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The Effect of Carboxymethylating a Single Methionine Residue on the Subunit Interactions of Glycophorin A†

Michael Silverberg*, Heinz Furthmayr, and Vincent T. Marchesi

ABSTRACT: Human red cell glycophorin A shows an equilibrium between dimeric and monomeric forms which have been designated PAS-1 and PAS-2, respectively. This equilibrium, which is dependent upon protein concentration, is achieved by incubation in sodium dodecyl sulfate solutions at elevated temperatures and is assayed by sodium dodecyl sulfate gel electrophoresis. Carboxymethylation of glycophorin A in guanidine hydrochloride or urea alters the interactions between polypeptide chains so that the lower molecular weight form (PAS-2) is obtained much more readily. If the carboxymethylation is performed at pH 3.0, the reaction is limited to the two methionine residues of glycophorin A which are located at positions 8 and 81 in the sequence. In the presence of sodium dodecyl sulfate, only one of the two methionine residues is carboxymethylated, and glycoprotein modified under these conditions does not exhibit the change in electrophoretic mobility. Experiments with [1-^{14}C]iodoacetic acid demonstrated that Met-81, located in the hydrophobic domain of the protein, is the residue protected by sodium dodecyl sulfate. Modification of Met-81 destabilizes the dimeric form relative to the monomer by weakening the interactions between polypeptide chains. The experiments described in this paper confirm that the hydrophobic domain of glycophorin A is involved in subunit interactions and that Met-81 plays a critical role in those interactions.

The sialoglycoproteins of human erythrocyte membranes migrate as three PAS-positive bands when either ghost membranes (Fairbanks et al., 1971) or partially purified preparations (Furthmayr et al., 1975) are analyzed by sodium dodecyl sulfate gel electrophoresis. Recent studies from several laboratories indicate that two of these electrophoretic forms (PAS-1 and PAS-2) are interconvertible (Marton and Garvin, 1973; Tuech and Morrison, 1974; Furthmayr and Marchesi, 1976). This conclusion was based on experiments in which membranes or glycoprotein preparations were dissolved in sodium dodecyl sulfate solutions and heated prior to electrophoresis. After heating there is a shift in material from PAS-1, which is the major band in unheated preparations, to the position of the band of lower apparent molecular weight, PAS-2. A similar shift can be observed by gel filtration. The decrease in apparent molecular weight was interpreted as the breakdown of an oligomer to a smaller subunit (Marton and Garvin, 1973; Ji and Ji, 1974; Garvin et al., 1975). The extent of the conversion of PAS-1 to PAS-2 induced by heating the glycoprotein in sodium dodecyl sulfate solutions is inversely dependent on the protein concentration and is reversible (Furthmayr and Marchesi, 1976). These observations appear to exclude other explanations of the band shift such as peptide bond cleavage or conformational changes. The experiments described in this paper demonstrate that the same change in electrophoretic mobility, and presumably therefore the same dissociation of subunits, can be induced by chemical modification. It is further shown that a single residue of methionine is the critical site of the modification and that conversion of this methionine to a sulfonium salt destabilizes the dimeric form of gly-

† From the Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510. *Received October 28, 1975.* This work was supported by a grant from the American Cancer Society BC-102B and Membrane Center Grant No. P17-GM 21714.

cophorin A, enabling denaturing procedures to disrupt the interactions between subunits more readily than in the case of unmodified glycoprotein. These experiments also provide additional evidence for the suggestion of Furthmayr and Marchesi (1976) that the association between subunits occurs via interactions between the hydrophobic domains of glycophorin A polypeptide chains.

The resolution of the red cell sialoglycoprotein preparation called glycophorin (Marchesi et al., 1972) into two chemically distinct species, glycophorin A and B (Furthmayr et al., 1975), was achieved while the work described in this paper was in progress. Glycophorin A is the component which accounts for most of the PAS-1 band in unresolved glycoprotein preparations and is the component responsible for the changes in electrophoretic pattern obtained after heating or carboxymethylation. The carboxymethylation experiments described in the first part of this paper were done using the unresolved mixture. However, the presence of glycophorin B as a minor component in these preparations does not alter the results obtained or the conclusions drawn from them.

Materials and Methods

Methods

Reagent grade chemicals were used throughout and all water was double distilled. Dialysis tubing was boiled in EDTA at pH 8–9 and thoroughly rinsed in distilled water before use. Tris and urea were "ultra pure" from Schwarz/Mann. Guanidine hydrochloride was "extra pure" from Heico. The lithium salt of diiodosalicylic acid was prepared from the free acid obtained from Eastman Kodak and recrystallized twice from anhydrous methanol. Basic fuchsin for the preparation of PAS staining reagent was from Matheson, Coleman and Bell.

