Biochimica et Biophysica Acta, 595 (1980) 235—243

BBA 78605

REVERSIBLE TRIFLUOROACETIC ACID-INDUCED CONFORMATIONAL CHANGES IN GLYCOPHORIN A AS DETECTED BY PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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(Received June 8th, 1979)

Key words: Glycophorin A; Conformation change; ^{1}H-NMR; Trifluoroacetic acid

Summary

High-field (270 MHz) ^{1}H-NMR has been employed to study the solution conformation of glycophorin A, a sialoglycoprotein which spans the human erythrocyte membrane. Glycophorin A is one of the most fully characterized integral membrane proteins known, making it an excellent model for the study of membrane-bound proteins. This protein consists of three distinct domains: a glycosylated extracellular N-terminus, a hydrophobic intramembranous segment, and a polar cytoplasmic C-terminus. These domains contain aromatic residues which serve as convenient ^{1}H-NMR conformational probes. The aromatic region of the NMR spectrum of glycophorin A in ^2H_2O shows single, well-resolved His and Tyr resonances. No resonances are observed, however, for the Phe residues which are located in or near the hydrophobic domain. These observations suggest that considerable heterogeneity with respect to segmental motions exists within the protein. This is consistent with circular dichroism data showing the intramembranous segment to be completely helical with the extremities of the protein being predominantly random coils. The helix of the hydrophrobic domain is remarkably resistant to conventional denaturing conditions including variations in pH, and temperature, and treatment with guanidine hydrochloride. However, in trifluoroacetic acid, which strongly solvates peptide backbones, there is extensive reversible unfolding of the helical structure as evidenced by the appearance of Phe resonances. Solvent titration experiments indicate that approximately a 1 : 1 volume ratio of trifluoroacetic acid to ^2H_2O is required to initiate unfolding of the helix.

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Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

Conformational studies of water-soluble membrane proteins in solution are required in order to understand the structure-function relationships of these proteins in the membrane-bound state. Glycophorin A, the major glycoprotein of the human erythrocyte membrane, is one of the most fully characterized integral membrane proteins known and is an excellent model system for the study of other membrane-bound proteins. This water-soluble protein, which has a monomer molecular weight of 31 000, spans the red cell membrane with the orientation depicted in Fig. 1. The primary structure of glycophorin A is known and indicates that the protein consists of three distinct chemical domains: a heavily glycosylated N-terminal region located extracellularly, a hydrophobic intramembranous segment consisting of 23 nonpolar amino acid residues, and a hydrophilic C-terminus which extends into the cytoplasm [1]. The glycosylated N-terminal segment contains 16 oligosaccharide chains which comprise 60% of the protein's mass. As yet, no major functional role has been assigned to glycophorin A other than carrying MN blood group antigen activities and influenza virus binding sites.

Polyacrylamide gel electrophoresis studies indicate that glycophorin A exists primarily as a dimer when dissolved in SDS solution [2]. Retention of this structure has been proposed for the membrane-bound state [3]. Evidence has been obtained indicating that carboxymethylation of the Met-81 residue, which is located in the hydrophobic segment, facilitates the dissociation of the dimer to monomer if the protein is heated in the presence of SDS [2,4]. Byers and Verpoorte have also demonstrated that glycophorin A is remarkably stable in guanidine hydrochloride or after heating [5]. These authors have concluded that glycophorin A is neither converted to monomer by guanidine

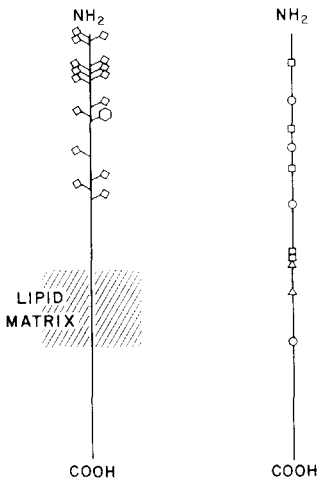

Fig. 1. Schematic diagram of the arrangement of glycophorin A with respect to the cell membrane and of the position of the aromatic residues within the protein. The drawing on the left illustrates the sites of glycosylation of the protein. The drawing on the right shows the positions of the aromatic residues and corresponds to equivalent positions on the left with respect to the lipid matrix: ○, Tyr; □, His; △, Phe.

