Magnetic resonance study of glycophorin A-containing ^{13}C-enriched methionines

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Methionine-81 and/or -8 of the transmembrane sialoglycoprotein, glycophorin A, have been specifically alkylated with $^{13}CH_3I$ to produce the sulfonium ion derivatives [*S*-[^{13}C]methylmethionine-8]glycophorin A and [*S*-[^{13}C]methylmethionine-8 and -81]glycophorin A. ^{13}C NMR spectra of these species show that the resonances of the methyl groups of the modified glycophorins occur at 26.1 ppm downfield from Me_4Si. A spin-lattice relaxation time of 0.4 s was observed for the ^{13}C-enriched methyl resonances of the sulfonium ion derivatives of Met-8 and -81, which corresponds to an effective correlation time of $< 2 \times 10^{-10}$ s. Demethylation of the 2 glycophorin A sulfonium ion species with 2-mercaptoethanol produces native glycophorin A which now has the ε-carbon of the methionine residue(s) 45% isotopically enriched. The ε-carbon of Met-8 was found to occur at 15.7 ppm downfield from Me_4Si whereas the ε-carbon of Met-81 exhibited an unusual chemical shift of 2.0 ppm downfield from Me_4Si. The spin-lattice relaxation time of both resonances was found to be ~0.3 s.

^{13}C-NMR *Glycophorin A* *^{13}C-enriched methionines* *[S-[methyl-^{13}C]methionine] glycophorin A*

1. INTRODUCTION

Glycophorin A (M_r 31 000) is the major component of the family of glycophorins, which are integral sialoglycoproteins of the human erythrocyte membrane [1–5]. Glycophorin A is composed of 131 amino acid residues [1,4,5] and also contains a large number of carbohydrate residues (60% carbohydrate, by wt); ~½ of these are α-D-NeuAc residues. The primary structures of the protein and carbohydrate portions of glycophorin A have been determined [1,4–7].

At this time, the exact function of glycophorin A in the erythrocyte membrane is not known. However, it is known that glycophorin A serves as a receptor for influenza virus [1,4,8,9], Portuguese man-of-war toxin [10], and also expresses the MN blood group determinants [3]. Moreover, glycophorin A may play a role in the binding of such important metal ions as Mg^{2+} and Ca^{2+} to the red cell membrane [11,12], and may be implicated in the aging mechanism of the red cell [13–15].

Because of the immense interest in the structure–function relationship of glycophorin A, it would be beneficial if one could gain same structural and dynamic information about this protein in aqueous solution. Here, we report data in which ^{13}C labels have been introduced into the ε-carbon of either Met-8 or Met-8 and Met-81 in the amino acid sequence (fig.1). Our ^{13}C NMR spectral results along with spin lattice relaxation (T_1) data for the ^{13}C labels indicate that these ^{13}C labels may be further used to gain structural and dynamic information about specific localities of glycophorin A in aqueous solution and also when reconstituted into lipid bilayers.

Abbreviations: α-D-NeuAc, α-D-*N*-acetylneuraminic acid; ^{13}C-NMR, carbon-13 nuclear magnetic resonance spectroscopy; PRFT, partially relaxed Fourier transform; SDS, sodium dodecylsulfate; LIS, lithium diiodosalicylate

2. MATERIALS AND METHODS

$^{13}CH_3I$ (90% ^{13}C-enriched) was purchased from Stohler Isotope Chemicals. Lithium diiodosalicylate was purchased from Sigma Chemical Co. Electrophoresis grade SDS and Biol-Gel A 0.5m resin

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were obtained from Bio-Rad Lab. All other chemicals were at least reagent grade quality.

Glycophorins were isolated from outdated homozygous N blood by LIS-phenol method [16]. Glycophorin A was then isolated as in [2]. The final removal of residual detergent, after dialysis, was accomplished by a chloroform–methanol extraction as in [17]. Sample purity was checked by SDS electrophoresis [18]. Amino acid analyses were performed on a Dionex amino acid–peptide analyzer (model MBN/SS), and were used to check the modifications of the methionines. Native and modified glycophorin samples were hydrolyzed in methanesulfonic acid as in [19]. These conditions do not convert the sulfonium ion salt to methionine [19].

