and the assigned serine βH resonances. Figures 3 and 4 show these contacts for BGH. Similar data for TGH were obtained. The chemical shifts of the galactose moieties are degenerate in both glycopeptides, suggesting they have similar conformations. We inferred the galactose assignments from the NOE between the xylose H5 equatorial protons and the galactose H1 protons as in Fig. 4.

To help confirm our assignments we acquired a 3D NOESY-TOCSY spectrum of BGH in D_2O, at 278 K. We used this solvent system to avoid technical difficulties caused by residual protons from TFE in the 3D spectra. A slice of the spectrum is shown in Fig. 5, whereby the Xyl 5 (H1) resonance is on the body-diagonal. A diagonal line denotes TOCSY connectivities between the Xyl 5 protons, and a horizontal line denotes NOE contacts with the Ser 5 protons, in addition to NOEs with other the Xyl 5 protons. The vertical line denotes back-transfer peaks, whereby magnetization is transferred back to the Xyl 5 (H1) proton in two steps; first by NOESY, then by TOCSY. In this figure the peak marked 'A' connects the Gal 5 (H1) resonance to the Xyl 5 (H1) resonance *via* the Xyl 5 (H4) resonance. Observation of this connectivity confirmed our sequential assignments of the Gal 5 (H1) resonances. Similar observations confirmed the sequential assignments of the galactose 1 resonances. Table 1 lists the complete ^{1}H chemical shifts of BGH and TGH.

Stereospecific assignments

Table 2 lists the ^3J-couplings of BGH obtained from 1D NMR and PH-COSY experiments. The $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ couplings of the three serines are each about 4 to 5 Hz, suggesting that the predominant rotamer corresponds to $\chi_1 = 60°$. We stereospecifically assigned the Ser 3 and Ser 5 β-proton resonances of BGH by comparing the NOEs between NH-βH and NH-βH′ in a 200 ms NOESY spectrum of the peptide, using standard methods [29]. The exocyclic angles $\theta = \theta$(O5′-C5′-C6′-O6′) of the galactose residues are also near 60°, based upon the small $^3J_{56}$ and $^3J_{56'}$ couplings obtained from an analysis of a

