Carbohydrate Research, 128 (1984) 183–191
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

^{13}C-N.M.R.-SPECTRAL STUDY OF THE pH BEHAVIOR OF REDUCTIVELY [^{13}C]METHYLATED, GLYCOPHORIN A GLYCO-OCTAPEPTIDES AND A RELATED GLYCOPENTAPEPTIDE

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(Received November 12th, 1983; accepted for publication, December 5th, 1983)

ABSTRACT

The pH dependence of the labeled-carbon resonances of reductively [^{13}C] methylated compounds tri-L-Ser, glyco-octapeptide A^M, asialoglyco-octapeptide A^M, glyco-octapeptide A^N, asialoglyco-octapeptide A^N, and a glycopentapeptide was investigated. The results are discussed relative to those previously observed for reductively [^{13}C]methylated, intact glycophorins A^M and A^N, and in terms of the mode of display of the MN blood-group specificities by these related glycoproteins. The results indicated that the α-D-NeuAc groups appear to affect the pH-titration results of glyco-octapeptides A^M and A^N. Moreover, comparison of the pH-titration results for reductively [^{13}C]methylated glyco-octapeptide A^M and reductively [^{13}C]methylated asialoglyco-octapeptide A^M with those of a reductively [^{13}C]methylated glycopentapeptide and reductively [^{13}C]methylated tri-L-Ser indicated that the other carbohydrate residues present (α-D-GalNAc and β-D-Gal) may also affect the pH-titration results. The reductive-methylation modification appears to affect the chemical shifts of the carbohydrate and peptide carbon atoms of the glycopentapeptide minimally.

INTRODUCTION

The mode of display of the MN blood-group specificities by glycophorins A^M and A^N has been a point of controversy in the literature, with several types of residues on the glycoprotein being implicated in their display. The residues that are presumed to be involved in the display of the MN specificities are the α-D-NeuAc groups[1–4], the lysine residue(s)[5–7], and the N-terminal amino acid residues[8–11] of glycophorins A^M and A^N. Much of the focus on the structural differences between

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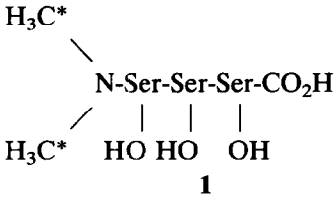
0008-6215/84/$ 03.00



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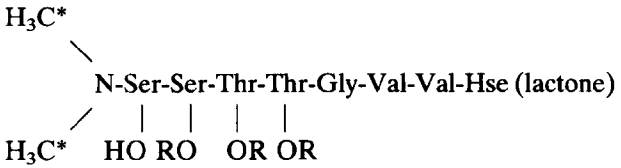
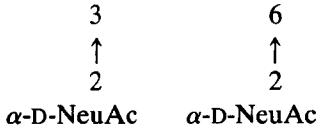
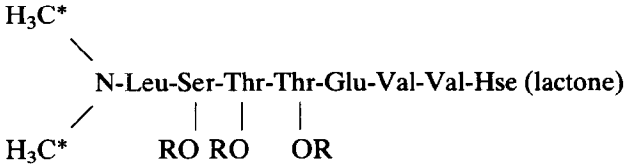


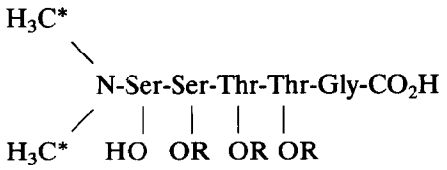
2 R = β-D-Gal-(1→3)-α-D-GalNAc→



3 R = β-D-Gal-(1→3)-α-D-GalNAc→



4 R = same as for **2**
5 R = same as for **3**



6 R = α-D-GalNAc→

*Indicates that the carbon atom is 90% ^{13}C-enriched.

glycophorins A^M and A^N has recently centered about the N-terminal amino acids of glycophorins A^M and A^N. This is because Furthmayr and co-workers[8–11] have conclusively shown that amino acid differences exist between glycophorins A^M and A^N at positions 1 (Ser/Leu) and 5 (Gly/Glu) in their respective amino acid sequences. Moreover, Lisowska and co-workers[7,12] have monitored the serological activity of glycophorin A as a function of the chemical modification of the amino groups of the glycoprotein; they found that the amino groups, especially the N-terminal-NH_2, are crucial for the display of the MN specificities.