hydrochloride nor does it undergo a reversible, two-state denaturation. The only spectroscopic technique employed to date in studies of the solution conformation of glycophorin A has been circular dichroism [5,6]. Results of these investigations indicate that the secondary structure of the hydrophobic domain is largely α-helical, whereas both the intra- and extracellular segments of the protein have little structure and approximate unordered random coils. A molecular model of the hydrophobic domain indicates that the helix has a relatively 'flat' side due to the presence of appropriately spaced glycine residues. Such an arrangement has been suggested to account for the proposed dimeric structure of the protein in detergent. If this arrangement also occurs in vivo, favorable protein-protein interaction might occur along the flat side of the helix, leaving the branched side chains on the external faces to interact with the membrane lipid.

High-field proton NMR is particularly well suited for protein conformational studies due to the large magnetogyric ratio of the proton, its great abundance in proteins and the dominance of the dipole-dipole relaxation mechanism. In addition to the structural information contained in the chemical shifts for individual protons and when resolved, the spin-spin couplings to adjacent protons, an assessment of the dynamics of the molecular motions can be obtained directly from an analysis of the linewidths for individual resonances. Line broadening will result when molecular motions are restricted as is the case in ordered conformations such as the α-helix. In contrast, conformations which have little or no order, such as random polypeptide coils, are usually associated with much greater motional freedom and hence result in narrower linewidths. However, the large number of hydrogen atoms present in even small proteins often makes the resolution and assignment of individual resonances difficult or unfeasible. This problem has been ameliorated by the increasing fields available with superconducting magnets [7]. Furthermore, there are only four aromatic amino acids (His, Tyr, Phe and Trp) that have resonances which are well resolved from those of the more abundant aliphatic protons. Thus, many studies have been performed in which resonances from aromatic residues have been utilized as probes of protein conformation [8,9].

As depicted in Fig. 1, glycophorin A contains five His, four Tyr, two Phe, and no Trp residues. These amino acids are distributed in the glycosylated region of the protein as well as in the intramembranous domain. We report here results of an NMR study of glycophorin A in which these amino acids were employed as conformational probes of both local and overall structure. A primary goal in this study was to determine a set of NMR denominators which may have general applicability to other membrane-bound proteins.

Materials and Methods

Protein preparation. Glycophorin A was isolated and delipidated by procedures which have been previously described [10,11]. Prior to NMR analysis the exchangeable hydrogens in the protein were replaced with deuterium by dissolving the lyophilized protein three to five times in successive amounts of ^2H_2O.

NMR methods. Proton NMR spectra were recorded at 270 MHz with a

Bruker HX 270 superconducting spectrometer operating in the Fourier transform mode. Typical spectra were obtained from approximately 0.3 to 1.0 mM protein solutions using quadrature detection and gated decoupled solvent suppression. Chemical shifts for ^2H_2O and trifluoroacetic acid spectra are referenced relative to H_2O at 4.8 ppm and trifluoroacetic acid at 11.47 ppm, respectively. All pH values are direct pH meter readings with no isotope corrections.

Results and Discussion

The ^1H NMR spectrum of glycophorin A in ^2H_2O is shown in Fig. 2. The prominent resonance at 2 ppm corresponds to the 50 N-acetylmethyl groups of the 16 oligosaccharides located on the N-terminus of the protein. The aromatic resonances (6—9 ppm) appear very sharp and have been assigned to the C-2 and C-4 His protons, 8.7 ppm and 7.3 ppm, respectively, and C-2,6 and C-3,5 Tyr protons, 7.1 ppm and 6.8 ppm, respectively. Aromatic resonances from the two Phe residues located in or near the protein's hydrophobic region are not evident. The remarkable equivalence of the His and Tyr resonances, coupled with the absence of Phe resonances indicates that considerable heterogeneity in segmental motion exists within the protein structure. The missing resonances may be plausibly explained by assuming that these peaks have been extensively broadened by dipole-dipole relaxation effects. Since the Phe residues fall in or directly adjacent to the hydrophobic domain, one can conclude that this region of the protein is more motionally restricted than the N-terminus. This conclusion is consistent with the previously mentioned