Iodoacetic acid-*1*-^{14}C was obtained from New England Nuclear at 5.2 mCi/mM.

Isolation of Glycoprotein. Membranes were prepared from human red cells as described by Furthmayr and Marchesi (1976). The glycoprotein fraction of freeze-dried membranes was prepared by suspending them in a solution of lithium diiodosalicylate and partitioning them in a phenol–water mixture according to the procedure of Marchesi and Andrews (1971). Glycophorin A was isolated by gel filtration in Ammonyx-Lo (Furthmayr et al., 1975). Glycoprotein preparations were delipidated by treatment with cold chloroform–methanol (2:1) for 1 h followed by chloroform–methanol–HCl (200:100:1) for 15 min to remove phosphoinositides.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out by the modification of the system of Fairbanks et al. (1971) described by Furthmayr and Marchesi (1976). Unless described otherwise, samples were prepared by dissolving them at 1 mg/ml in loading buffer containing 7 mM Tris-HCl, 0.7 mM EDTA, 8 M urea, and 2% sodium dodecyl sulfate at pH 7.2, and heating them at 80 °C for 15 min. A maximum of 100 μl was put onto each gel (6 mm × 11 cm) together with 2 μl of Pyronin Y solution (0.5 mg/ml). Gels were fixed in 25% 2-propanol, 10% acetic acid for 3 h at 37 °C. Gels were stained overnight with 0.05% Coomassie brilliant blue in 50% methanol, 10% acetic acid and destained in 15% methanol, 10% acetic acid followed by 10% acetic acid. For PAS staining, the fixed gels were washed overnight in fresh fixing solution and then stained as described by Furthmayr and Marchesi (1976). Identical gel patterns were obtained using either staining procedure. The stained gels were scanned with a Gilford spectrophotometer equipped with the linear transport attachment using 560 nm for PAS and 550 nm for Coomassie blue. Peak areas were estimated as height above baseline multiplied by peak width at half height.

For the determination of radioactivity, gels were sliced into 1-mm sections and the sections placed in scintillation vials with 10 ml of toluene-based scintillation fluid containing 5% protosol (New England Nuclear). After incubation for 24 h at 60 °C, the vials were transferred to a Beckman LS-250 scintillation counter for determination of cpm.

Carboxymethylation. Glycoprotein preparations were carboxymethylated at protein concentrations of 1 mg/ml. Unless otherwise stated, 0.3 M iodoacetic acid was used in 0.2 M citrate containing 7 M guanidine hydrochloride. The pH used in each experiment is noted in the text. The reaction was stopped by the addition of a slight molar excess of 2-mercaptoethanol. When carboxymethylation was performed at pH 3.0, 20 mM sodium thiosulfate was included to remove any iodine formed by decomposition of iodoacetic acid (Lee and Westheimer, 1966), and the mercaptoethanol was added together with sufficient NaOH and Tris to achieve a final pH of 7–8. The quenched reaction samples were routinely dialyzed against several changes of distilled water at room temperature and lyophilized.

Amino Acid Analysis. The amino acid composition of samples was determined with a Durrum D-500 analyzer after hydrolysis by HCl vapor as described by Tomita and Marchesi (1975). Performic acid oxidation was performed according to Schram et al. (1954); the reagents were removed by lyophilization after dilution with distilled water.

Results

The unresolved mixture of glycophorin A and glycophorin B gives the electrophoretic pattern shown in Figure 1a. When this preparation was treated with 0.3 M iodoacetic acid for 1 h at 37 °C in buffer containing 7 M guanidine hydrochloride at pH 10.5, there is a change in the electrophoretic pattern with PAS-2 becoming the major component (Figure 1c–f). As shown in Figure 1, the ratios of stained material found in PAS-1 and PAS-2 vary with the severity of the denaturing conditions used in preparing the samples for electrophoresis. The conditions used are described in the legend to Figure 1. Figure 1b shows a control of unmodified glycoprotein which had been subjected to the most severe denaturing regime. Although it shows some band shift, this is not nearly so great as that induced by carboxymethylation. These experiments showed that the association of glycophorin subunits is profoundly altered by chemical modification.