To obtain [*S*-[^{13}C]methylmethionine-8 and -81]glycophorin A, 56 mg native glycophorin A were added to a capped vial containing 10 ml solution of 8 M urea at about pH 4.0, 25°C. Aliquots of ^{13}C enriched methyliodide were added every 12 h to the stirred solution for a period of 3 days (100-fold excess added each time). The solution was then extensively dialysed against distilled water and freeze-dried.

[*S*-[^{13}C]methylmethionine-8]Glycophorin A was prepared by adding 60 mg glycophorin A to a capped vial containing 10 ml 2% aqueous SDS solution at about pH 4.0, 25°C. Aliquots of $^{13}CH_3I$ (100-fold excess) were added to the stirred solution at intervals of 12 h for 3 days. The solution was then extensively dialysed against distilled water, extracted with chloroform–methanol, and freeze-dried.

All ^{13}C enriched *S*-methylmethionine glycophorin A species were demethylated according to the following procedure: the labeled glycophorin A species was dissolved in 20 ml aqueous solution containing 8 M urea, and 0.5 M 2-mercaptoethanol at pH 10.5. The solution was then incubated at 37°C for 24 h. The glycoprotein solution was then extensively dialysed against distilled water, centrifuged to remove precipitate, and freeze-dried.

^{13}C NMR spectra were recorded with a JEOL-FX90Q instrument operating at 21 kG (22.5 MHz for ^{13}C) in the Favier transform mode by use of quadrature detection. Samples (1.5 ml) were contained in a 10-mm tube having a concentrically inserted 5-mm tube containing D_2O to serve as the field-frequency lock. The probe temperature was maintained at 25°C for all samples. For ^{13}C excitation, 90° radio-frequency pulses of 19.5 μs were used, and the carrier frequency was set 90 ppm downfield from the ^{13}C resonance of Me_4Si. A spectral window of 5.5 kHz was used for recording the spectra. Fully proton-decoupled spectra were obtained when the noise-modulated, ^{1}H irradiation, having a bandwidth of 1.0 kHz, was centered 4 ppm downfield from Me_4Si. Spin lattice relaxation times were determined using the PRFT method [20].

Chemical shifts are given relative to a trace of internal 1,4-dioxane (added only when chemical shifts were determined), whose chemical shift was taken to be 67.86 ppm downfield from Me_4Si.

3. RESULTS AND DISCUSSION

Glycophorin A is known to contain only two methionine residues in its amino acid sequence; these occur at positions 8 and 81 [1]. The methionine at position 8 is in the hydrophilic region of the molecule which contains all the carbohydrate residues and protrudes into the external environment. On the other hand Met-81 resides within the membrane structure [1].

Previous methionine alkylation studies of glycophorin A using such alkylating agents as iodoacetamide and iodoacetic acid [21] indicated that under certain conditions (either in the presence of SDS or in 8 M urea) alkylation could occur at position 8 or at positions 8 and 81. We have used these conditions (section 2) in order to specifically alkylate one or both of the methionine residues of glycophorin with $^{13}CH_3I$ (fig.1). The reaction scheme indicates that

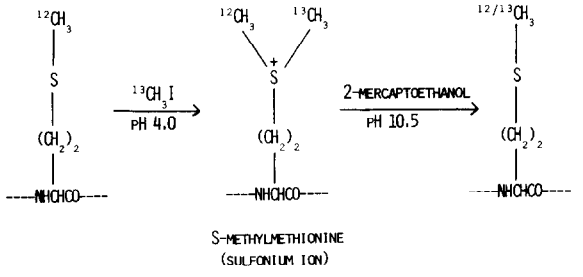

Fig.1. Reaction scheme for the $^{13}CH_3I$ isotopic labeling of the methionine residues of glycophorin A. For the specific details of the modifications of methionine-8 and -81, see section 2.

the stable sulfonium ion (*S*-[^{13}C]methylmethionine species) is produced initially and demethylation occurs in the presence of 2-mercaptoethanol at pH 10.5. The extent of alkylation was monitored using amino acid analysis [19]. Demethylation produces a methionine residue which now has the ε-carbon 45% isotopically enriched. The electrophoretic results for [[ε-^{13}C]methionine-8] and [[ε-^{13}C]methionine-8 and -81]glycophorin A samples were identical to that found for virgin glycophorin A. Therefore, this procedure allows one to obtain glycophorin A which has the ε-carbon of the methionine residue(s) specifically enriched.