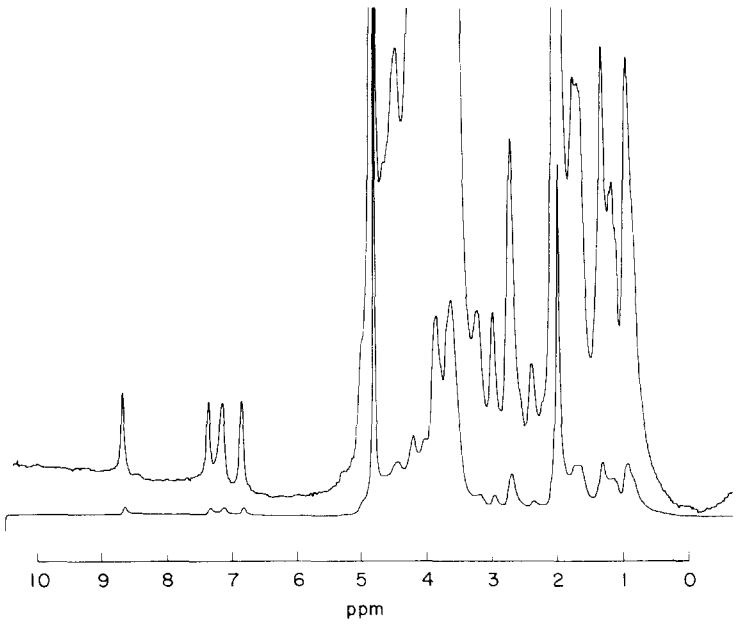

Fig. 2. The 270 MHz ^1H NMR spectrum of glycophorin A in ^2H_2O, pH $\approx$ 3.

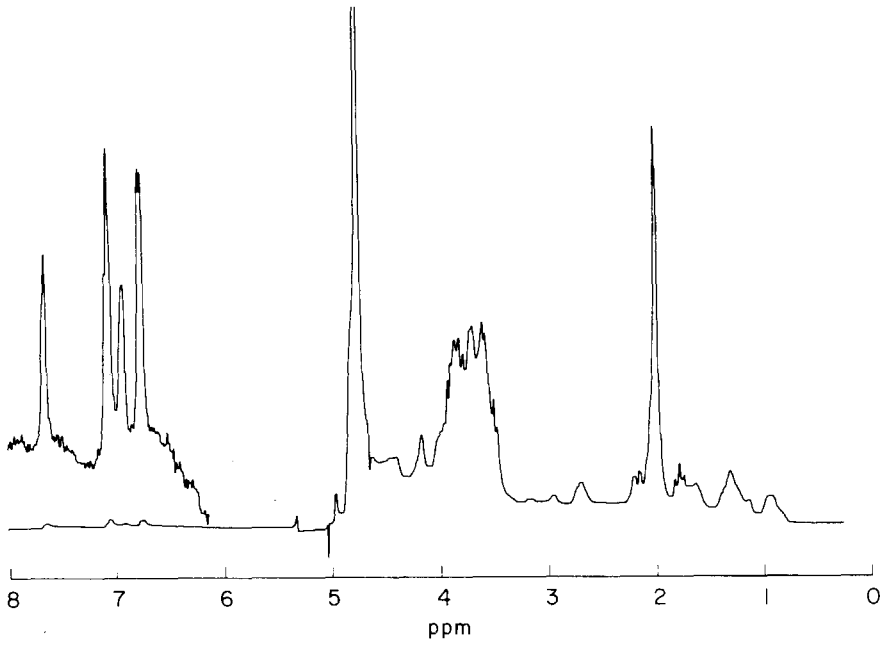

Fig. 3. The 270 MHz ^1H NMR spectrum of the tryptic digest N-terminal peptide corresponding to amino acid residues 1—39 of glycophorin A in ^2H_2O, pH 9.8.

circular dichroism data which indicate that the intramembranous segment is an α-helix and the N-terminal segment essentially a random coil. Other ^1H NMR studies have demonstrated that resonances from a polypeptide helix are broad compared to those of a random coil due to the restricted motion of the helix [12].

