Subunit Structure of Human Erythrocyte Glycophorin A†

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ABSTRACT: Glycophorin A is a sialoglycoprotein isolated from human erythrocyte membranes which seems to exist as stable dimeric complexes in the presence of sodium dodecyl sulfate. When analyzed by dodecyl sulfate acrylamide electrophoresis this molecule forms two PAS-stainable bands (PAS-1 and PAS-2) which are reversibly interconvertible. This change in electrophoretic mobility is dependent on the concentration of dodecyl sulfate, the use of Tris-buffer systems, the protein concentration in the incubation mixture, and the duration and temperature of incubation before electrophoresis. Reducing agents do not influence the results. Chromatography of the sialoglycopeptides on Sepharose columns in dodecyl sulfate before and after heat treatment gave similar results. A small hydrophobic peptide (T-6) derived from glycophorin A was able to prevent reassociation of the monomeric subunits back to the higher molecular weight form. This peptide was able to bind to the subunit of glycophorin A, but not to the high molecular weight complex. These results are consistent with a model of glycophorin A composed of two subunits which can dissociate and reassociate in the presence of detergents. These subunits may interact via the hydrophobic portions of the polypeptide chains.

For many years the erythrocyte membrane has been used as a model for studying membrane structure and function. Part of this effort has been directed toward a better understanding of membrane proteins and their interactions with the lipid bilayer and special attention has been directed to the unique sialoglycoproteins, which constitute approximately 5% of the total membrane proteins (Winzler, 1969). When these glycopeptides are analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, up to four bands can be detected after staining with PAS[1] (Steck, 1974). Despite numerous efforts, previous attempts to isolate a single component on a large scale have been unsuccessful. In addition, uncertainty still exists concerning the molecular weights of the polypeptides in glycoprotein fractions which have been isolated by various methods (Winzler, 1969; Blumenfeld et al., 1970; Marchesi and Andrews, 1971; Cleve et al., 1972; Fukuda and Osawa, 1973; Grefrath and Reynolds, 1974). The molecular weights found for the major component in different preparations have ranged from 20 000 to 61 000 as determined by ultracentrifugation, chromatography, or polyacrylamide gel electrophoresis.

Recently it was found that the glycoprotein fractions obtained by various procedures contain at least two separate sialoglycopeptides (Janado et al., 1973; Fujita and Cleve, 1973; Furthmayr et al., 1975). The major component designated glycophorin A by Furthmayr et al. (1975) constitutes approximately 75% of the total fraction and corresponds to the PAS-1 and PAS-2 bands on dodecyl sulfate gels. The amino acid sequence of the polypeptide portion of this molecule has recently been determined, and from these data a molecular weight of 31 000 was obtained (Tomita and Marchesi, 1975). A second peptide provisionally designated glycophorin B and chemically distinct from glycophorin A can also be isolated in a homogeneous form (Furthmayr et al., 1975).

This report deals specifically with the question of the subunit composition of glycophorin A and the possible site of subunit interaction. While this work was in progress, Marton and Garvin (1973) and Tuech and Morrison (1974) published preliminary reports dealing with this problem. Marton and Garvin made the critical observation that glycoprotein aggregates in dodecyl sulfate can be partially dissociated by heat treatment.

Materials and Methods

Materials. Reagent or equivalent grade chemicals were used throughout. Chemicals used to prepare polyacrylamide gels were acrylamide, *N,N'*-methylenebisacrylamide, and ammonium persulfate from Canalco; TEMED, Eastman 8178; sodium dodecyl sulfate, "Sipon", Alcolac Chem. Corporation; urea, ultra pure, Schwarz/Mann 909200; Tris, Sigma "Trizma base"; Coomassie Brilliant Blue, Schwarz/Mann 909207; pyronin Y, Baker, lot No. 1-9807; basic fuchsin, Matheson Coleman and Bell; HSEtOH, Eastman 4196; $PhCH_2SO_2F$, Calbiochem 52332. Lithium 3,5-diiodosalicylate (Marchesi and Andrews, 1971) was prepared from 3,5-diiodosalicylic acid (Eastman Kodak, 2166) after 2 × recrystallization from anhydrous methyl alcohol, Mallinckrodt 3016.