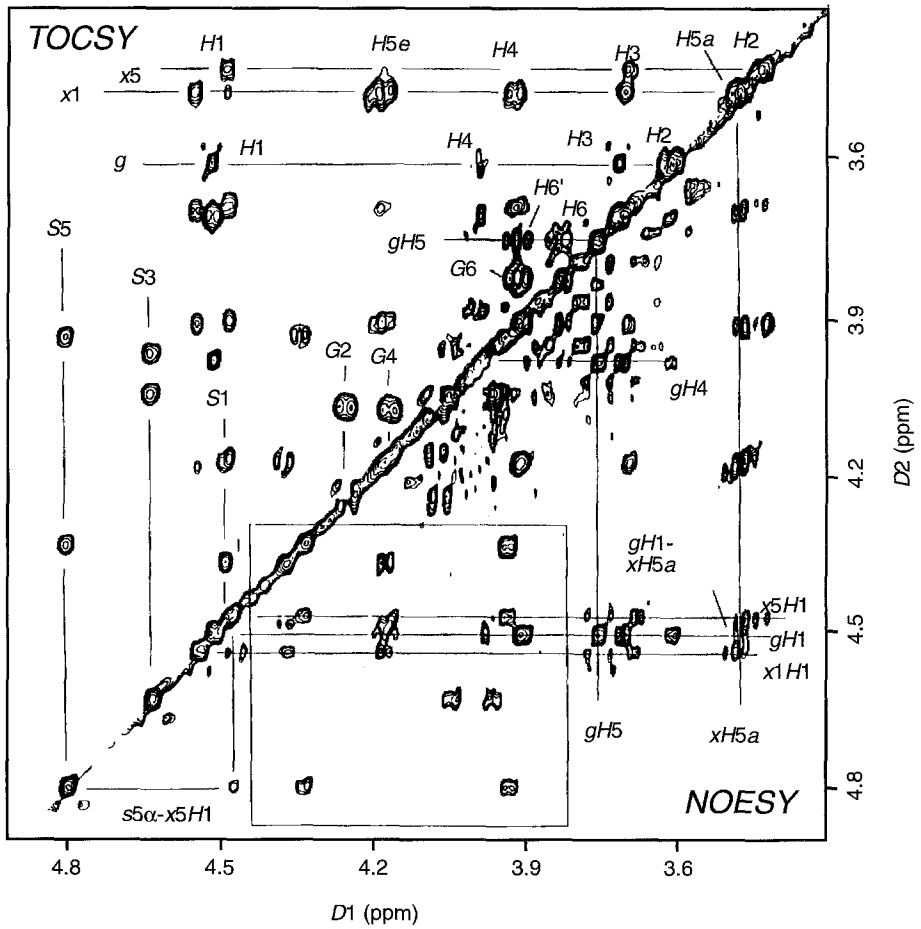

Figure 3. Aliphatic region of a 70 ms TOCSY spectrum of BGH, (above the diagonal) and 400 ms NOESY spectrum (below the diagonal) of BGH. Sample conditions are the same as in Fig. 2. Resonance assignments are indicated in the figure. Here, x1, and x5 denote sequentially assigned xylose 1 and xylose 5 residues, respectively, and g refers to both galactose residues.

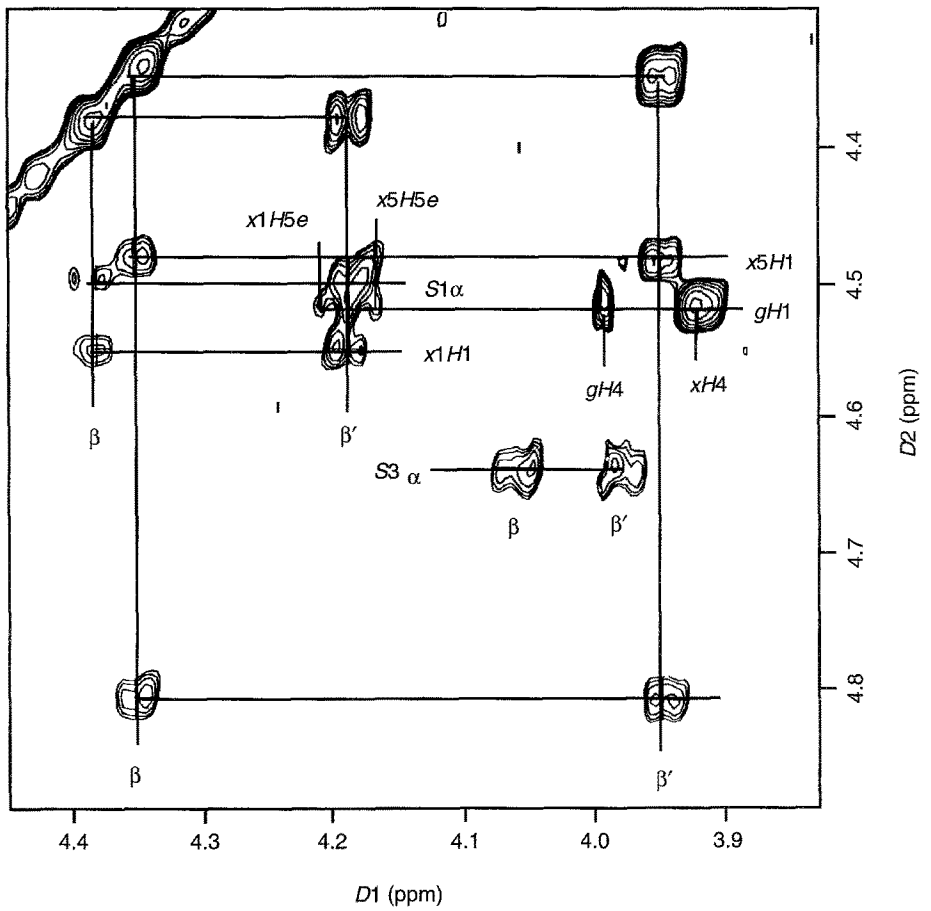

Figure 4. Expanded portion of Fig. 3 indicated by the box. Both serine residues show strong NOESY contacts between both β protons and the corresponding xylose (H1) protons.