In order to quantitate the numbers of His and Tyr protons represented by the resonances in Fig. 2, spin lattice relaxation times (T_1 values) were determined for these peaks and the N-acetylmethyl peak by the progressive saturation method. The T_1 values for all five resonances are approximately equivalent and range from 0.4 to 0.6 s. Cut and weigh integration of the aromatic resonances and the N-acetylmethyl peak, assuming that this latter resonance represents all 150 protons, revealed that approximately one Tyr and two His residues are missing from the spectrum. The fact that this same number of His and Tyr residues are located proximally to the hydrophobic segment at positions 66, 67 and 93 is consistent with the idea that this segment of the molecule has the restricted motion of a helix.

Fig. 3 shows the NMR spectrum of a tryptic digest peptide consisting of amino acid residues 1—39 of the glycosylated segment of glycophorin A. The aromatic region of the spectrum is strikingly similar to that of Fig. 2 except for chemical shift changes resulting from the different pH of the peptide sample. Integration of the aromatic resonances indicates that all of the expected aromatic residues (two His and two Tyr) are observed. Thus, even though the peptide is heavily glycosylated, it possesses enhanced molecular motions, both as a free peptide in solution and as the N-terminal segment of glycophorin A.

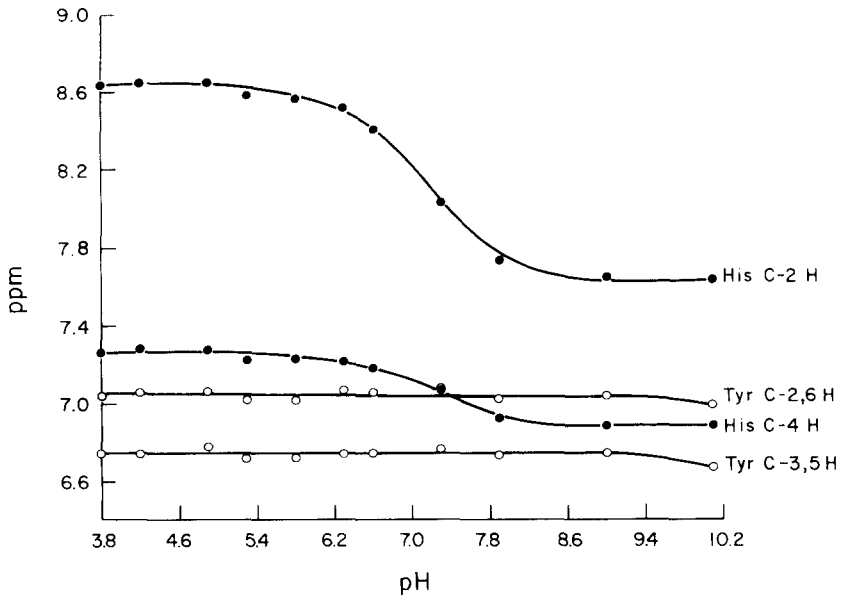

Fig. 4. The pH dependence of the chemical shifts of the His and Tyr resonances from glycophorin A in ^2H_2O.

Glycophorin spectra were determined over a range of pH values from 4 to 10. No significant alterations in the non-aromatic regions of the spectra were observed. The pH profile of the aromatic resonances is presented in Fig. 4. The pK_a of the His residues is approximately 7.1, whereas the phenolic side chains of the Tyr residues do not undergo appreciable ionization until the pH is raised above 9. These data agree with the pK_a values of His and Tyr residues in other proteins and provide confirmation of the peak assignments made in Fig. 2 and 3 [13]. No new aromatic resonances appear as the pH is altered; however, the C-2 His resonance does broaden somewhat at pH 6—8. This may reflect small differences in the His pK_a values or possibly intermediate exchange of C-2 protons with solvent. Thus it may be concluded that changes in pH do not significantly alter the structure of glycophorin A.

In order to assess the thermal stability of glycophorin A in ^2H_2O, NMR spectra were recorded at pH 8 over a temperature range from 295 to 350 K. Although some resonances became sharper with increasing temperature, no new resonances, in particular the Phe residues, were observed. Both the C-2 and C-4 His resonances show considerable broadening by this treatment. 5 mol guanidine hydrochloride was demonstrated to have little effect on the NMR spectrum even at 350 K. These data indicate that glycophorin A is an unusually stable protein with respect to normal denaturing conditions in agreement with the data of Byers and Verpoorte [5].