In order to interpret properly the effect of carboxymethylation on the subunit interactions of glycophorin, it was necessary to obtain further information on the conversion of PAS-1 to PAS-2 induced by heating the protein at low concentration in sodium dodecyl sulfate solution. Glycophorin A was dissolved in gel loading buffer to different concentrations and heated at 80 °C for 15 min. Figure 2 shows the percentage of PAS-2, obtained after electrophoresis of the various samples, as a function of protein concentration. Essentially the same concentration effect was found as was reported by Furthmayr and Marchesi (1976). When the gels were loaded with double the volume of sample, the proportions of PAS-1 and PAS-2 are unaltered. Thus, increasing

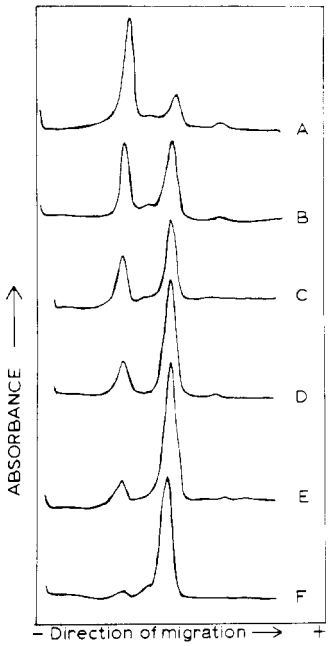

FIGURE 1: Sodium dodecyl sulfate polyacryladmide gel electrophoresis of the sialoglycoprotein preparation from human red cell membranes. The gels were stained with PAS as described in Materials and Methods and scanned at 560 nm. (A) Fresh material dissolved in gel loading buffer and heated at 80 °C for 15 min. (B) Glycoprotein incubated at 37 °C for 2 h in 7 mM Tris-HCl, 0.7 mM EDTA, pH 10.5, containing 7 M guanidine hydrochloride. The sample was prepared for electrophoresis by dialysis against 10 mM Tris-HCl, 1 mM EDTA, pH 7.0, containing 8 M urea with the addition of 2% sodium dodecyl sulfate to the final change of buffer, and then heated at 80 °C for 15 min before electrophoresis. (C) Glycoprotein carboxymethylated as in (B) but then dialyzed against buffer containing 1.3 M urea and without heating before electrophoresis. (D) Same as (C) but heated to 80 °C before electrophoresis. (E) Same as (C) but dialyzed against 8 M urea. (F) Glycoprotein carboxymethylated, dialyzed against 8 M urea, and heated to 80 °C before electrophoresis.

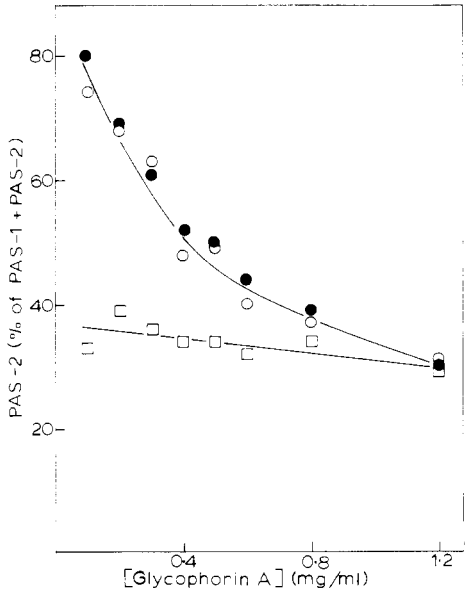

FIGURE 2: Glycophorin A was dissolved in gel loading buffer and heated at 80 °C for 15 min before electrophoresis. Gels were scanned and peak areas calculated as described in Materials and Methods. The amount of PAS-2, expressed as a percentage of the total of PAS-1 and PAS-2, is plotted against the concentration of glycoprotein in the sample loaded onto the gel. (○) Protein diluted before heating to the concentrations shown; 50 μl of sample per gel. (●) Protein diluted before heating; 100 μl of sample per gel. (□) Protein heated at 1.25 mg/ml and then diluted to the concentrations shown; 50 μl of sample per gel.

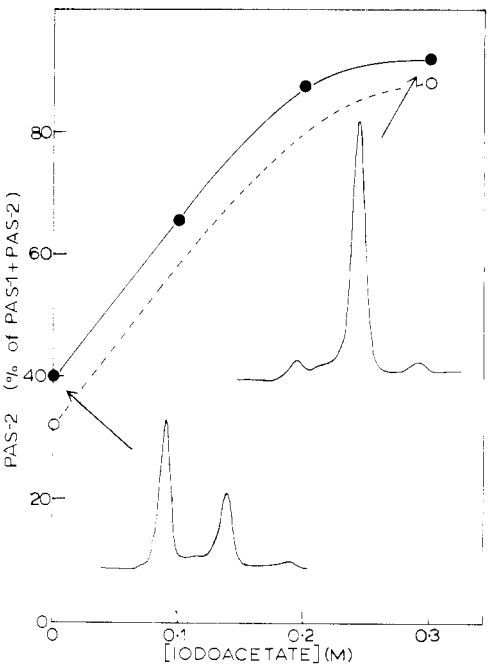

FIGURE 3: Glycoprotein carboxymethylated in 0.2 M Tris-HCl, pH 8.0 (●) or pH 10.0 (○) containing 7.0 M guanidine hydrochloride for 1 h at 37 °C using the concentrations of iodoacetic acid shown. The samples were prepared for electrophoresis by dialysis against distilled water, lyophilization, solubilization in gel loading buffer at 1 mg/ml, and heating at 80 °C for 15 min. The inset scans represent gels of glycoprotein treated with 0 or 0.3 M iodoacetic acid.

