Preferential sulfoxidation of the methionine residues of glycophorin A

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Carbon-13 nuclear magnetic resonance spectroscopy was used to monitor the preferential sulfoxidation of the two methionine residues (8 and 81) of glycophorin A. In urea Met-8 is readily oxidized. However, Met-81 can only be oxidized in trifluoroacetic acid containing hydrogen peroxide. Our results also give some insight into the reagent accessibility of different portions of the protein molecule and the general stability of this glycoprotein.

^{13}C-NMR *Glycophorin A* *Methionine sulfoxidation* *^{13}C-enriched methionine*

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 known to serve as a receptor for influenza virus [1,2,6,7], certain lectins [1,4,6,7], portuguese man-of-war toxin [8], and also expresses the MN blood group determinants [2,5].

This glycoprotein of 131 amino acids (60% carbohydrate, by wt) contains only 2 methionine residues which are strategically located within the sequence:

Met-8 occurs within the hydrophilic portion of the molecule (containing all the carbohydrate residues) which protrudes into the external environment [1];

Met-81 occurs in the hydrophobic portion of the molecule that resides within the red cell membrane [1].

Isolated glycophorin A in aqueous solution is known to aggregate [9–11] and it has been postulated that the site of aggregation involves the hydrophobic portion of the molecule (residues 71–92) [9]. Moreover, it is also known that modification of Met-81 affects the extent of glycophorin A aggregation [12].

Here, we present data concerning the preferential sulfoxidation of the methionine residues of glycophorin A. Moreover, our data gives some insight into the reagent accessibility of different portions of the molecule and the general stability of this glycoprotein. This work was accomplished by monitoring the ^{13}C labels of [[ε-^{13}C]*methionine*-8 and -81]glycophorin A by ^{13}C-NMR.

2. MATERIALS AND METHODS

The preparation of [[ε-^{13}C]*methionine*-8 and -81]glycophorin A has been described [13]. The oxidation of the methionine residues to methionine sulfone was monitored by amino acid analysis using a Dionex amino acid-peptide analyzer (model MBN/ss). ^{13}C spectra were recorded with a JEOL-FX90Q instrument operating at 21 kG in the FT mode by use of quadrature detection. 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 noise-modulated, ^{1}H irradiation, having a band width of 1.0 kHz, was centered 4 ppm downfield from Me_4Si.

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Abbreviations: ^{13}C-NMR, carbon-13 nuclear magnetic resonance spectroscopy; TFA, trifluoroacetic acid; H_2O_2, hydrogen peroxide; ^{1}H-NMR, proton magnetic resonance spectroscopy; α-D-NeuAc, α-D-*N*-acetylneuraminic acid

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3. RESULTS AND DISCUSSION

Fig.1A shows the proton-decoupled natural abundance ^{13}C-NMR spectrum of virgin glycophorin A. The intense peaks in the spectrum are not due to the protein but correspond to the carbon atoms of α-D-NeuAc [14,15], the predominant carbohydrate found in glycophorin A [1]. Fig.1B shows the proton-decoupled ^{13}C spectrum of [[ε-^{13}C]*methionine*-8 and -81]glycophorin A. In [13] we assigned the new resonances appearing in fig.1B (relative to fig.1A) at 15.7 ppm and 2.0 ppm to the ^{13}C-enriched ε-carbon of Met-8 and Met-81, respectively.

Fig.1C shows the proton-decoupled ^{13}C-NMR spectrum of a [[ε-^{13}C]*methionine*-8 and -81]glycophorin A in an aqueous solution of 8 M urea. Instead of observing a ^{13}C spectrum of denatured glycoprotein containing well-resolved sharp resonances [16], we observe a 'washed-out' spectrum which probably results from glycophorin A which is in a high state of aggregation. It is known that urea, a common denaturing agent, does not have the ability to denature glycophorin A [10,12].

After treatment of this sample for ~7 days in concentrated urea, it was extensively dialyzed, then freeze-dried. A ^{13}C-NMR spectrum of this sample was taken and a portion of the aliphatic region (0–50 ppm) is shown in fig.2B. For comparison the same aliphatic region of the spectrum of [[ε-^{13}C]*methionine*-8 and -81]glycophorin A is depicted in fig.2A. The resonance corresponding to the ε-carbon of Met-8 (15.7 ppm) in fig.2A has now shifted to 38.1 ppm in fig.2B. The resonance of the ε-carbon of Met-81 remains unaffected. The chemical shift of the resonance at 38.1 ppm is consistent with the chemical shift of an ε-carbon of a methionine residue which has undergone sulfoxidation [17,18] (to produce methionine sulfoxide). It would appear then that the treatment of glycophorin A in denaturing agents may, in time, cause air oxidation of the Met-8 residue to methionine sulfoxide.

