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POSSIBLE ROLE OF THE CARBOHYDRATE RESIDUES ON THE STRUCTURE OF THE N-TERMINUS OF GLYCOPHORIN A^M

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ABSTRACT

Natural-abundance ^{13}C nuclear magnetic resonance (^{13}C-n.m.r.) was used to study the effect of monoglycosylation on the structure and dynamics of a pentapeptide related to the N-terminus of glycophorin A^M. The results of this study indicate that a single point of glycosylation, on the pentapeptide, can significantly affect its structure. Moreover, glycosylation of this pentapeptide also affects its dynamic motion in solution. This study further defines the role that the carbohydrate residue plays in determining the structure about the N-terminus of glycophorin A^M.

INTRODUCTION

The effects of glycosylation on the structure and function of glycoproteins has been of interest for some time[1]. Of recent particular interest are the effects of glycosylation and amino acid substitution on the structures of glycophorins A^N and A^M near the N-terminus of these glycoproteins and their antigenic properties[2–13]. They are known to carry the MN blood group determinants found on the human erythrocyte membrane[5,14–16]. The MN antigenic properties of these molecules appear to be localized within the first six amino acid residues of the glycoconjugates, and also appear to involve the carbohydrate residues attached to some of these amino acid residues[3,12,13].

To obtain structural information on the N-terminus of glycophorins A^N and A^M in solution and within the red-cell membrane, we have undertaken extensive ^{13}C-labeling studies[17–26] of the N-terminus of glycophorins A^M and A^N, and B. By monitoring these ^{13}C labels, we have been able to show that glycosylation at residues 2 and 3 seems to perturb the structure about the N-terminus[25], whereas substitution of amino acid residues at residues 4 and 5 appears to have a limited

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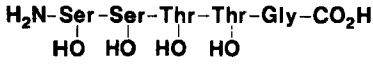
effect. Moreover, from studies on reductively (^{13}C)methylated glycophorin A^M, we observed unexpected behavior for this glycoprotein that indicated an unusual form of aggregation in solution[17,18,23,26].

Unfortunately, addition of C-13 labels to a macromolecule, by modification of one of the functional groups, has the potential of altering the molecular structure of the macromolecule. In view of this, and the necessity of knowing whether glycosylation of glycophorin A^M affects its structure and hence its antigenicity, we decided to investigate the structure and dynamics of pentapeptide **1** and variously monoglycosylated pentapeptides (**2–4**) that are identical in sequence to the first 5 N-terminal residues of glycophorin A^M.

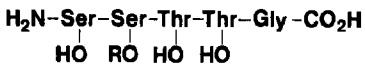
Information on the structure and segmental motions of compounds **1–4** is provided by ^{13}C chemical-shift data, pH-titration information, and the T_1 values of the various carbon atoms.

EXPERIMENTAL

Syntheses of model compounds. — Peptide **1** and glycopeptides **2–4** were synthesized by the conventional liquid-phase procedure[27,28] according to Schemes I–IV. In general, the benzyl group (Bzl) was chosen as the protecting group for the carboxyl group of glycine, the hydroxyl group of serine and threonine, and the hydroxyl groups of the galactosyl group. The *tert*-butyloxycarbonyl group (Boc) was used for temporary protection of the N-terminal α-amino group during the

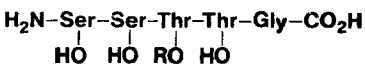


1



2

R = α-D-GalNAc



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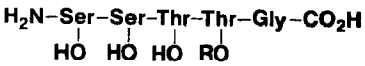
R = same as for 2



4

R = same as for 2

syntheses, except for Ser[1], for which the benzyloxycarbonyl (Z) group was used. *N*-Hydroxysuccinimyl (OSu) and *p*-nitrophenyl (ONp) active esters were used as acylating agents.

Some of the simple compounds were purchased; the more-complex derivatives were synthesized. Glycine benzyl ester and the active esters of the protected serine and threonine were purchased from Bachem, Switzerland. *N*-Hydroxysuccinimyl *O*-(2-azido-2-deoxy-3,4,6-tri-*O*-benzyl-α-D-galactopyranosyl)-*N*-(fluorenylmethoxycarbonyl)-L-threoninate and *p*-nitrophenyl *O*-(2-azido-2-deoxy-3,4,6-tri-*O*-benzyl-α-galactopyranosyl)-*N*-(fluorenylmethoxycarbonyl)-L-serinate were prepared as described by Ferrari and Pavia[27]. Removal of the fluorenylmethoxycarbonyl (Fmoc) protecting group was achieved, in 1 h, in dichloromethane containing 10% piperidine. The Boc protecting group was removed by the action of 1:1 trifluoroacetic acid–dichloromethane.