the amount of material loaded onto the gel does not cause greater aggregation which suggests that any uncontrolled concentrating effect at the top of the gel does not distort the electrophoretic pattern. When glycophorin A was heated at the high concentration of 1.25 mg/ml and then diluted before electrophoresis, the proportions of PAS-1 and PAS-2 found showed very little concentration dependence (Figure 2). We conclude that at 80 °C, an equilibrium exists between PAS-1 and PAS-2 which we postulate to be dimeric and monomeric forms of the same polypeptide chain. The position of equilibrium is dependent upon the total protein concentration during the incubation at 80 °C but is fixed when the temperature is lowered. Comparison of Figure 1 with the data in Figure 2 indicates that, at high concentrations of glycoprotein (>1 mg/ml), the native structure exists mostly in the PAS-1 form when heated in sodium dodecyl sulfate solutions whereas the carboxymethylated protein forms PAS-2. Thus carboxymethylated glycoprotein does not exhibit the same equilibrium between monomer and dimer found in unmodified glycoprotein. Rather, it seems that the dimer is destabilized relative to the monomer by carboxymethylation so denaturation procedures have greater effect.

If the hypothesis of destabilization of the dimer is correct, the continued maintenance of denaturing conditions would be unnecessary. A maximum conversion of PAS-1 to PAS-2 should occur provided only that the carboxymethylated protein is subjected to severe denaturing conditions before electrophoresis. This was found to be the case. Thus, after carboxymethylation in 7 M guanidine hydrochloride, excess reagents and guanidine were removed by dialysis against distilled water followed by lyophilization. When the dry material was dissolved in sample buffer containing 2% sodium dodecyl sulfate and 8 M urea and heated for 15 min

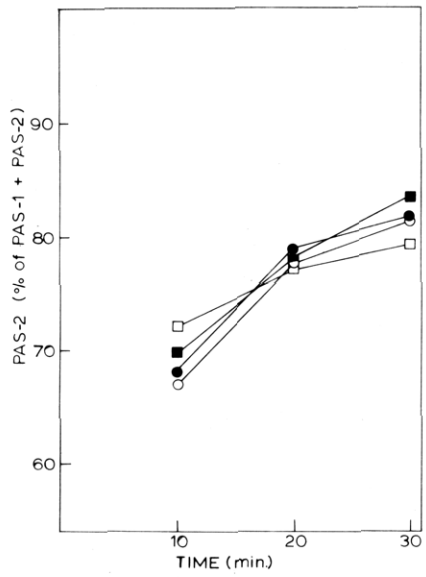

FIGURE 4: Glycoprotein was treated at 37 °C with 0.3 M iodoacetic acid in the following buffers, all containing 7 M guanidine hydrochloride: (○) 0.2 M citrate, pH 5.0; (□) 0.2 M citrate, pH 6.0; (■) 0.1 M phosphate, pH 7.0; (●) 0.1 M phosphate, pH 8.0. Samples were prepared for electrophoresis as described in Materials and Methods.

at 80 °C, over 90% of the material migrated as PAS-2 (Figure 3). Under these conditions, control samples incubated without iodoacetic acid showed the same electrophoretic pattern as starting material. The protocol used in this experiment was adopted as standard procedure in subsequent experiments.

As shown in Figure 3, the extent of conversion of PAS-1 to PAS-2 obtained after 1 h incubation with iodoacetic acid was strongly dependent on the concentration of iodoacetic acid and independent of a change of pH from 10 to 8. Parallel incubations with and without 10 mM dithiothreitol gave identical results, in accord with the absence of cysteine or cystine in glycophorin (Furthmayr et al., 1975).

Iodoacetic acid can react with various amino acid side chains and the dependence of the reaction on pH could give an indication of which residues are critical in the modification reported here. When glycophorin was incubated with 0.3 M iodoacetic acid for 35 min at pH values between 5 and 10 in 0.5 unit intervals, all the samples have identical gel patterns in which 90% or more of the glycoprotein migrated as PAS-2. Although this experiment suggested that there was no great dependence on pH in the reaction responsible for the band shift, it was still possible that the rate of reaction had indeed varied with pH but that even the slowest reaction was complete by the end of the incubation. Figure 4 shows the results of an experiment in which incubations at pH 5, 6, 7, and 8 were sampled at 10, 20, and 30 min. It is clear that no significant variation of rate can be discerned in this pH range. The insensitivity of the reaction to pH suggests that the shift in electrophoretic mobility results from the modification of one or more methionine residues (Gundlach et al., 1959a).