PH-COSY experiment in D_2O (Table 2). We stereospecifically assigned the galactose H6 and H6′ protons by comparing their NOEs to the neighbouring H4 protons. The $^3J_{N\alpha}$ couplings of the backbone protons of TGH are essentially identical to those of BGH. We deduced the stereospecific assignments of the TGH carbohydrate resonances by comparing their chemical-shifts to BGH.

Galactose-xylose-serine conformations

Spectra of the peptides in 60:40 (by vol) H_2O/TFE-d_3 were sharp and well-resolved at 273 K, and did not show additional line broadening compared to spectra of the peptides in aqueous solution at 278 K. Under these conditions the NOESY intensities of the peptides are negative and increase linearly in magnitude with mixing time up to 400 ms, at least. These observations are consistent with the notion that the peptides are not aggregated and that they have correlation times that are only slightly longer than the null correlation time (0.296 ns) of the 600 MHz spectrometer. Cross-peaks observed in the 400 ms NOESY spectra of the peptides under these conditions are not due to spin-diffusion and can be used for structural analysis.

Conformations of the galactose-xylose linkages of BGH and TGH are essentially identical to those of galactosyl-xylosyl-serine (GXS) as previously described [10]. The NOESY interactions between xylose and galactose protons are similar in all three compounds (Fig. 3). Significantly, however, conformations of the xylose-serine linkages of both glycopeptides differ from GXS. A portion of a 400 ms NOESY spectrum of BGH in the organic solvent is shown in Fig. 4. For each glycosylated side-chain, both serineβ protons show equally intense 'strong' NOEs to the corresponding xylose H1 proton. This result was also observed in 200 ms mixing time NOESY spectra of BGH (data not shown). For comparison, we acquired a 200 ms mixing time NOESY spectrum of GXS under identical conditions at 400 MHz. A portion of the spectrum is shown in Fig. 6. Under these conditions, NOESY cross-peaks of GXS are the same sign as the diagonal, indicating that the correlation time of the fragment is in the slow tumbling regime. The spectrum indicates that for GXS the β-H1 intensity is 'weak' and the β'-H1 intensity is 'strong'. The contrast between the NOESY spectra of the peptides and GXS suggests that the peptide backbone influences the conformation of the xylose-serine linkages.

The results are less clear-cut for the peptides in water.