Amino acid coupling-reactions, using the active ester compounds, were performed in dichloromethane containing 1.5 equivalents of ethyldiisopropylamine. Reduction and subsequent acetylation of the 2-azido derivative was performed by the procedure of Ferrari and Pavia[28].

Final deprotection of pentapeptide **1** and glycopentapeptides **2–4** employed catalytic reductive hydrogenation in methanol with 10% palladium-on-charcoal and hydrogen (5 bar) for 15 h at room temperature. The deprotected model-compounds were purified by gel filtration on Sephadex G-10 (Pharmacia). These methods afforded compounds **1**, $[\alpha]_D^{20}$ −35.8° (*c* 1.0, H_2O) [lit.[28] −38.9° (*c* 1.0, H_2O)]; **2** $[\alpha]_D^{20}$ +40° (*c* 1.0, H_2O); **3**, $[\alpha]_D^{20}$ +48° (*c* 1.0, H_2O); and **4** $[\alpha]_D^{20}$ +28° (*c* 1.0, H_2O).

All reactions were monitored by t.l.c. during the syntheses. The peptides and

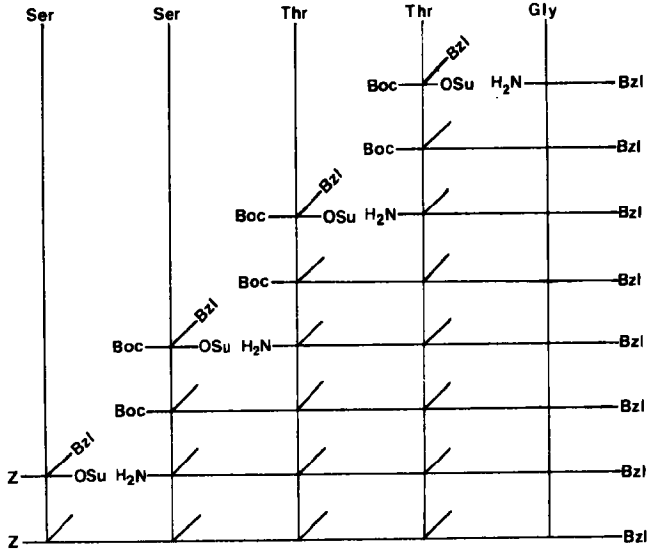


Chart I. Synthesis of compound **1**.

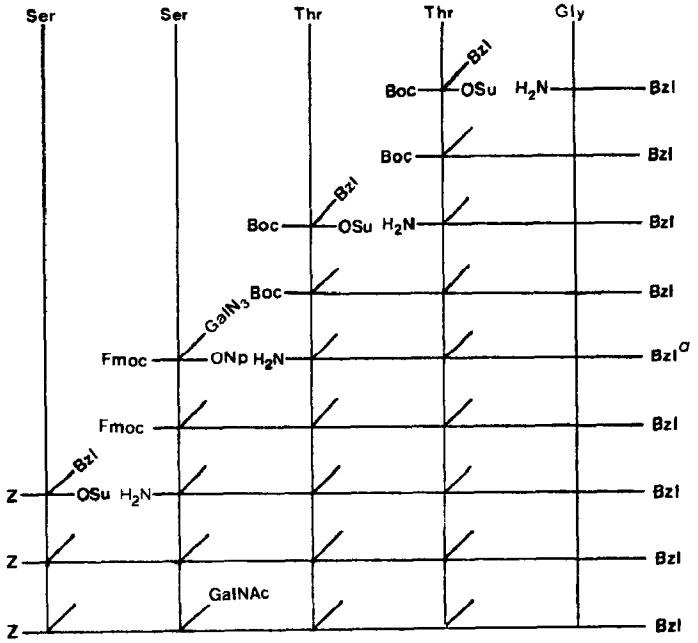


Chart II. Synthesis of compound **2**.
[a]Pure α anomer of the glycosyl active ester was used in this coupling step.