Amino acid analysis disclosed that at pH 5.0 some histidine was modified as well as methionine. In order to restrict the reaction with iodoacetic acid to methionine side chains, the pH was lowered to 3.0. Purified glycophorin A is shifted almost entirely to the PAS-2 position when treated with iodoacetic acid at pH 3.0 (Figure 5c,d). At this pH, only methionine residues are lost during the reaction with iodoacetic acid (Table I).

Table I: Amino Acid Analysis of Glycophorin A and Carboxymethylated Glycophorin A.

Residue	GPA	CM-GPA[a]
Aspartic acid (+ amide)	8.0[b]	8.0
Threonine	12.8	12.6
Serine	11.8	12.3
Glutamic acid (+ amide)	13.2	13.7
Proline	8.8	9.2
Glycine	6.2	6.4
Alanine	7.6	7.4
Valine	10.7	10.6
Methionine	2.3	0.9
Isoleucine	9.4	9.1
Leucine	8.1	8.0
Tyrosine	2.3	2.3
Phenylalanine	2.4	2.3
Histidine	5.2	5.0
Lysine	5.0	4.9
Arginine	5.3	5.3

[a] CM-GPA is glycophorin A carboxymethylated at pH 3.0 as described in Materials and Methods. [b] Values for each residue are expressed relative to aspartic acid (+ amide) = 8.0.

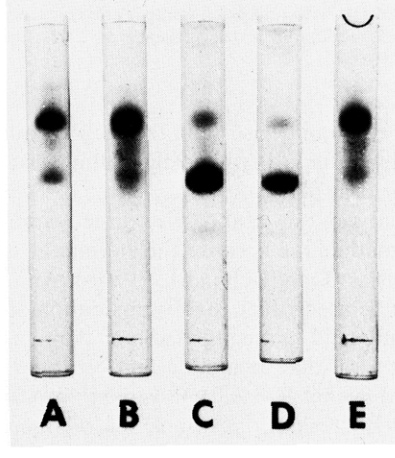

FIGURE 5: Coomassie blue stained gels of glycophorin A carboxymethylated for 2 h in citrate buffer at pH 3.0. (A) Unmodified control. (B) Carboxymethylated in 2% sodium dodecyl sulfate. (C) Carboxymethylated in 7 M guanidine hydrochloride. (D) Carboxymethylated in 10 M urea. (E) Carboxymethylated in 10 M urea + 2% sodium dodecyl sulfate.

It is significant that the band shift obtained from the reaction of glycophorin A with iodoacetic acid at pH 3.0 is contingent upon the use of glycophorin which has been delipidated by treatment with chloroform–methanol and acidified chloroform–methanol. If this treatment is omitted, little or no change in the electrophoretic pattern is observed after carboxymethylation at pH 3.0, although some shift is obtained after reaction at high pH.

A similar inhibitory effect is found with sodium dodecyl sulfate itself. Figure 5 shows that the change in electrophoretic mobility occurs to the same extent after carboxymethylation in either guanidine or urea, but that it does not occur after carboxymethylation in sodium dodecyl sulfate, even when 10 M urea is also present. Thus, sodium dodecyl sulfate and lipid may bind to the glycoprotein in a similar manner, preventing the reaction of some methionine residues with iodoacetic acid. Sodium dodecyl sulfate is less effective as an inhibitor of the reaction at pH values greater

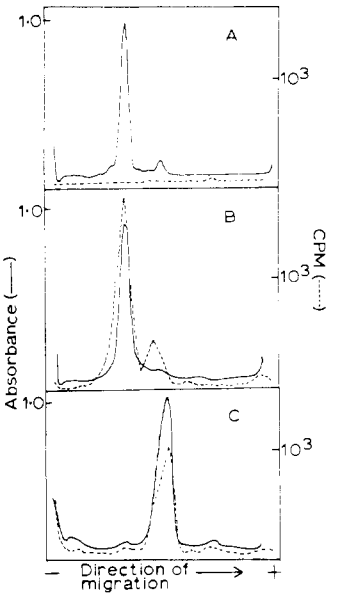

FIGURE 6: (A) Unmodified glycophorin A; (B) glycophorin A carboxymethylated with [1-^{14}C]iodoacetic acid for 4 h at 37 °C in the presence of 2% sodium dodecyl sulfate; (C) glycophorin A carboxymethylated as in (B) with unlabeled reagent and re-carboxymethylated with [1-^{14}C]iodoacetic acid in the presence of 7 M guanidine hydrochloride.

than 8, as was found with lipid. At neutral pH or below, carboxymethylation is inhibited by sodium dodecyl sulfate as effectively as at pH 3.0.