In order to clarify some of the possible discrepancies in the literature concerning the display of the MN blood-group specificities by glycophorin A, we had previously employed[13–17] the reductive [^{13}C]methylation (^{13}C-r.m.) technique, in order to introduce ^{13}C labels into the lysine and N-terminal amino acid residues of glycophorins A^M and A^N. This technique modifies the lysine residues, to afford N^{ε},*N*-di[^{13}C]methyllysine, and the N-terminal acid residues, to afford N^{α},*N*-di[^{13}C]methylSer and N^{α},*N*-di[^{13}C]methylLeu of glycophorins A^M and A^N, respectively.

From our previous studies, we have already been able to determine the following facts. (*i*) A structural difference does exist[13] at, or near, or both, the N-terminal amino acid residues of glycophorins A^M and A^N. (*ii*) Two structural states exist[13–16] for glycophorin A^M. (*iii*) Removal of the α-D-NeuAc groups has little effect on the structure about the N-terminal residue of glycophorins A^M and A^N. However, the near total removal of the carbohydrate residues produces[14,15] a pronounced effect on the structure about the N-terminal amino acid residue of glycophorins A^M and A^N. (*iv*) The methylation of one (or more) of the lysine residues has a structural effect[14,15] on the N^{α},*N*-di[^{13}C]methylSer residue of glycophorin A^M. (*v*) The removal of ~75% of the amino acid residues from the C-terminal portion of ^{13}C-r.m. glycophorins A^M and A^N appears to have little effect on the structure about the N-terminal amino acid residues of these species[16]. Further removal of the amino acid residues from the C-terminal portion of this molecule (to form ^{13}C-r.m. glyco-octapeptides) results[17] in the loss of one structural state of glycophorin A^M.

In order to investigate further the structures of the MN blood-group determinants at, or near, or both, the N-terminal amino acid residues, we decided to monitor the pH dependence of the ^{13}C resonances of ^{13}C-r.m. tri-L-Ser (**1**), ^{13}C-r.m. glyco-octapeptides A^M and A^N in various degrees of glycosylation (**2–5**), and a related glycopentapeptide (**6**). The information gained from the titration work (pK_a values and Hill coefficients) may give some insight into the possible steric arrangements or electrostatic interactions that may occur in these molecules, and which may influence their display of the MN blood-specificities.

EXPERIMENTAL

Materials and methods. — Tri-L-Ser and sodium cyanoborohydride were purchased from Sigma Chemical Co., St. Louis, MO. ^{13}C-Enriched formaldehyde

(90% ^{13}C-enriched) was obtained from Merck as a 14% aqueous solution. The unblocked glycopentapeptide was synthesized by Ferrari and Pavia as described previously[18]. ^{13}C-r.m. glycophorin glyco-octapeptides A^M and A^N, and the ^{13}C-r.m. asialoglyco-octapeptides were obtained as described previously[17]. Tri-L-Ser and the glycopentapeptide were reductively [^{13}C]methylated using ^{13}C-enriched formaldehyde and cyanoborohydride as described earlier[13].

For the pH-titration studies, measurements of pH were made by using a radiometer PHM63, digital pH-meter. Sample pH values were checked before and after each n.m.r. run, and the data were used if the pH drift was less than or equal to 0.1 pH unit.

^{13}C-N.m.r. spectra were recorded with a JEOL-FX90Q spectrometer operated at 22.5 MHz, as described previously[19]. Time-domain data were collected in 8,192 addresses or, in some cases, 16,384 addresses. Chemical shifts are given relative to internal 1,4-dioxane, whose chemical shift was taken to be 67.86 p.p.m. from Me_4Si.