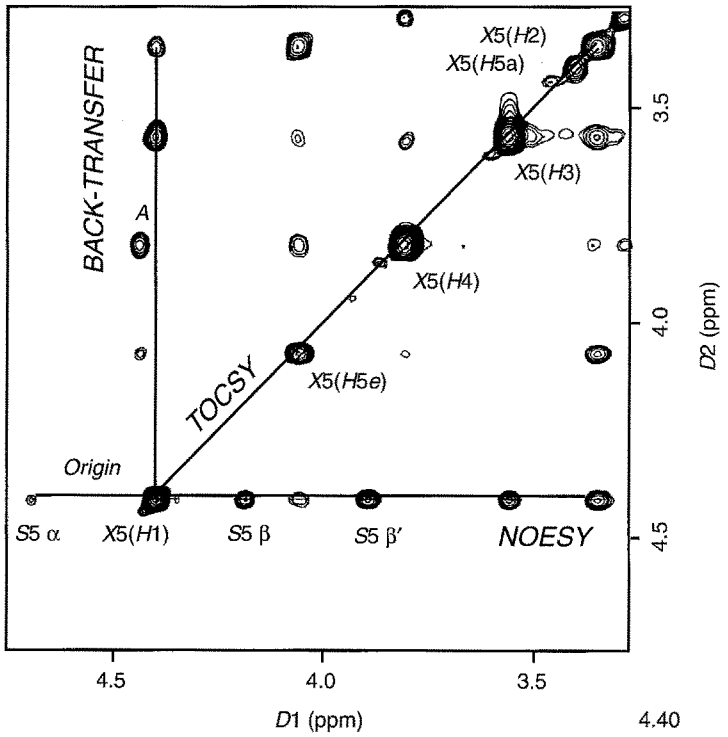

Figure 5. Portions of a 3D NOESY-TOCSY spectrum of BGH. The sample was 1.0 mM BGH, in D_2O at 278 K, pH 4.2. The spectrum was acquired with a 800 ms NOESY mixing time and a 74 ms TOCSY mixing time. The Xyl 5 (H1) resonance is on the body diagonal. The horizontal line indicates NOESY connectivities and the diagonal line denotes TOCSY connectivities. The vertical line denotes back-transfer connectivities. Resonance assignments are described in the text.

The NOESY interactions of the glycosylated serine 5 side-chain of BGH are consistent in both water and organic solvent, but the glycosylated serine 1 side-chain behaves more like GXS (data not shown). This contrast may arise because the N-terminal glycosylated serine 1 side-chain is more like GXS, whereas the glycosylated serine 5 side-chain is situated between two glycines, and is less like GXS. This interpretation is consistent with the notion that the peptide backbone influences xylose-serine linkage conformation. These results also suggest that the organic solvent may help stabilize the peptide backbone conformations, compared to water.

Secondary structure

A preliminary examination of the NMR data suggests that the peptides exist in equilibrium between extended and folded structures. The latter probably involve some closed-loop structures, such that small populations of the peptides maintain an intramolecular salt-bridge between their N and C termini. This hypothesis is based on the following observations. (1) We measured chemical-shift changes in BGH and TGH amide protons as a function of pH, in 60:40 (by vol) H_2O/TFE-d_3, 273 K. Amides near the N and C terminals of both peptides show small chemical-shift changes, but in opposite directions. Curve fits performed on the titration data indicate that the N and C terminals of each peptide sense a pK_a near 3.5, suggesting the presence of a salt-bridge between these moieties in both glycopeptides. However, the rather small chemical-shift changes (~0.005 ppm in both peptides) observed for the Gly 2 NH proton suggests that the populations of these salt-bridged conformations are small. (2) The chemical-shift-index (CSI) has become a useful means of identifying secondary structure in proteins [30]. We measured the CSI values of the BGH and TGH α protons with respect to coil values in 60:40 (by vol) H_2O/TFE-d_3 (Table 1). All of the serine residues in BGH and TGH have positive CSI values. The observation that serine 3 from BGH which is non-glycosylated also shows positive CSI suggests that these shifts are not a consequence of inductive effects associated with glycosylation. Under these same conditions serines from non-glycosylated model compounds, such as $(Ser\text{-}Gly)_7$, have zero CSI values (data not shown). This suggests that the positive CSI values of the serines in the two glycopeptides are a consequence of specific conformations different from that of the control peptide. Interpretation of the CSI values requires some caution since less common secondary structures can sometimes exhibit consecutive positive CSI values that are not β-strand [31]. (3) Random coil $^3J_{\alpha\alpha'}$ geminal-couplings of glycine residues are near −15 Hz [32] whereas, glycines in both glycopeptides have

Table 1. ^{1}H Chemical shifts of BGH and TGH, in ppm (± 0.003), referenced to DSS.