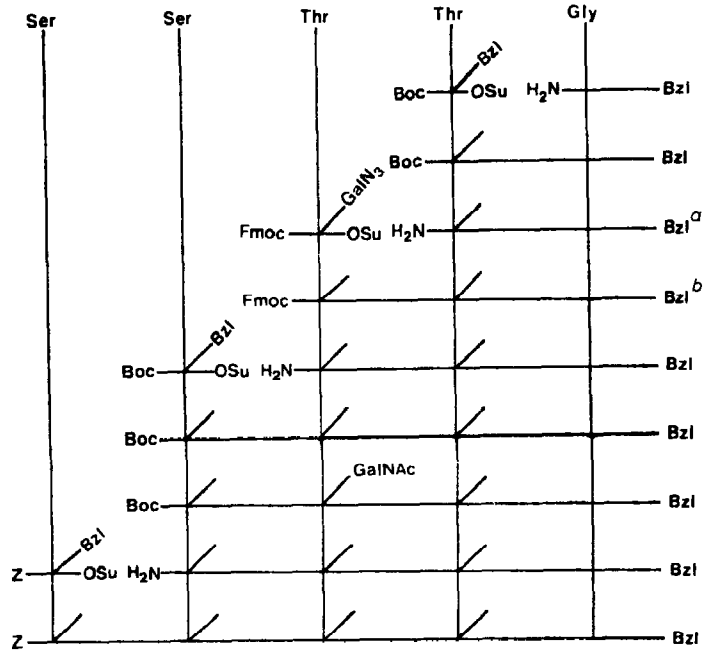


Chart III. Synthesis of compound **3**.
[a]Coupling was performed with the anomeric mixture of α-(2-azido-2-deoxy-D-galactopyranosyl)-*N*-Fmoc-L-threonine active ester. [b]Pure α anomer was obtained after liquid-chromatographic separation, on silica gel, of the glycosyl tripeptide (7:3 v/v hexane–ethyl acetate).

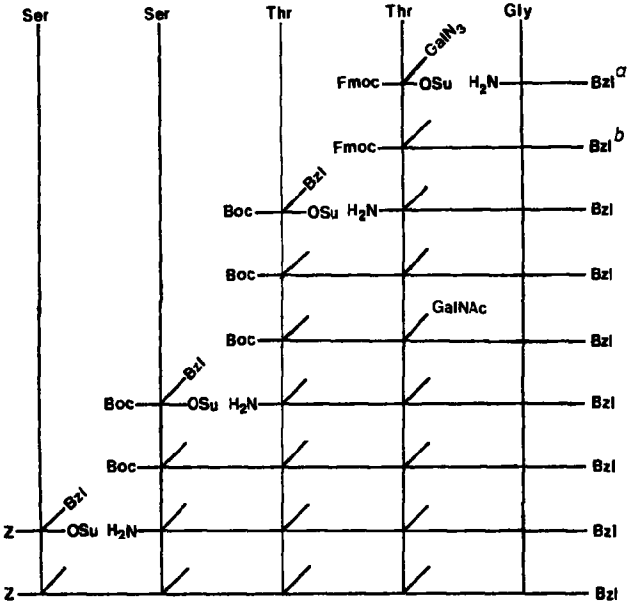


Chart IV. Synthesis of compound **4**.
[a]Coupling was performed with the anomeric mixture of α-(2-azido-2-deoxy-D-galactopyranosyl)-*N*-Fmoc-L-threonine active ester. [b]Pure α anomer was obtained after liquid-chromatographic separation, on silica gel, of the glycosyl dipeptide (725:275 v/v hexane–ethyl acetate).

glycopeptides were purified by liquid chromatography after each coupling step. The structure of the intermediates and the final products were confirmed by ^{13}C-n.m.r. spectroscopy.

Sample preparation. — Model compound samples were treated with Chelex-100 (H^+) to remove trace metals and were freeze-dried before any data, especially T_1 data, were recorded. For n.m.r. use, these samples were dissolved in deionized-distilled water and the pH of the sample adjusted using, M NaOH or M HCl. pH Measurements were made with a Radiometer PHM63 digital pH meter. The pH values of the samples were checked before and after each n.m.r. run and the data were used if the pH drift was ≤0.1 pH units.