The data obtained for ^{13}C-r.m. tri-L-Ser, ^{13}C-r.m. glyco-octapeptide A^N, and ^{13}C-r.m. asialoglyco-octapeptide were analyzed for the best pK_a values and Hill coefficients (n) by using the following equation[20]:

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

In this case, δ_T is the best-fit, theoretical ^{13}C-chemical shift, δ_B is the chemical shift of the di-[^{13}C]methylamino group in the nonprotonated form, and Δ is the shift difference between the di-[^{13}C]methylamino group in the nonprotonated and protonated forms. The best fit was obtained when $\Sigma_i[\delta_{T(i)} - \delta_{obs(i)}]^2$ was minimized; δ_{obs} is the observed chemical shift at that given pH value.

RESULTS AND DISCUSSION

The aim of this work was to obtain structural information about the N-terminal portion of glycophorins A^M and A^N, and possibly to relate this information to the mode of display of the MN blood-group specificities by these glycoproteins. This information was obtained by monitoring the pH dependence of the ^{13}C resonances of ^{13}C-r.m. glyco-octapeptides **2–5** derived from glycophorins A^M and A^N in two states of glycosylation. Also presented is the pH dependence of the ^{13}C resonances of ^{13}C-r.m. tri-L-Ser and a ^{13}C-r.m. glycopentapeptide. The first two residues of tri-L-Ser (**1**) resemble the sequence of glyco-octapeptide A^M (nonglycosylated), and the glycopentapeptide (**6**) is identical in sequence to the first five residues of glyco-octapeptide A^M. However, the glycopentapeptide is only monoglycosylated (by α-D-GalNAc) at positions 2, 3, and 4. Thus, these two compounds were chosen for these studies, because they resemble the N-terminal structure of glycophorin A^M, and this ^{13}C-r.m. glycoprotein had previously given some

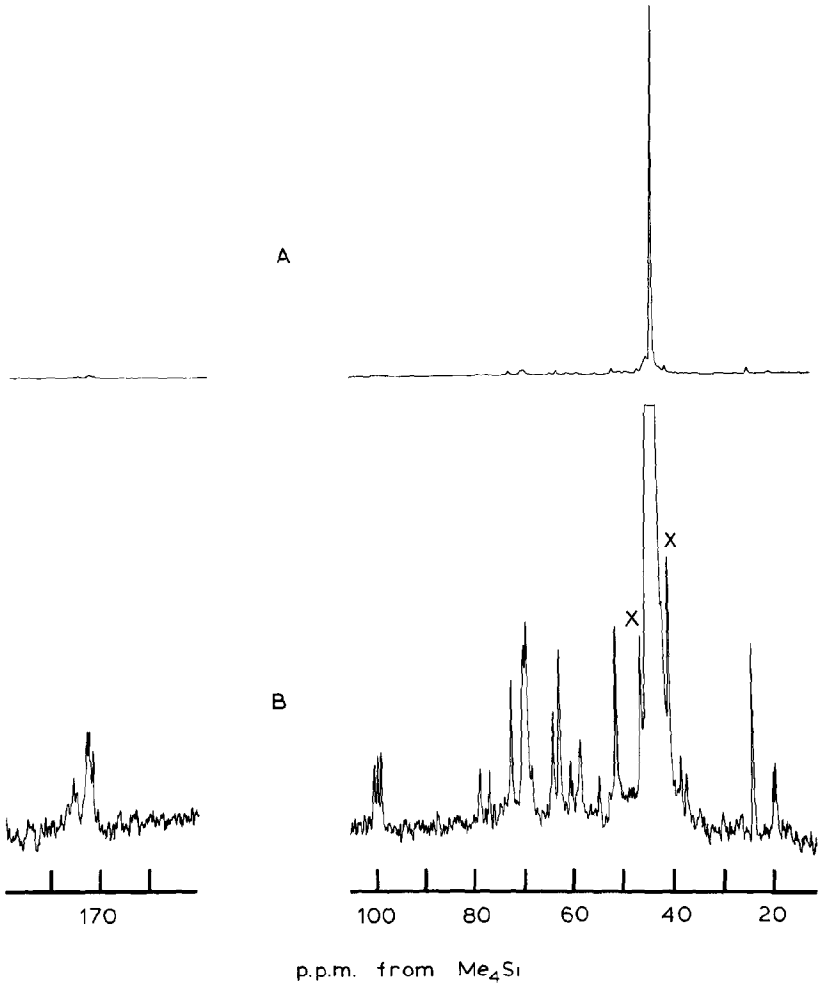


Fig. 1. Proton-decoupled, ^{13}C-n.m.r. spectrum of reductively [^{13}C]methylated glycopentapeptide **6** (~17mM, in H_2O; pH 7.1) at 30°, after 24,000 accumulations. [Time-domain data were accumulated in 8,192 addresses, with a recycle time of 1.5 s. A digital broadening of 2.8 Hz was applied. (A) Normal spectrum, (B) vertically expanded spectrum.]