An attempt was made to oxidize Met-81 in the presence of 8 M urea and hydrogen peroxide (~0.5 M). After incubation of the sample (37°C) for 2 h, the sample was dialyzed and freeze-dried. A ^{13}C-NMR spectrum of the sample indicated that even though the carbohydrate residues appeared to have lost some intensity (broadened), the resonances at 38.1 ppm and 2.0 ppm remained intact. The only reagent which has been postulated to completely denature glycophorin A is trifluoroacetic acid [10]. Fig.2C shows a spectrum of the

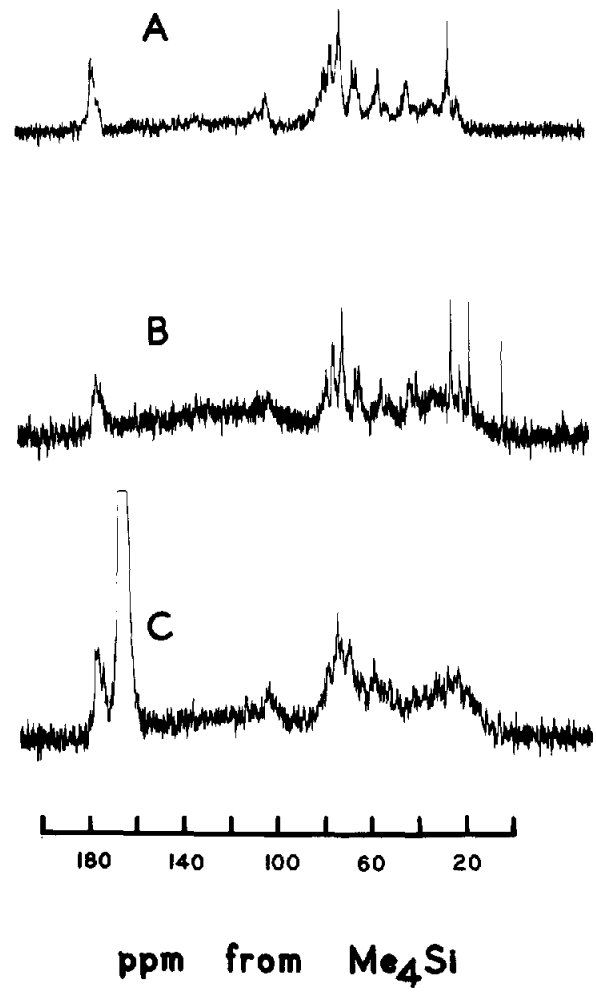
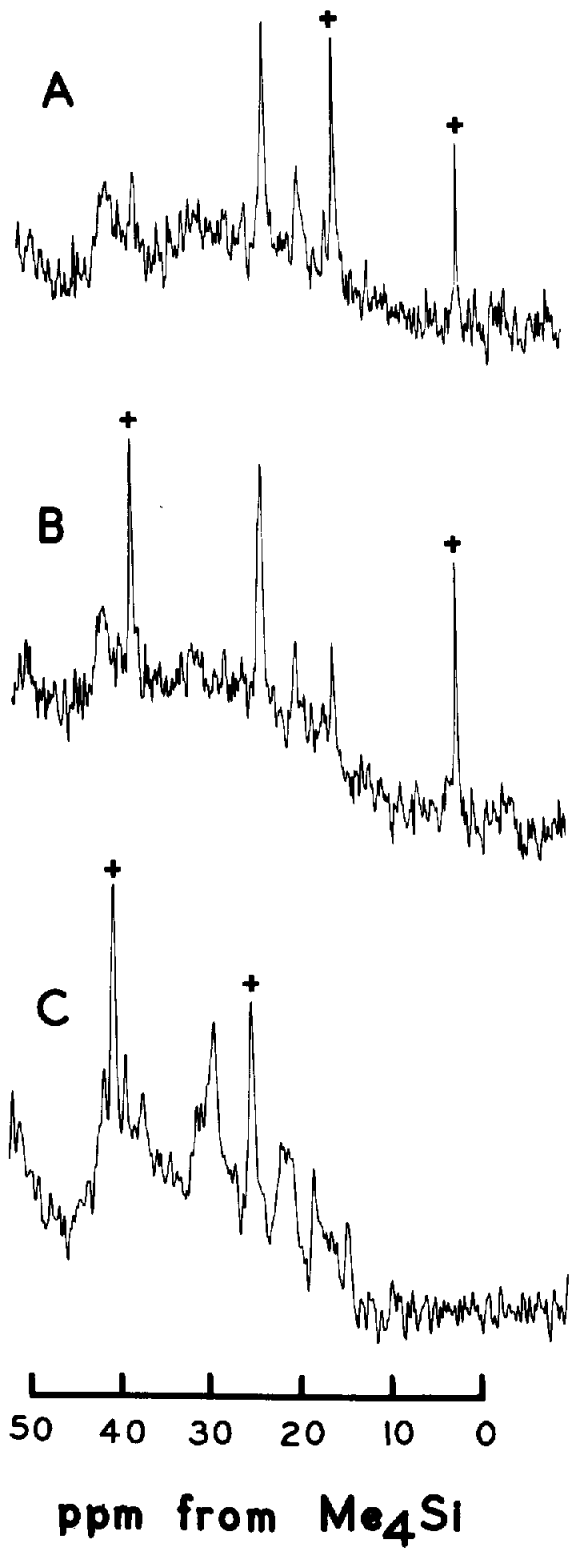