Amino acid analysis cannot give direct estimates of carboxymethylmethionine because the sulfonium salt is unstable to hydrolysis (Gundlach et al., 1959b). An indirect estimation must be used which involves oxidation of the protein with performic acid and comparison of the methionine sulfone content with that of unmodified control samples (Vithayathil and Richards, 1960). Glycophorin A was found to contain 2.3–2.5 residues per mole of methionine sulfone after performic acid oxidation. The yield of sulfone was reduced to 1.1–1.5 after carboxymethylation of nondelipidated samples or of delipidated samples in the presence of sodium dodecyl sulfate or sodium dodecyl sulfate + urea. After carboxymethylation of delipidated samples in the presence of guanidine or urea, the yield of sulfone was 0.6 residue. The persistence of methionine sulfone after rigorous carboxymethylation is attributed to the presence of methionine sulfoxide in the native glycophorin; methionine sulfoxide does not react with iodoacetic acid (Neumann, 1972). In spite of the difficulty of obtaining precise analytical data of this kind, it is clear that in the presence of sodium dodecyl sulfate or residual lipid there is less reaction with iodoacetic acid and the protein shows a greater amount of sulfone than after carboxymethylation in guanidine or urea. Thus the change in electrophoretic mobility is associated with the modification of only a portion of the methionine content of glycophorin A.

Since sodium dodecyl sulfate and many of the common membrane phospholipids carry negative charges on their hydrophilic head groups, it was conceivable that, when these substances were bound to glycophorin, iodoacetate anions could not approach some areas of the protein because of electrostatic repulsion. This explanation for the effects of sodium dodecyl sulfate and lipid on the carboxymethylation was ruled out by using iodoacetamide in place of iodoacetic acid. The experimental results reported above were duplicated with iodoacetamide, the electrophoretic mobility of glycophorin was increased and this change did not occur when the reaction was performed in sodium dodecyl sulfate. This experiment also indicated that the band shift that occurs as a result of carboxymethylation was not due to the introduction of a negative charge on the carboxymethyl group because the carboxamido derivative formed from iodoacetamide has no such charge. Thus, it appears that the cause of the destabilization of the glycophorin A dimer is the conversion of some of the methionine residues to positively charged sulfonium ions.

The sequence of glycophorin A contains two methionine residues (Tomita and Marchesi, 1975). One of these, residue 8, is located in a heavily glycosylated part of the polypeptide chain; the other is residue 81 which is situated in the middle of the hydrophobic region. The results of peptide competition studies (Furthmayr and Marchesi, 1976) implicated the hydrophobic region as the site of the interactions holding two glycophorin A subunits together. The breaking of this contact is presumed to be responsible for the shift in electrophoretic mobility to PAS-2. If this hypothesis is correct, methionine 81 would be implicated as the site of the critical modification which is protected by sodium dodecyl sulfate. We attempted to confirm this hypothesis by the following experiments.

Glycophorin A was carboxymethylated with iodoacetic acid-*1*-^{14}*C* in the presence of 2% sodium dodecyl sulfate at pH 3.0. Another sample of glycophorin A was carboxymethylated under the same conditions but with unlabeled iodoacetic acid. After the two samples had been dialyzed exhaustively to remove sodium dodecyl sulfate, the unlabeled sample was carboxymethylated again, this time using iodoacetic acid-*1*-^{14}*C* in the presence of 7 M guanidine hydrochloride at pH 3.0. After dialysis and lyophilization, aliquots of these two samples were electrophoresed. Figure 6 shows that the sample labeled in sodium dodecyl sulfate migrated as PAS-1 predominantly, whereas the sample labeled in guanidine hydrochloride migrated as PAS-2. In each case the patterns of stain and label coincide. The rest of each sample was digested with trypsin according to the procedure of Furthmayr et al. (1975). The peptide T6 formed by the tryptic cleavage of glycophorin A contains the hydrophobic domain of the polypeptide chain and is obtained as a precipitate after acidification of the peptide mixture; the glycopeptides and the C-terminal peptides remain in solution.