anomalous results (pH behavior of the ^{13}C resonances of N-terminal di-[^{13}C]methylSer residue, and multiple states)[13–17]

We have previously used circular dichroism spectra to show that the reductive [^{13}C]methylation of glycophorin A does not severely perturb the structure of that molecule[17]. However, this technique provides only an overall picture of the possible structural perturbations, and not a localized picture. Therefore, presented in Fig. 1 and Table I are ^{13}C-n.m.r.-spectral data for the ^{13}C-r.m. glycopentapeptide **6** and for the unmodified glycopentapeptide. Figs. 1A and 1B depict the same

TABLE I

SELECTED ^{13}C-N M.R CHEMICAL-SHIFT DATA[a] FOR THE SYNTHETIC GLYCOPENTAPEPTIDE[b] AND ITS ^{13}C-R M DERIVATIVE (**6**)

Carbon atom[c]	*Chemical shift*	
	Glycopentapeptide	*^{13}C-r.m. Glycopentapeptide*
1′	100.6 99.9 99.3	100.6 99.9 99.2
2′	51.2	51.2
3′ 4′	70.0 69.2	70.0 69.6
5′	72.8	72.6
6′	62.6	62.7
CH_3(Ac-2′)	23.5	23.6
Ser C-2	54.9 56.4	54.8 60.1
Ser C-3	68.5 —	68.5 60.5
Thr C-2	58.8 58.4	58.8 58.5
Thr C-3	77.04 78.83	77.1 79.0
Thr C-4	19.5 19.1	19.6 19.2
Gly C-2	44.8	—[d]

[a]Not all the spectral data are given. [b]The synthesis of the glycopentapeptide was reported by Ferrari and Pavia[18]. [c]More-specific assignments of the resonances to specific carbon atoms of the glycopentapeptide were provided by Pavia and Ferrari[21] for the protected glycopentapeptide. Our assignments are based on their published results. [d]The ^{13}C-enriched-methyl resonance of the di-[^{13}C]methylSer residue overlaps this resonance.

spectrum, except that 1B is vertically expanded. In Fig. 1A, the single resonance for the N^{α},*N*-di-[^{13}C]methylSer residue can be clearly observed. Fig. 1B shows the ^{13}C resonances of all of the carbohydrate and peptide carbon atoms. The anomeric carbon atoms (~100 p.p.m.), and the two C-3 atoms (~78 p.p.m.) and the two C-4 atoms of glycosylated threonine (~19.5 p.p.m.) are clearly seen. The assignments of most of the resonances to specific carbon atoms of the ^{13}C-r.m. glycopentapeptide are given in Table I; also provided in Table I are the ^{13}C-chemical shift data for the unmodified glycopentapeptide. The resonance assignments indicated in Table I are based on the ^{13}C-n.m.r.-spectral data published by Pavia and Ferrari[21] for the blocked glycopentapeptide. It may be seen that reductive [^{13}C]methylation of the glycopentapeptide does not greatly affect the chemical shifts of the carbon atoms of the glycopentapeptide. Therefore, it may also be concluded that this methylation does not greatly perturb the structure of the glycopentapeptide.