Clearly, much harsher conditions were required to unfold the protein and this led to the choice of trifluoroacetic acid. In synthetic polypeptides, extensive studies have been made of the helix-to-coil transition-inducing powers of trifluoroacetic acid [14]. Mechanistic studies of this very potent disrupter of

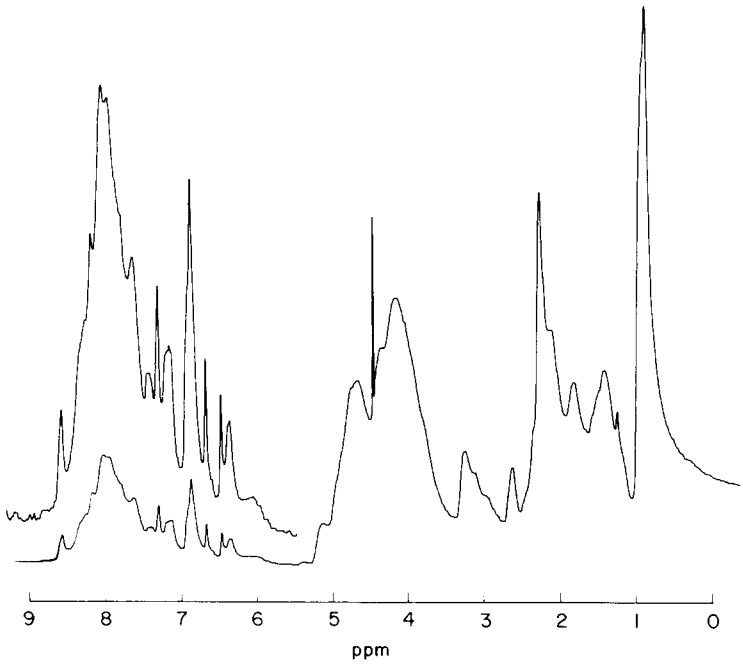

Fig. 5. The 270 MHz ^{1}H NMR spectrum of glycophorin A in protium trifluoroacetic acid.

protein secondary and tertiary structure suggest that it acts by forming hydrogen bonds with essentially all the carbonyl groups of the peptide backbone and side chains [15].

The spectrum of glycophorin A dissolved in 100% trifluoroacetic acid is presented in Fig. 5. Several major differences are apparent in this spectrum compared to the ^2H_2O spectrum in Fig. 2. The most prominent resonance is from the protein methyl groups at 1 ppm. The collapse of these methyl groups into a single peak indicates that trifluoroacetic acid induces extensive unfolding of the protein's conformational structure. The many resonances from 6 to 9 ppm correspond to slowly exchanging amide NH protons in addition to aromatic protons.

In order to determine whether the effects of trifluoroacetic acid are reversible, the glycophorin A/trifluoroacetic acid solution was lyophilized and the protein dissolved in ^2H_2O. The NMR spectrum of the ^2H_2O solution was essentially the same as in Fig. 2 except for possible perturbations around 4—5 ppm where most of the carbohydrate resonances occur. Thus the induced unfolding by trifluoroacetic acid is reversible. In order to more fully characterize the effects of trifluoroacetic acid, a solvent titration was performed as summarized in Fig. 6. In this study deuterated trifluoroacetic acid was employed in order to permit the resolution and assignment of aromatic resonances. The spectra in Fig. 6 demonstrate that as much as 50% trifluoroacetic acid (by vol.) is required to resolve resonances which are not apparent in the ^2H_2O spectrum.