Fig.2A shows the ^{13}C spectrum of virgin glycophorin A. Fig.2B, C show the spectra of [*S*-[^{13}C]-methylmethionine-8]glycophorin A and [*S*-[^{13}C]-methylmethionine-8 and -81]glycophorin A, respectively. The intense peaks observed in fig.2A are not due to the protein but correspond mainly to the carbon atoms of α-D-NeuAc [22], the preç dominant carbohydrate found in glycophorin A. The only noticeable difference between fig.2B, C and 2A is the new peak arising at 26.1 ppm. The spectral region of 26 ppm is where one expects to find the ^{13}C resonance of the ε-carbons of *S*-[^{13}C]methylmethionine [23,24]. In going from fig.2B–2C the resonance at 26.1 ppm has nearly doubled in intensity. Therefore, in fig.2B this resonance must correspond to the *S*-[^{13}C]methylmethionine-8 and -81.

One can gain an estimate of the amount of ^{13}C label incorporated into glycophorin by comparing the integrated intensity of the *S*-[^{13}C]methylmethionine resonance with that of one of carbohydrate carbon resonances (providing peak intensities are not attenuated by short recycle times coupled with differential T_1-values and nuclear Overhauser enhancements). The resonance at 23.4 ppm represents ~ 52 methyl carbons of the acetyl moieties (whose T_1 is similar to that of the *S*-[^{13}C]methylmethionine carbon(s)) of α-D-NeuAc, α-D-GalNAc, and β-D-GlcNAc found in glycophorin A [1,6,7]. A comparison of these relative integrated intensities in fig.2B yields a value of ~ 100 methyl carbons (±20) for the 90% enriched *S*-[^{13}C]methylmethionine-8. This value is close to what is expected for mono-alkylation and agrees with amino acid analysis data. Comparison of the integrated intensities of the acetyl methyl moieties and *S*-[^{13}C]methylmethionine residues in fig.2C yields a value of

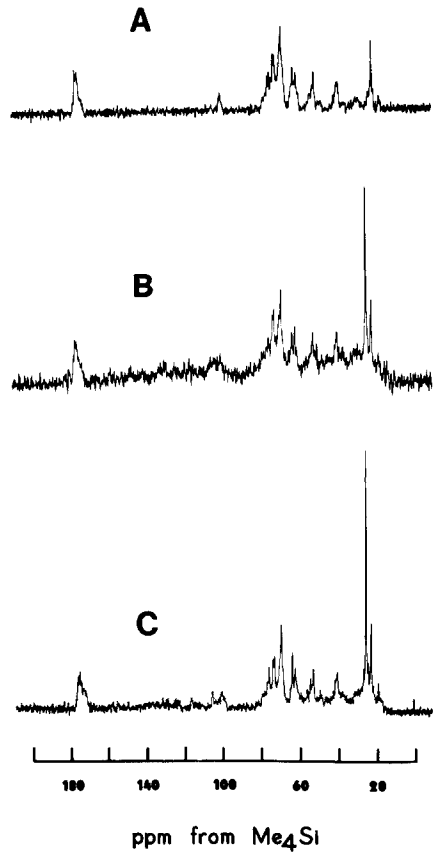

Fig.2. Proton-decoupled ^{13}C NMR spectra of native and ^{13}C-enriched methylated glycophorin A (in H_2O, 25°C). Time-domain data were accumulated in 8192 addresses, with a recycle time of 2.0 s. A digital broadening of 2.8 Hz was applied: (A) 1.9 mM virgin glycophorin A, at pH 6.5, after 50 000 accumulations; (B) 1.8 mM [*S*-[^{13}C]methylmethionine-8]glycophorin A, at pH 4.8, after 50 000 accumulations; (C) 2.0 mM [*S*-^{13}C]methylmethionine-8 and -81]glycophorin A, at pH 5.5, after 40 000 accumulations.

~ 150 carbons (±20). This value is slightly smaller than the 182 carbons expected. Amino acid analysis showed that we did obtain the dialkylated species.