Residue	*^1H**	*TGH^A*	*BGH^A*	*BGH^B*	*BGH^C*
Ser 1	α	4.48(+)	4.50(+)	4.28(–)	4.29(0)
	β	4.36	4.39	4.23	4.24
	β′	4.17	4.19	4.03	4.07
Gly 2	N	8.99	9.00	–	–
	α′	4.26(+)	4.26(+)	4.11(+)	4.15(+)
	α	4.05(+)	4.09(+)	4.00(+)	4.06(+)
Ser 3	N	8.78	8.57	–	–
	α	4.79(+)	4.64(+)	4.51(+)	4.56(+)
	β	4.36	4.06	3.90	3.95
	β′	3.97	3.98	3.85	3.90
Gly 4	N	8.64	8.73	–	–
	α′	4.14(+)	4.17(+)	4.04(+)	4.08(+)
	α	4.09(+)	4.08(+)	3.98(0)	4.03
Ser 5	N	8.61	8.61	–	–
	α	4.80(+)	4.81(+)	4.70(+)	4.73(+)
	β	4.33	4.35	4.19	4.21
	β′	3.97	3.95	3.90	3.96
Gly 6	N	8.26	8.16	–	–
	α	3.98(+)	3.94(0)	3.79(0)	3.85(0)
	α′	3.91(0)	3.84(0)	3.71(–)	3.77(–)
Xyl 1	H1	4.54	4.55	4.43	4.49
	H2	3.47	3.48	3.33	3.38
	H3	3.69	3.70	3.57	3.62
	H4	3.89	3.92	3.81	3.86
	H5 eq	4.17	4.19	4.07	4.12
	H5 ax	3.47	3.48	3.35	3.42
Gal 1, (3)†, 5	H1	4.51	4.52	4.43	4.49
	H2	3.60	3.62	3.47	3.52
	H3	3.71	3.72	3.61	3.65
	H4	3.98	3.99	3.87	3.95
	H5	3.75	3.77	3.67	3.72
	H6′	3.91	3.92	3.76	3.82
	H6	3.82	3.83	3.71	3.76
Xyl (3)†, 5	H1	4.49	4.49	4.40	4.45
	H2	3.44	3.44	3.29	3.35
	H3	3.69	3.70	3.56	3.62
	H4	3.91	3.92	3.81	3.86
	H5 eq	4.17	4.18	4.06	4.12
	H5 ax	3.44	3.49	3.36	3.42

*The prime denotes the H3 proton in the IUPAC convention.
†for TGH.
[A]60:40 (by vol) H_2O/TFE, pH 4.2, 273 K (2D TOCSY and NOESY).
[B]100% D_2O, pH 4.2, 278 K (3D NOESY-TOCSY).
[C]100% D_2O, pH 4.2, 298 K (2D PH-COSY).
CSI are denoted by (+) (0) (–); random coil ppm, Ser 4.38, Gly 3.88.

Table 2. *J*-Coupling of ^{1}H resonances of BGH, in Hz (Resolution 0.6 Hz, 1.3 Hz).

Residue	*^1H–^1H*	*J^A*	*J^B*
Ser 1	α–β	5.4	5.3
	α–β′	3.6	4.5
	β–β′	–11.1	–11.3
Gly 2	N–α	–	5.9
	N–α′	–	5.2
	α–α′	–17.0	–17.1
Ser 3	N–α	–	7.0
	α–β	5.7	5.2
	α–β′	4.4	4.5
	β–β′	–11.7	–11.3
Gly 4	N–α	–	5.5
	N–α′	–	5.5
	α–α′	–17.0	–17.0
Ser 5	N–α	–	7.7
	α–β	5.8	4.6
	α–β′	4.1	5.1
	β–β′	–11.0	–11.0
Gly 6	N–α	–	5.9
	N–α′	–	5.0
	α–α′	–17.3	–17.3
Xyl 1,5	1–2	7.5	7.7
	2–3	9.8	10.2
	3–4	8.0	7.9
	4–5eq	5.3	5.4
	4–5ax	10.0	10.7
	5eq–5ax	–11.8	–11.9
Gal 1,5	1–2	7.8	7.9
	2–3	9.9	10.4
	3–4	3.1	3.5
	4–5	1.9	1.3
	5–6′	8.4	8.3
	5–6	3.7	4.5
	6–6′	–11.7	–12.6

A 100% D_2O, pH 4.2 278 K.
B (40/60) TFE/H_2O, 278 K.

relatively large geminal-couplings near −17.0 Hz (error ± 0.6 Hz) (Table 2), suggesting the presence of stable secondary structures. Although the $^3J_{N\alpha}$ vicinal-couplings of the peptides are near random coil values (Table 2), a loop model of the peptides can be constructed that is also compatible with the vicinal and geminal couplings and the salt-bridge data; with ϕ torsion angles of serines 3 and 5 near −155°, and ϕ and ψ torsion angles of the glycines near 125° and −160°, respectively. (4) Sequential d_{NN} connectivities of the peptides in 60:40 (by vol) H_2O/TFE-d_3 and $d_{\alpha\alpha}$ connectivities in 60:40 (by vol) D_2O/TFE-d_3 were observed in NOESY spectra of the glycopeptides (data not shown) which are also consistent with a loop structure model and are not expected for a β-strand structure, as in the silk polymer. We have refrained from