Molecular weight markers for calibration of the gel filtration columns were bovine serum albumin (Miles 81-001), horse myoglobin (Miles 95-062), and [^{3}H]cholesterol (New England Nuclear, lot No. 635-158, specific activity 10 Ci/mmol). Sodium [^{3}H]borohydride was purchased from New England Nuclear. Several batches were used with specific radioactivities ranging from 142 to 245 mCi/mmol. Sodium borohydride (lot No. 21 C-03519) and sodium metaperiodate (lot. No. 62C-2260) were from Sigma. Carrier free [^{125}I]iodide was purchased from New England Nuclear.

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[1] Abbreviations used are: PAS, periodic acid–Schiff's reagent; PAS-1–3, the three major glycopeptide components demonstrated by electrophoresis (Fairbanks et al., 1971); TEMED, *N,N,N',N'*-tetramethylethylenediamine; HSEtOH, β-mercaptoethanol; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

Preparation of Erythrocyte Ghosts. Ghosts were prepared from fresh human blood drawn in citrate dextrose anticoagulant (U.S.P. formula A), following the procedure used by Fairbanks et al. (1971). The buffer used for lysis of the cells contained 5 mM phosphate (pH 8.0) and 5.22 mg of $PhCH_2SOF$/l.; lysis was performed strictly at ice bath temperature, and after two washes in the same buffer, the membranes were washed once in 25 mM Tris (pH 7.4). Labeling of intact cells after periodate oxidation and treatment with [^{3}H]$NaBH_4$ was done according to Blumenfeld et al. (1972) and membranes were prepared from these cells as above.

Assays. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Sialic acid in the column effluent was determined by the procedure of Warren (1959) after hydrolysis of 0.9 ml of column effluent in 0.1 N H_2SO_4 for 1 h at 80 °C.

Sialidase treatment of the isolated glycoprotein fraction was done with an enzyme obtained from Sigma (*Clostridium perfringens,* Type VI, lot 31C-8100-1), which was purified by affinity chromatography according to Cuatrecasas and Illiano (1971). ^{125}I-labeled glycoprotein (see below) (1×10^6 cpm/mg) (2.5 mg) was mixed with 2.5 mg of cold glycoprotein and dissolved in 1 ml of buffer containing 0.05 M sodium acetate (pH 5.5), 0.2 mM EDTA, and 2 mM $CaCl_2$. After addition of 50 μl of neuraminidase in 0.1 M NaCl (0.127 OD_{280} units/ml) the mixture was incubated for 4 h at 37 °C. Release of sialic acid was monitored using Warren's procedure. The desialated glycoprotein was recovered free from sialic acid after chromatography on Bio-Gel P-2 in 0.1 N acetic acid. The final analysis using gas chromatography revealed 1 mol of sialic acid/30 000 or release of 95% of sialic acid.

Isolation of Sialoglycopeptides. The sialoglycoprotein fraction was isolated as described previously (Marchesi and Andrews, 1971). To remove phosphoinositides, the glycoprotein fraction was extracted for 1 h at room temperature with acidic chloroform–methanol (chloroform–methanol–HCl, 200:100:1) as described by Wells and Dittmer (1965). [^{125}I]Glycoprotein was prepared by iodination with purified lactoperoxidase as described by Phillips and Morrison (1971) and Segrest et al. (1973). [^{125}I]Glycoprotein and iodinated peptides derived from it were also prepared by iodination with insolubilized glucose oxidase and lactoperoxidase on CNBr-activated Sepharose (J. P. Kraehenbuhl, personal communication). Tryptic peptides were prepared from the isolated glycoprotein fractions and purified as described elsewhere (M. Tomita, H. Furthmayr, and V. T. Marchesi, in preparation). Glycophorin A was isolated and trypic peptides were prepared as described (Furthmayr et al., 1975).