Fig.1. Proton-decoupled ^{13}C-NMR spectra of virgin, ^{13}C-enriched, and denatured glycophorin A (at ~25°C). Time-domain data were collected in 8192 addresses, with a recycle time of 2.0 s. A digital broadening of 2.8 Hz was appplied, except in (C) where 4.5 Hz was applied. (A) 1.9 mM virgin glycophorin A in H_2O, at pH 6.5, after 50 000 accumulations; (B) 1.3 mM [[ε-^{13}C]*methionine*-8 and -81]glycophorin A in H_2O, at pH 5.5, after 100 000 accumulations; (C) 1.3 mM [[ε-^{13}C]*methionine*-8 and -81]glycophorin A in 8 M urea, at pH 7.4, after 71 300 accumulations.



sample shown in fig.2B* which is now in TFA containing ~0.5 M H_2O_2 (also 2.0 M H_2O). The peak at 38.1 ppm has now shifted to 40.5 ppm (due to further oxidation, see below) and the resonance at 2.0 ppm appears to have shifted to 25.0 ppm. The acetyl-methyl resonance (23.4 ppm) has also shifted to 29.3 ppm; this was determined from a ^{13}C spectrum of virgin glycophorin A in the same media. Under these conditions methionine and methionine sulfoxide will be converted to methionine sulfone. This was observed in the ^{13}C-NMR spectra of methionine, methionine sulfoxide, and methionine sulfone in TFA/H_2O_2 solutions. Moreover, methionine sulfone was only detected in the amino acid analysis trace of the glycophorin A sample which had been in the TFA/H_2O_2 solution. Under the HCl hydrolysis conditions used for the glycoprotein, methionine sulfoxide would revert to methionine [12].

As a result of the chemical shift non-equivalence of the 2 enriched ε-carbons, our data indicates that even in trifluoroacetic acid the hydrophobic region of glycophorin is not denatured. Previous researchers had predominantly monitored the well-resolved proton resonances of the aromatic amino acids (in the ^1H-NMR spectra of glycophorin A) in order to postulate that TFA fully denatures glycophorin A; however, the glycoprotein does not contain any aromatic amino acid residues in the hydrophobic portion of the molecule. In contrast, in our studies we have a direct probe within the hydrophobic portion of the molecule.

Our ^{13}C-NMR data conclusively show that Met-8 of glycophorin A can readily be oxidized; caution must be used in the treatment of glycophorin A, as one may unsuspectingly oxidize

* This is the same sample that was treated with H_2O_2 in 8 M urea

Fig.2. A portion of the aliphatic region (0–50 ppm) of the proton-decoupled ^{13}C-NMR spectra of ^{13}C-enriched glycophorin A and various oxidized species of glycophorin A. The markings on top the peaks indicate a ^{13}C-label: (A) as fig.1B; (B) sample shown in fig.1B and 2A which was in urea for 7 days (~1.3 mM in H_2O, pH 4.72), after 33 247 accumulations; (C) ~1.3 mM sample depicted in (C) now in trifluoroacetic acid containing ~0.5 M H_2O_2 (also 2.0 M H_2O) after 53 100 accumulations.

Met-8. It would appear that Met-81 is exceedingly difficult to oxidize; this may be due to the fact that this residue is buried in the hydrophobic region of the molecule which may not be permeable to certain reagents. We have also shown, contrary to earlier reports, that glycophorin A does not totally denature in TFA.

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