Fig.3A–C show the ^{13}C NMR spectra of virgin glycophorin A, [[ε-^{13}C]methionine-8] glycophorin A and [[ε-^{13}C]methionine-8 and -81]glycophorin A, respectively, in aqueous solution. The spectral difference between fig.3A and 3B is a peak that occurs in spectrum 3B at 15.7 ppm. Its chemical shift is consistent with the chemical shift for ε-carbon of

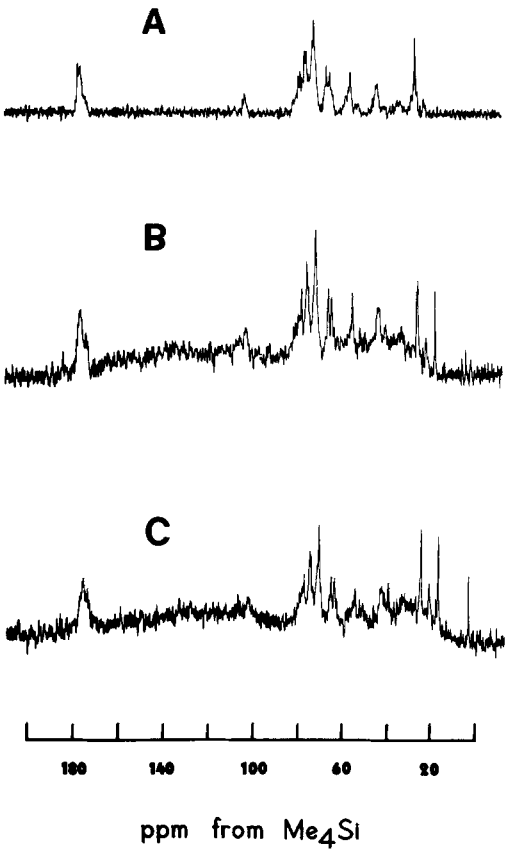

Fig.3. Proton-decoupled ^{13}C NMR spectra of native and [ε-^{13}C]methionine glycophorin A (in H_2O, 25°C). Time-domain data were accumulated in 8192 addresses, with recycle time of 2.0 s. A digital broadening of 2.8 Hz was applied: (A) same as fig.2A; (B) ~1.3 mM [[ε-^{13}C]-methionine- 8]glycophorin A, at pH 5.5, after 100 000 accumulations; (C) ~ 1.3 mM [[ε-^{13}C]methionine-8 and -81]glycophorin A, at pH 4.71, after 79 409 accumulations.

methionines [19,25]. The spectral differences between fig.3C and 3A are 2 peaks in 3C which occur at 15.7 ppm and 2.0 ppm. The resonance at 15.7 ppm can definitely be assigned to the ε-carbon of Met-8 and the one at 2.0 ppm must then be assigned to the ε-carbon of Met-81.

The resonance of the ε-carbon of Met-81 exhibits an unusual chemical shift which may be attributed to the fact that it is buried in an hydrophobic region of the molecule. Note the intensity similarities between the ε-carbons of Met-8 and -81, and the methyl resonance of the acetyl moieties. At this stage the resonances should represent approximately an equal number of carbons.

Because of the large difference in solvent exposure of the two methionine residues of glycophorin A, we have been able to preferentially oxidize Met-8 to methionine sulfoxide. This results in a loss of the resonance at 15.7 ppm and the appearance of a new resonance at 38.1 ppm. The chemical shift of the new resonance is consistent with the chemical shift expected for the ε-carbon of methionines which have undergone sulfoxidation [26,27]. The oxidation of Met-81 to the sulfone (with a concomitant 23 ppm downfield shift of the resonance at 2 ppm) did not occur until the sample was placed into trifluoroacetic acid and hydrogen peroxide [28]. These results corroborate our earlier assignments of the resonances of ^{13}C enriched ε-carbons to specific methionine residues.

The spin lattice relaxation times of the ^{13}C-enriched methyl groups of *S*-[^{13}C]methylmethionine-8 and/or -81 are 0.4 s. Moreover, T_1-values of the ε-carbons of ^{13}C-enriched native methionine residues of glycophorin A were found to be 0.3 s. Assuming a rigid rotor, these values correspond to an effective correlation time (τ_R) of $< 2 \times 10^{-10}$ s. This value indicates that these residues probably have a high degree of motional freedom. This degree of motional freedom has been observed for the spin-labeled oligosaccharides [29] of glycophorin.

These results show that the 2 methionines of glycophorin A can be specifically ^{13}C-enriched at the ε-carbon. Moreover, these residues would appear to exhibit a high degree of motional freedom, resulting in sharp resonances and making them excellent protein conformation and membrane structural probes. Further ^{13}C-NMR work is in progress with these ^{13}C enriched species in order to gain structural and dynamic information about this transmembrane glycoprotein in aqueous solution and in lipid bilayers.

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