Polyacrylamide Gel Electrophoresis in Dodecyl Sulfate. Gels were prepared essentially as described by Fairbanks et al. (1971) in 6 mm × 12.5 cm tubes with the following modifications: 0.5 M urea was included into the polymerization mixture, the gels were overlaid with distilled water, and the sample buffer contained 6% dodecyl sulfate, 4 M urea, 20 mM Tris-HCl (pH 6.8), 2 mM EDTA, 3% HSEtOH, and 30 μg of Pyronin Y/ml. Equal volumes of sample and sample buffer were mixed and incubated for 30 min at 37 °C. Further treatment is indicated under Results. The samples were applied onto the gels without prior dialysis.

Gels were also prepared according to Shapiro et al. (1967) and Maizel (1971) as described by Segrest et al. (1971), and by Hubbard and Cohn (1972), respectively. 3.5% agarose-acrylamide gels were adapted from Dingman and Peacock (1968), using the buffer system of Fairbanks et al. (1971). Gels were stained with Coomassie Blue as described by Fairbanks et al. (1971); destaining was done in 25% isopropyl alcohol containing 10% acetic acid overnight and was completed in 10% acetic acid. After complete removal of dodecyl sulfate by fixation for 2 days in isopropyl alcohol–acetic acid, the gels were treated for 2 h in 0.7% periodic acid in 5% acetic acid, followed by a 2-h treatment with 0.2% sodium metabisulfate in 5% acetic acid and the gels were transferred to tubes and stained with the PAS reagent. Destaining was done as recommended by Fairbanks et al. (1971). Gels were scanned at 560 nm using a Gilford spectrophotometer and scanning device. The peak areas were determined by triangulation (Matthieu and Quarles, 1973). Linearity was found for amounts of 1–100 μg of glycoprotein per gel for the sum of the peak areas of the three major PAS-stainable bands. The positions of the three glycopeptides were determined under standard conditions. Slices of 3 mm containing these peptides were cut out of the unfixed gel and rerun on another set of gels with or without prior treatment. When slices were incubated, 50 μl of sample buffer (diluted 1:2) was added.

Determination of Radioactivity. ^{125}I was determined by using a gamma counter from Beckman Instruments Co., Model Biogamma. Gel slices of 1 or 2 mm thickness obtained with a homemade instrument were counted directly. ^{3}H was counted by liquid scintillation spectroscopy in a Beckman LS-250 scintillation counter. Gel slices usually of 2 mm thickness were dissolved in 8 ml of toluene-based scintillation liquid containing 5% Protosol (New England Nuclear) by shaking at 40–50 °C for 12 h.

Nomenclature. The three major glycoproteins were designated PAS-1, PAS-2, and PAS-3 according to Fairbanks et al. (1971). A fourth, rather faint band, migrating between PAS-1 and PAS-2, was found in all membrane preparations and also in the isolated glycoprotein fraction (PAS-4 of Tanner and Boxer, 1972; 1a of Tuech and Morrison, 1974). It is not clear whether this band represents a minor species of glycoprotein or an aggregated form of the glycophorin components (Furthmayr et al., 1975). Since this band was not very well resolved on the gel systems used, it was not included in the calculations.

Results

I. Glycophorin A Undergoes Heat-Induced Dissociation in the Presence of Dodecyl Sulfate. Pretreatment of the dodecyl sulfate membrane protein mixture at higher temperature prior to electrophoresis changes the electrophoretic pattern (cf. Figure 1a and b). Only two polypeptide species are involved in this change regardless of the conditions employed for electrophoresis, i.e., concentrations of polyacrylamide between 3.5 and 7.5% or of dodecyl sulfate between 0.2 and 1%. This change was not observed when phosphate buffer systems were used (data not shown). Triangulation of the peak areas obtained after scanning of PAS-stained gels (Matthieu and Quarles, 1973) was found to be a reliable way to quantitate the amount of glycoprotein in the gels. Approximately 60, 30, and 10% of the total PAS-stainable material were found in the PAS-1, PAS-2, and PAS-3 positions, respectively, when standard amounts of total membrane proteins were analyzed using this method. Quantitation of incorporated ^{125}I or ^{3}H using the appropriate systems for labeling (Segrest et al., 1973; Blumenfeld et al., 1972) gives essentially the same results (Figure 2) except