Fig. 2 shows the pH dependence of the N^{α},*N*-di-[^{13}C]methylLeu residues of ^{13}C-r.m. glyco-octapeptide A^N and ^{13}C-r.m. asialoglyco-octapeptide A^N. The ^{13}C-

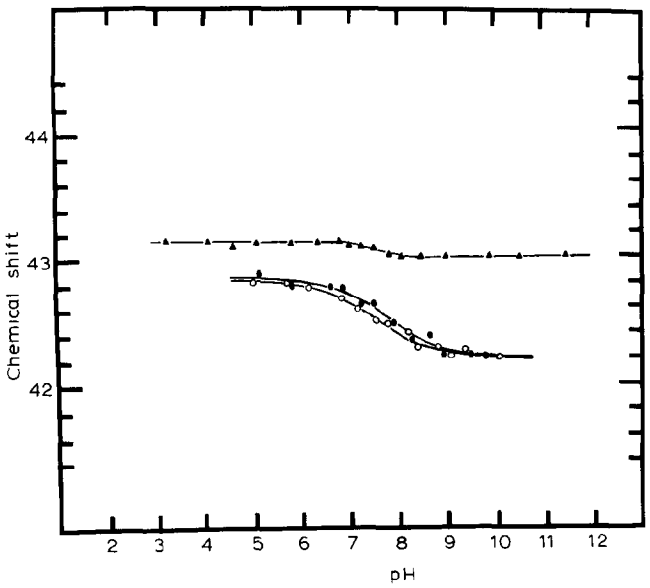


Fig. 2. pH-Dependence of the ^{13}C resonances for the [^{13}C]methylated N-terminal amino groups of tri-L-Ser (▲), glyco-octapeptide A^N (●), and asialoglyco-octapeptide A^N (○).

TABLE II

^{13}C-CHEMICAL SHIFT AND TITRATION DATA FOR THE N-TERMINAL DI-[^{13}C]METHYLAMINO GROUPS OF COMPOUNDS **1–6** AND GLYCOPHORINS A^M AND A^N

Species	*pK_a*	*Hill coefficient*	*Chemical shift[a]*
Intact glycophorin A^N[b]	7.2_0[c]	0.89[c]	42.7[c,d]
Intact glycophorin A^M[b]	—	—	43.3
Glyco-octapeptide A^N	7.7_2	0.85	42.8[d]
Glyco-octapeptide A^M	—	—	43.3
Asialoglyco-octapeptide A^N	7.4_4	0.80	42.6[d]
Asialoglyco-octapeptide A^M	—	—	43.3
Tri-L-serine	7.4_0	1.41[e]	43.1
Glycopentapeptide	—	—	43.1

[a]The chemical shift is given for the resonance at pH 7.3. [b]Obtained from ref. 13. [c]These values were recalculated from the data taken from ref. 13 (with a few additional points) by using the equation given in the text. [d]Determined from the theoretical fit of the data, using the equation given in the text. [e]The titration values for the tri-L-Ser may be error-prone, because of the small shift ($\Delta \approx 0.12$ p.p.m.) of the [^{13}C]methyl resonance when going from the protonated to the nonprotonated form of the dimethylamino group.

chemical shift data for these compounds, and the pK_a and Hill coefficient calculations for the data, are given in Table II. The N^{α},N-di-[^{13}C]methylLeu residue of glyco-octapeptide A^N exhibits a pK_a of 7.7 ($n = 0.85$), and that of asialoglyco-octapeptide A^N, a pK_a of 7.4 ($n = 0.80$). These values are somewhat different from