N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were obtained with a JEOL-FX90Q spectrometer operating at 22.5 MHz (2.1 T) in the F.t. mode as described previously[29]. Time-domain data were collected in 8,192 addresses. Chemical shifts are given relative to Me_4Si.

Spin–lattice relaxation times of the various carbon atoms were determined by the p.r.f.t. method[30] with up to two sets of 6 τ values. Estimated precision was ±15%.

pH-Titration analysis. — The pH-titration data for the various carbon atoms were analyzed for the best pK_a values and Hill coefficients (n) by the equation:

$$\delta_T = \delta_B + \frac{\Delta 10^{n(pK-pH)}}{1 + 10^{n(pK-pH)}}$$

In this case, δ_T is the best theoretical ^{13}C chemical-shift value, δ_B is the chemical-shift value of the species in the base form, and Δ is the chemical-shift difference between the system in the protonated and the non-protonated forms. The best fit for the data was obtained when the following equation was minimized: $[\delta_T(i) - \delta_{obs}(i)]^2$, where $\delta_T(i)$ is the theoretical chemical shift at a given pH and $\delta_{obs}(i)$ is the observed chemical shift at that pH.

RESULTS AND DISCUSSION

The structural differences that exist near the N-terminus of the glycophorins and also result in display of the MN blood-group antigens have been of interest for some time. A recent, detailed n.m.r. study has focused on the tertiary structure of the N-terminal portion of these glycoproteins, with particular emphasis on possible hydrogen-bonding schemes involving some of the carbohydrate[31] and amino acid residues. The evidence suggests that both types of residue affect the structure about the N-terminus.

Our recent work on the ^{13}C methylation of the N-terminal amino group also indicates that some of the carbohydrate residues have a pronounced effect on the structure about the N-terminus[18,19,25]. The labeling studies also show[25] that, for glycophorin A^M, a unique, intramolecular hydrogen-bond may exist at the N-terminus of this glycoprotein involving the O^γ and N^α-H moieties of Ser^1. Recent work by Aubry and coworkers[32,33] indicates that intermolecular hydrogen-bonding involving O^γ of one residue and N^α-H of the neighboring residue may also exist. These results indicate that glycophorin A^M may possess a rather unique structure.

Natural-abundance ^{13}C-n.m.r. was used here to investigate the structure and dynamics of compounds **1–4**. These compounds are identical in sequence to glycophorin A^M, which also has tetrasaccharides glycosylated to Ser^2, Thr^3, and Thr^4. Compounds **2–4** are variously monoglycosylated with an α-D-GalNAc residue attached to either Ser^2, Thr^3, or Thr^4. α-D-GalNAc is also the linkage carbohydrate residue of the native tetrasaccharide. The change of position of the monosaccharide was expected to show whether monoglycosylation by a monosaccharide, and variation of its position near the N-terminus, can influence the structure about the N-terminus. The single glycosylation does not confer the steric bulk and hydrogen bonding that may occur with a tetrasaccharide, but it does provide a simple basis for further study of more-complex systems. Moreover, evidence from our laboratory[34] has previously shown that monoglycosylation of a simple peptide may result in minor conformational changes of the peptide that can be monitored by ^{13}C-n.m.r. spectroscopy.

The effects of glycosylation on the structure and dynamics of peptide **1** may be determined from the T_1 values of the various carbon atoms, chemical-shift

differences between compounds **1–4**. and changes in the titration parameters (electrostatics). These data are provided in Tables I–IV. The ^{13}C resonance assignments to specific carbohydrate and amino acid carbon atoms were based on our previous, extensive n.m.r. work on glycopeptides containing Thr, Ser, Gal, and GalNAc[22,34–40]. The anomeric coupling constant, $^1J_{C\text{-}H'}$ was ~170 Hz in **2–4**, indicating the α-pyranose configuration for the GalNAc residue[35].