Fig. 6A shows the aromatic region of the spectrum in 100% trifluoroacetic acid (^{2}H-labelled). The peaks at 6.9 and 7.2 ppm were assigned to C-3,5 and C-2,6 Tyr protons, respectively, by homonuclear decoupling. In this experi-

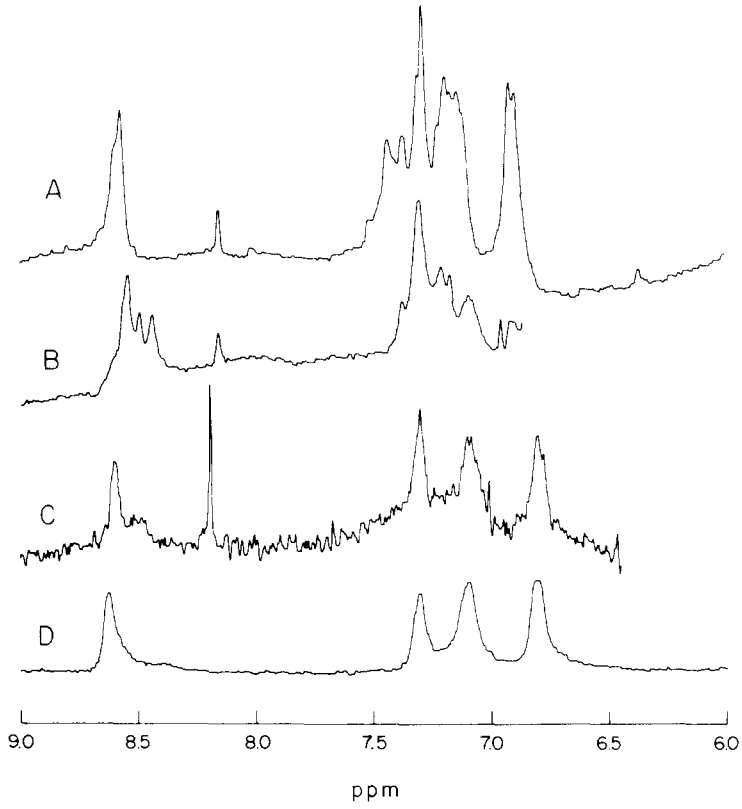

Fig. 6. The aromatic region of the ^1H NMR spectrum of glycophorin A in four different solvents: (A) ^2H-labelled-trifluoroacetic acid, (B) 1 : 1 (by vol.) ^2H-labelled-trifluoroacetic acid/^2H_2O, (C) 1 : 3 ^2H-labelled-trifluoroacetic acid/^2H_2O, and (D) ^2H_2O.

ment irradiation of the 6.9 ppm C-3,5 resonance produced a partial collapse of the 7.2 ppm resonance. The C-2 His resonances occur at 8.6 ppm while the C-4 peaks probably occur at approximately 7.4 ppm. The prominent resonance centered at 7.3 ppm most likely corresponds to the two Phe residues. The area ratio of the Tyr C-3,5 peak to the His C-2 resonance is roughly 8 : 5 which indicates that all four Tyr and five His of glycophorin A appear in this spectrum. A new resonance is observed at approx. 8.1 ppm in the presence of ^2H-labelled trifluoroacetic acid (Fig. 6A—C), the identity of which is unknown.

In an effort to examine the ^1H spectrum of glycophorin A in the monomeric state, the protein was carboxymethylated using a modification of the procedure described by Silverberg et al. [4]. Based upon SDS-polyacrylamide gel electrophoresis analysis, greater than 95% of the protein was converted to monomer. Preliminary ^1H NMR studies of this derivative indicate that at 80° C a new resonance at approximately 7.6 ppm is resolved which is absent in the ^2H_2O spectrum.

Further investigations of the subunit interactions and segmental motions of glycophorin A are planned in which ^{13}C labels will be introduced into the protein by the carboxymethylation of Met-8 and Met-81 residues using ^{13}C-

labelled iodoacetic acid. Line shape analysis of the proton-coupled ^{13}C NMR spectra should permit an assessment of anisotropic segmental motions of these widely separated regions of the molecule.

Note added in proof (Received November 2nd, 1979)

Similar findings to those presented here have recently been reported by Egmond et al. [16] in their ^{1}H-NMR studies of this protein.

Acknowledgements

We thank Dr. James D. Otvos for his participation in acquiring the proton pH data and for helpful discussions. This work was supported by Grant AM 18778-03 from the National Institutes of Health. The 270 MHz ^{1}H NMR work was supported in part by Grant PR 00798 from the Division of Research Resources, National Institutes of Health.

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