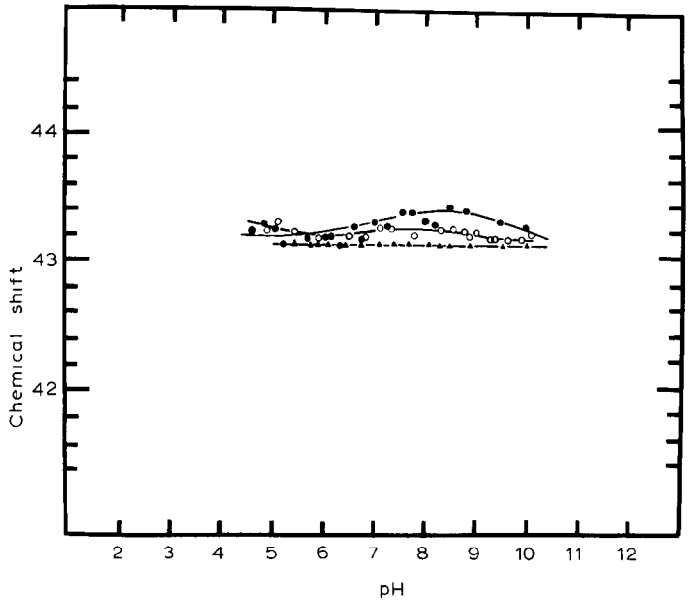


Fig. 3. pH-Dependence of the ^{13}C resonances for the [^{13}C]methylated N-terminal amino groups of glyco-octapeptide A^M (●), asialoglyco-octapeptide A^M (○), and glycopentapeptide **6** (▲).

those obtained for N^{α},*N*-di-[^{13}C]methylLeu of intact glycophorin A^N (pK_a =7.2, n = 0.89). Although the titration data for the A^N glyco-octapeptides are similar (see Fig. 2 and Table II), the results do, however, suggest that the structure about the N-terminal Leu residue of glycophorin A^N may be affected very slightly by removal of (*i*) most of the amino acids of the glycoprotein from the C-terminus, and (*ii*) the α-D-NeuAc groups.

Fig. 3 shows the pH dependence of the N^{α},*N*-di-[^{13}C]methylSer residue of ^{13}C-r.m. glyco-octapeptide A^M and ^{13}C-r.m. asialoglyco-octapeptide A^M. The pH dependence of the ^{13}C resonance of N^{α},*N*-di-[^{13}C]methylSer of glyco-octapeptide A^M resembles that of the major resonance observed[13] for the N^{α},*N*-di-[^{13}C]methylSer residue of intact ^{13}C-r.m. glycophorin A^M. It may be noted that loss of the α-D-NeuAc groups appears to affect the titration behavior of the ^{13}C resonance of the N^{α},*N*-di-[^{13}C]methylSer residue of glyco-octapeptide A^M.

In order to investigate whether the further removal of carbohydrate residues may possibly affect the pH dependence of the ^{13}C-r.m. N-terminal Ser residue of ^{13}C-r.m. glyco-octapeptide A^M, we investigated the pH dependence of the ^{13}C-r.m. N-terminal Ser residue of ^{13}C-r.m. tri-L-Ser (**1**; see Fig. 2) and of a related ^{13}C-r.m. glycopentapeptide (**6**; see Fig. 3). Clearly, the ^{13}C-r.m. tri-L-Ser species exhibits an upfield shift of 0.12 p.p.m. when going from the protonated form of N^{α},*N*-di-[^{13}C]methylSer to the nonprotonated form of this residue. This is somewhat different from what was observed for the pH dependence of N^{α},*N*-di-[^{13}C]methylSer of ^{13}C-r.m. glyco-octapeptide A^M.

The ^{13}C-chemical shift of the methyl carbon atom of the N^{α},*N*-di-[^{13}C]methylSer residue of ^{13}C-r.m. glycopentapeptide **6** is essentially invariant with pH (see Fig. 3). This glycopentapeptide has the same amino acid sequence as the glyco-octapeptide, but is only monoglycosylated by an α-D-GalNAc group at O-2, 3, and 4.

In conclusion, it would appear that, for the N-terminal portion of glycophorin A^N, removal of most of the C-terminal amino acids and α-D-NeuAc groups has a small, but noticeable, effect on the pH behavior of the N-terminal N^{α},*N*-di-[^{13}C]methylLeu residue. The results are more pronounced for glycophorin A^M. Although little difference is observed for the pH behavior of the N-terminal N^{α},*N*-di-[^{13}C]methylSer residue of ^{13}C-r.m. intact glycophorin A^M and ^{13}C-r.m. glyco-octapeptide A^M, more-substantial differences are noticed when the carbohydrates are removed. This result may indicate that, for glycophorin A^M, steric or electrostatic constraints may exist in the N-terminal portion of this molecule.

ACKNOWLEDGMENTS

This work was supported by grants from the South Carolina Affiliate of the American Heart Association (to K.D.) and the Institut de la Santé et de la Recherche Medicale (to A.A.P.). We thank the Carolina Blood Center for outdated, human blood.

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