Table I lists ^{13}C chemical-shift and titration data for compounds **1–4**, and Table II gives the chemical-shift differences between the carbohydrate residues of compounds **2–4**. Only two carbohydrate carbon atoms appear to be substantially affected by varying the point of glycosylation; these are C-1 of α-D-GalNAc and C-3 or C-4 (C-3 and C-4 were not specifically differentiated). The chemical-shift differences for C-1 of α-D-GalNAc merely results from glycosylation to either Ser or Thr[35]. The difference in the chemical shift of either C-3 or C-4 of α-D-GalNAc upon glycosylation to Ser^2, Thr^3, or Thr^4 is more difficult to explain in view of the fact that there is even a significant difference between the sugar attached to Thr^3 or Thr^4. The chemical shifts for C-3 and C-4 of α-D-GalNAc of compound **4** are more consistent with the chemical-shift values of 70.0 and 69.1 p.p.m. observed[35] (but not differentiated) for C-3 and C-4 for the α-D-GalNAc group of the glycopeptide α-D-GalNAc→Thr. The unusual findings for α-D-GalNAc attached to Thr^3 can thus be rationalized only if the α-D-GalNAc residue is sensitive to the local environment (steric, hydrogen bonding, etc.) because of interaction with the attached Thr^3 residue or with the neighboring Ser or Thr residues. We could not, however, observe any differences in the resonances of the carbohydrate residues in a related glycopeptide[22] A^M where α-D-GalNAc was attached to amino acids 2, 3, and 4. This was because of overlap of resonances and indicates that information on individual carbohydrate residues is obscured when the glycopeptide A^M is glycosylated in more than one position. This appears to be the case, because unusual chemical shifts and pH behavior is also observed for the C^α and C^β of Ser^1 when Thr^3 is glycosylated (see later).

Another way to monitor the effects of glycosylation on protein structure is to focus on the chemical-shift changes of the peptide residues, if any, that occur upon glycosylation of the peptide these data are given in Table III. Appreciably affected upon glycosylation are C^α and C^β of the glycosylated amino acid. *O*-Glycosylation of the amino acid typically results in a ~1–2 p.p.m. upfield shift of C^α and a ~6–9 p.p.m. downfield shift of C^β. The C^γ atom of the glycosylated Thr is also shifted ~1 p.p.m. upfield. The chemical shifts of some of the neighboring carbon atoms are minimally affected. For instance, α-glycosylation of Ser^2 shifts C^β of Ser^1 ~0.3 p.p.m. upfield. *O*-Glycosylation of Thr^3 shifts C^β of Thr^4 ~0.3 p.p.m. downfield and also appears to affect the titration of the Ser^1 α-amino group (see later). Clearly, the results indicate that even a single carbohydrate residue has the ability to affect the structure about a neighboring residue, and hence the structure of the glycopeptide.

The effect of glycosylation on the structure of the pentapeptide was also

TABLE I

CHEMICAL SHIFT[a] AND TITRATION DATA FOR THE ALIPHATIC CARBON ATOMS OF COMPOUNDS **1–4**

Carbon atoms	*Compound*															
	1				**2**				**3**				**4**			
	Chemical shift[b]	pK_a	n	Δ	*Chemical shift*[b]	pK_a	n	Δ	*Chemical shift*[b]	pK_a	n	Δ	*Chemical shift*[b]	pK_a	n	Δ
Gly^5 C$^\alpha$	44.81	3.99	1.5	2.04	44.93	4.27[c]	0.66	−1.21	44.99	4.07[c]	0.90	−2.00	44.75	3.5[c]	0.99	−1.45
Thr^4 C$^\alpha$	60.51[d]			−0.18	60.48[g]			0	60.92			−0.18	58.93			−0.06
Thr^3 C$^\alpha$	60.51[d]			−0.18	60.48[g]			0	58.81			−0.12	60.48			0.06
Thr^4 C$^\beta$	68.40[e]			−0.11	68.53[h]			0	68.71			−0.05	77.11			0.36
Thr^3 C$^\beta$	68.40[e]			−0.11	68.53[h]			0	77.53			−0.16	68.42			0.36
Thr^4 C$^\gamma$	20.20[f]			0.00	20.26[i]			0	20.14			−0.06	19.21			0.24
Thr^3 C$^\gamma$	20.20[f]			0.00	20.26[i]			0	19.37			−0.06	20.26			0.06
Ser^2 C$^\alpha$	57.05			−0.30	55.06			−0.11	56.73[j]			−0.12	56.85			0.06
Ser^1 C$^\alpha$	56.19	8.14	0.9	−1.13	56.23	7.62	0.99	−1.13	56.73[j]			−0.65[l]	56.41	7.29[m]	1.1	−0.66
Ser^2 C$^\beta$	62.56			0.00	68.71			0	62.63[k]			−0.12	62.74			0.06
Ser^1 C$^\beta$	61.83	8.26	1.2	−3.58	61.52	7.70	0.74	−3.58	64.36			−3.15[n]	62.24	7.76[m]	1.1	−3.48
α-GalNAc C-1					99.10			0	100.17			−0.12	100.05			0.12
α-GalNAc C-2					51.13			0	51.19			−0.06	51.37			0.12
α-GalNAc C-3					69.96			0	70.02			−0.12	70.03			0.24
α-GalNAc C-4									69.42			−0.06	69.00			0.35
α-GalNAc C-5					72.76			0.06	72.70			−0.12	72.64			0.06
α-GalNAc C-6					62.69			0.06	62.63[k]			−0.12	62.69			0.06
-CH_3 (Ac)					23.72			−0.09	23.66			−0.18	23.73			0.18