Table II shows the radioactivity appearing in the supernatant and in the precipitate after tryptic digestion of the two samples. It is clear that in sodium dodecyl sulfate only methionine 8 undergoes any extensive reaction with iodoacetic acid. As the undigested protein that had been carboxymethylated in sodium dodecyl sulfate only showed PAS-1 on gels (Figure 6), methionine 8 cannot be at the site of interaction between glycophorin subunits. When methionine 8 is blocked with unlabeled iodoacetic acid and the protein treated with labeled reagent in guanidine, the label is found in the hydrophobic peptide after tryptic digestion. Because our previous experiments demonstrated that only methionine residues react under the conditions used, we can deduce that the carboxymethylation of methionine 81 is sufficient to cause the electrophoretic mobility to change from that of PAS-1 to PAS-2.

In a final experiment, the hydrophobic peptides prepared as just described were used in a competition experiment according to the procedure of Furthmayr and Marchesi

Table II: Radioactive Content of Soluble and Insoluble Fractions after Trypsin Digestion of Glycophorin A Carboxymethylated with Iodoacetic Acid-1-^{14}C.[a]

Fraction		cpm[b]	Ratio[c]
Glycophorin A labeled in guanidine hydrochloride:	Supernatant	434	1
	Precipitate	2800	6.5
Gycophorin A labeled in sodium dodecyl sulfate:	Supernatant	6190	10
	Precipitate	612	1

[a] See text for description of glycophorin labeling experiment. [b] The insoluble peptide was washed with acidified distilled water and dissolved in 0.5 ml of gel loading buffer. The soluble peptides were lyophilized and also dissolved in 0.5 ml loading buffer to give the same molar concentration as the solution of hydrophobic peptide. Aliquots (20 μl) of each solution were counted and these figures given in the table. [c] Ratio of larger to smaller figure obtained from the two fractions of each glycophorin sample.

(1975). Mixtures of glycophorin A and peptide in approximately equal amounts by weight were heated in loading buffer containing 2% SDS and 8 M urea and electrophoresed. The results, shown in Fig. 7, indicate that the unlabeled peptide from glycophorin carboxymethylated in SDS promoted almost complete conversion to PAS-2 in a manner similar to the peptide from native glycophorin (Furthmayr and Marchesi, 1976). The peptide from material carboxymethylated in guanidine produced a small shift in the electrophoretic pattern but this was much less than the other peptide. It is clear that modification of methionine 81 severely reduces the ability of the peptide to interact with the native molecule.

Discussion

The experiments described in this paper demonstrate that subunit interactions of glycophorin A are profoundly modified by the carboxymethylation of a single methionine residue. This methionine residue at position 81 is located in the hydrophobic domain of the molecule which is believed to be that part of the polypeptide chain which interacts with membrane lipids in vivo. Thus the discovery of a role for this methionine in the interactions between subunits is strong evidence for the participation of the intramembranous region of the protein in those interactions.

The interactions between unmodified subunits are usually strong. The experiments of Furthmayr and Marchesi (1976) and the results shown in Figure 2 indicate that dissociation of glycophorin subunits does not occur in solutions of sodium dodecyl sulfate unless strict conditions of temperature and protein concentration are fulfilled. One interpretation of these results is that the conversion of dimer to monomer is a reversible process which has a relatively high energy of activation barrier. Thus only at high temperatures can an equilibrium position be approached.

The experiments shown in Figure 1 show that carboxymethylated glycophorin is mostly in the PAS-2 form even without the use of high temperature. Thus the energy of activation barrier for the conversion of dimer to monomer must be appreciably lower. This experiment and that shown in Figure 3 also show that, at the concentrations where heated glycophorin remains as PAS-1, heated carboxymethylated glycophorin is wholly converted to PAS-2. Thus the position of equilibrium is presumably also shifted for the modified protein. We postulate on the basis of these conclusions that the dimeric form of the molecule is destabilized by carboxymethylation.

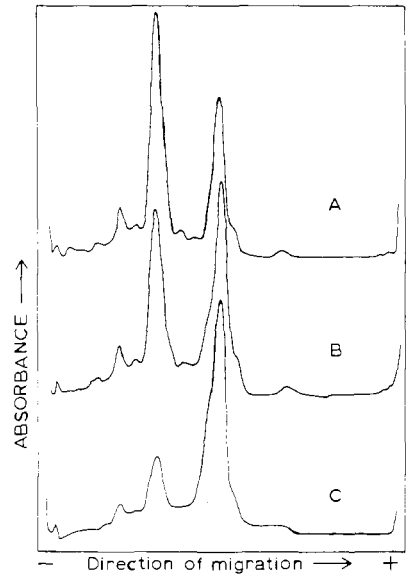

FIGURE 7: Glycophorin A dissolved in gel loading buffer at 0.8 mg/ml and heated at 80 °C for 15 min—(A) alone; (B) with 0.8 mg/ml of hydrophobic peptide from glycophorin A carboxymethylated in guanidine hydrochloride; (C) with 0.8 mg/ml of hydrophobic peptide from glycophorin A carboxymethylated in 2% sodium dodecyl sulfate.