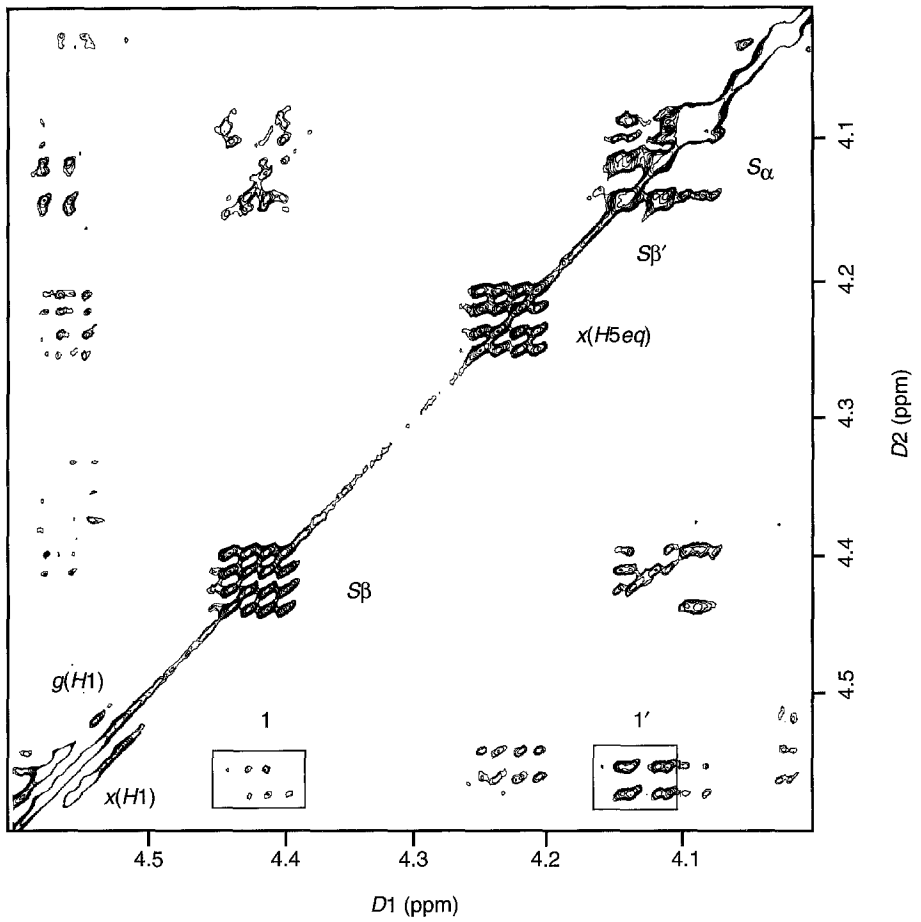

Figure 6. A portion of a 200 ms NOESY spectrum of 1.0 mM GXS acquired at 400 MHz in 60:40 (by vol) D_2O/TFE-d_3, pH 4.2 and 273 K. The boxed regions marked 1 and 1′ indicate interactions between the xylose H1 protons and the serine β and β' protons respectively.

deriving model structures of BGH and TGH because of difficulties in quantitating the NOESY data in terms of contributions from several populations of the various conformers. Structural analysis would benefit from the synthesis of longer serglycin glycopeptide sequences which should exhibit greater stability.

In conclusion complete chemical shift assignments and vicinal coupling data for two glycopeptides representative of the carbohydrate-protein linkage regions of serglycins have been presented. The evidence presented here argues for some folded structures for BGH and TGH. The observed differences in the NOESY spectra of these peptides with respect to GXS, provide evidence that the serine-xylose linkage is influenced by the peptide backbone. Our findings suggest an interaction between the core protein and the carbohydrate segment. Such interactions may play a role during the biosynthesis of proteoglycans.

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