[a]Estimated precision for the chemical shifts is ±0.05 p.p.m. [b]Chemical shifts were taken from samples at a pH of ≈7.0. The chemical shifts for the titrating resonances are determined from the theoretical fit of those data at pH 7.0. [c]Because the glycosidic linkage is labile, few data points were taken at low pH. As a result, the Δ value was allowed to float until $\Sigma_i[\delta_{\tau(i)} - \delta_{\mathrm{obs}(i)}]^2$ was minimized. [d–k]Overlap of resonances. [l]Resonance was small and broad, and overlapped with the resonance of C$^\alpha$ of Ser^2 at high pH. [m]The better pK_a value for the α-amino group of Ser^1 is that exhibited by C$^\beta$ of Ser^1. The value of Δ for C$^\alpha$ of Ser^1 is considerably smaller and the resonance also overlaps the resonance of C$^\alpha$ of Ser^2 at high pH. [n]Resonance was extremely broad and overlapped (or was a shoulder) with the resonance of C-6 of α-GalNAc and C$^\beta$ of Ser^2.

TABLE II

CHEMICAL-SHIFT DIFFERENCES BETWEEN THE CARBOHYDRATE RESIDUES OF COMPOUNDS **2–4**

Carbon atom	Δ(4 − 3)	Δ(4 − 2)	Δ(3 − 2)
α-GalNAc C-1	−0.12	+0.95	+1.07
α-GalNAc C-2	+0.18	+0.24	+0.06
α-GalNAc C-3	+0.42	−0.96	+0.54
α-GalNAc C-4	+0.01	+0.07	+0.06
α-GalNAc C-5	−0.06	−0.12	−0.04
α-GalNAc C-6	+0.06	0.00	−0.06
$-CH_3$ (Ac)	+0.06	+0.01	−0.06

TABLE III

CHEMICAL-SHIFT DIFFERENCE BETWEEN THE AMINO ACID CARBON ATOMS OF THE NONGLYCOSYLATED COMPOUND **1** AND THE VARIOUSLY GLYCOSYLATED SPECIES **2–4**

Carbon atom	*Glycosylalted compounds − nonglycosylated compounds*		
	2 − 1	**3 − 1**	**4 − 1**
Gly^5 C^α	+0.11	+0.18	−0.06
Thr^4 C^α	−0.03	−0.09	−1.58
Thr^3 C^α	−0.03	−1.70	−0.03
Thr^4 C^β	+0.13	−0.04	+8.71
Thr^3 C^β	+0.13	+9.13	+0.02
Thr^4 C^γ	+0.06	−0.31	−0.99
Thr^3 C^γ	+0.06	−0.83	+0.06
Ser^2 C^α	−1.99	−0.32	−0.20
Ser^1 C^α	−0.04	+0.53	+0.22
Ser^2 C^β	+6.15	+0.15	+0.18
Ser^1 C^β	−0.31	+2.53	+0.41

monitored by the change in titration parameters for the C- and N-terminus. Titration of the carboxyl group of the glycine residue appears to be significantly perturbed only when Thr^4 is glycosylated [$\Delta pK_a(4 - 1) = 0.46$] and this is to be expected. The finding for the α-amino group of the N-terminal Ser residue is somewhat puzzling but is consistent with our earlier findings[25] with the (^{13}C)methylated glycopeptides related to glycophorin A^M. The results suggest that the structure about the N-terminal serine residue is perturbed when α-D-GalNAc is attached either to Ser^2, Thr^3, or Thr^4. When attached to Ser^2, where the largest effect is expected, a noticeable change in pK_a(ΔpK_a ~0.55) of the α-amino group is observed. However, a similar result is also seen when Thr^4 is glycosylated. Moreover, *O*-glycosylation of Thr^3 also appears to affect the titration of the N-terminal α-amino group; this is observed by the fact that the resonance of C^α of Ser^1 coalesced with the resonance of C^α of Ser^2; the latter appears to shift minimally, if at all. The