It should be emphasized that we do not postulate that glycophorin is completely dissociated as a result of carboxymethylating methionine 81. Indeed, in guanidine or urea the protein may be in an highly aggregated state, and even in sodium dodecyl sulfate appreciable amounts of PAS-1 will be present if the solution has not been heated.

The experiments with hydrophobic peptides from modified glycophorin support this concept. When glycophorin was incubated at 80 °C with the peptide which contained an unmodified methionine residue, most of the material was shifted to the PAS-2 position (Figure 7). When the peptide which contained a modified methionine was used, there was some shift compared with glycophorin incubated alone. Thus it would appear that, when an excess of peptide is present, some binding can still occur, although this is to a much smaller extent than that found with unmodified peptide. This result is consistent with the idea that carboxymethylation of Met-81 does not completely abolish subunit interactions but weakens them, giving rise to an increased dissociation constant for the equilibrium between dimer and monomer. It is worthy of note that experiments with the modified peptide involve an interaction between one modified site and one unmodified site (on the intact glycophorin) and this heterogeneous "dimer" may be more stable than a homogeneous dimer with both components modified.

The major question raised by these results is the nature of these subunit interactions. In particular we would like to know whether they represent an alternative to interactions with membrane lipids for the glycophorin subunits or whether they exist even when protein is inserted into the membrane. To some extent the carboxymethylation reaction described in this paper can serve as a probe of the subunit interactions of glycophorin A. In this regard the most significant results are those showing that sodium dodecyl sulfate or residual lipid severely inhibit the carboxymethylation of methionine 81. This result combined with the experiments described above shows that sodium dodecyl sulfate not only fails to dissociate the subunits but can also protect the region of interaction from attack by low molecu-

lar weight reagents. This protective effect is presumably exerted through sodium dodecyl sulfate binding to the protein and covering up the hydrophobic region. The close parallel between the effects on the carboxymethylation of sodium dodecyl sulfate and of residual lipid lends weight to the suggestion that sodium dodecyl sulfate can act as an analogue of the amphiphilic phospholipids that comprise membranes. This inhibitory effect on carboxymethylation is a possible approach to further investigation of the interaction of a glycophorin with various amphiphiles.

The similarity between the effects of lipid and sodium dodecyl sulfate suggests that the glycoprotein may exist in an oligomeric form in the membranes as well as in sodium dodecyl sulfate. However, so far only circumstantial evidence has been obtained on this question, and the configuration of glycophorin in the membrane is essentially unknown.

In aqueous solution, glycophorin appears to exist in an highly aggregated form (V. T. Marchesi, unpublished observations), and it would appear that, in the absence of amphiphiles, the hydrophobic regions of the polypeptide chains can sequester themselves away from water to form micelle-like structures. These structures are presumably loose aggregates of dimer and are accessible to iodoacetic acid because carboxymethylation of glycoprotein in buffer can produce some shift (M. Silverberg, data not shown). Urea or guanidine significantly enhance the reaction with iodoacetic acid and incubation with guanidine alone can lead to some band shift if denaturing conditions are maintained throughout the procedure (Figure 1b). Thus these traditional protein denaturants do have some effect on the structure of this molecule but, as shown in Figure 3, this effect is reversible. Also, the data in Figure 5 show that, in the presence of 2% sodium dodecyl sulfate, the effect of 10 M urea on the carboxymethylation is completely negated.

It is clear that the traditional concepts of protein denaturation do not apply to a protein like glycophorin which may well be typical of many integral membrane proteins. If this is the case, the determination of the smallest molecular weight unit of a membrane protein should be made much more carefully than it normally is. In particular, incubation of proteins in sodium dodecyl sulfate at 37 °C does not appear to be a denaturing regime for membrane proteins.

The localization of the site of interchain interactions to the hydrophobic domain of glycophorin A has interesting implications for membrane biology. It has been generally assumed that events leading to changes in the aggregation state of membrane proteins in situ affect those portions of the membrane proteins which extend either to the exterior or the interior of the cell. Thus some intracellular event might bring together two membrane polypeptide chains by linking together their ends which are in contact with the cytoplasm. However, the data presented in this paper indicate that the intramembranous region is very important in determining the state of aggregation of one integral membrane protein. It would seem then that events occurring inside the lipid bilayer may also be important in the regulation of membrane processes.

Acknowledgment

We appreciate the dedicated technical assistance of Ms. Philomena Ostapchuk.

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