TABLE IV

^{13}C-SPIN-LATTICE RELAXATION TIMES[a] FOR THE ALIPHATIC CARBON ATOMS OF COMPOUNDS **1–4**[b]

Carbon atom	*Compound*			
	1	**2**	**3**	**4**
Gly^5 C$^\alpha$	813	315	184	217
Thr^4 C$^\alpha$	500[c]	264[f]	152	260
Thr^3 C$^\alpha$	500[c]	264[f]	161	290
Thr^4 C$^\beta$	508[d]	221[g]	292	191
Thr^3 C$^\beta$	508[d]	221[g]	137	321
Thr^4 C$^\gamma$	385[e]	310[h]	386	437
Thr^3 C$^\gamma$	385[e]	310[h]	316	385
Ser^2 C$^\alpha$	490	230	161[i]	323
Ser^1 C$^\alpha$	440	327	161[i]	544
Ser^2 C$^\beta$	394	234[j]	198[k]	253[l]
Ser^1 C$^\beta$	568	234[j]	—[n]	253[l,o]
α-GalNAc C-1		206	182	242
α-GalNAc C-2		183	184	238
α-GalNAc C-3		298[m]	279	198
α-GalNAc C-4			201	216
α-GalNAc C-5		323	225	262
α-GalNAc C-6		253	198[k]	253[l]
-CH_3 (Ac)		567[p]	974	833

[a]Observed T_1 values are given in ms. [b]The samples of **1–4** were 300, 200, 94, and 188mM, respectively in H_2O. Sample pH values were near 7.0. [c–m]Average T_1 values because of overlap of resonances. [n]Peak too small and broad to give an accurate T_1 value. [o]At the pH value that the T_1 values were determined, the resonances of Ser^1 C$^\beta$ overlapped with the resonance of α-GalNAc C-6 and Ser^2 C$^\beta$. [p]Overlap with a residual amount of acetate.

C$^\beta$ resonance of Ser^1 shifts, but the signal is exceedingly broad and difficult to observe, indicating that the proton released from the α-amino group is in "slow exchange" on the n.m.r. time-scale. This resonance also exhibits a differential isotope-shift of 0.4 p.p.m. (^{13}C data for sample in H_2O *vs.* D_2O). The results indicate that the amino acid residues interact strongly, possibly by hydrogen bonding, with the α-D-GalNAc residue. Interestingly the N-terminal structural model for glycophorin A, proposed by Prohaska *et al.*[31], has O-6 of the GalNAc residues at amino acid positions 2, 3, and 4 hydrogen bonded to the neighboring backbone amide (N-H) group.

Table IV provides information on the dynamics of the glycopeptide. Clearly, *O*-glycosylation of Ser^2, Thr^3, or Thr^4 produces a significant decrease in the T_1 values of most of the respective amino acid carbon atoms, especially those of the polypeptide backbone. For instance, all of the α-carbon resonances of the peptide show decreases of 200-300 ms when a carbohydrate is attached. The side-chain γ-carbon atom of Thr appears to be minimally affected. Moreover, in general, the T_1 values of the carbon atoms of the residues containing the α-D-GalNAc residue appear to be more affected than those that lack this group.

CONCLUSION

Attachment of an α-D-GalNAc residue to Ser^2, Thr^3, or Thr^4 of peptide **1** significantly decreases the T_1 values of the carbon atoms of that molecule, in particular the T_1 values of the carbon atoms of the glycosylated amino acid, indicating that the glycosylation significantly affects the conformational motions of peptide **1**. Chemical-shift and titration data for these molecules provide supporting data. The N-terminal structure, about Ser^1, appears to be most affected when Ser^2 is *O*-glycosylated. These results provide a basis for continued research of the structural effects of "complex" carbohydrates on the structures of such glycoproteins as glycophorin A.

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