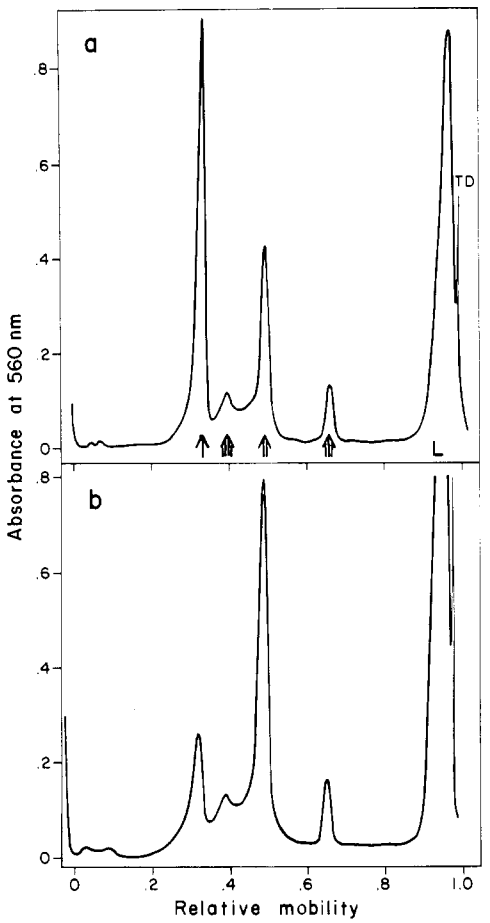

FIGURE 1: Mobilities of sialoglycopeptides of human erythrocyte membranes on dodecyl sulfate polyacrylamide gels as a function of heat treatment. 25 μl of packed ghosts was mixed with an equal volume of sample buffer and electrophoresed after incubation for 30 min at 37 °C (a) or an additional 2 min at 100 °C (b). The gels were stained with PAS reagent and scanned at 560 nm. Arrows denote the major sialoglycopeptides PAS-1 (↑), PAS-4 (⇑), PAS-2 (↑), PAS-3 (⇑). Lipid (L) migrates behind the tracking dye (TD).

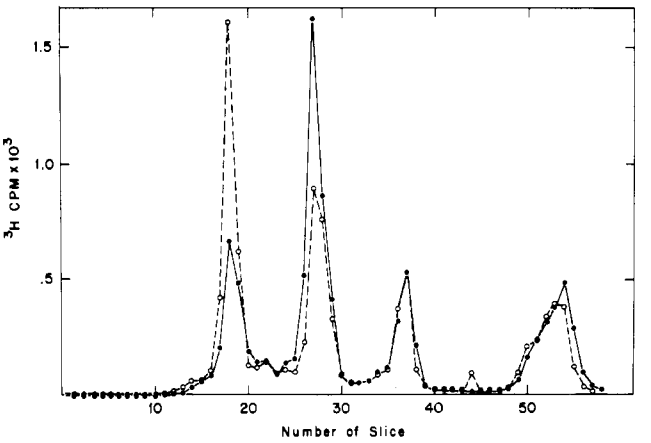

FIGURE 2: Quantitative distribution of labeled sialoglycopeptides. Intact human erythrocytes were tritiated and ghosts prepared from these cells were analyzed on dodecyl sulfate polyacrylamide gels after incubation at 37 °C (- - -) or 100 °C (—). The gels were sliced, solubilized, and counted as described in the Methods section. The labeled peaks correspond to the PAS-stainable bands in Figure 1.

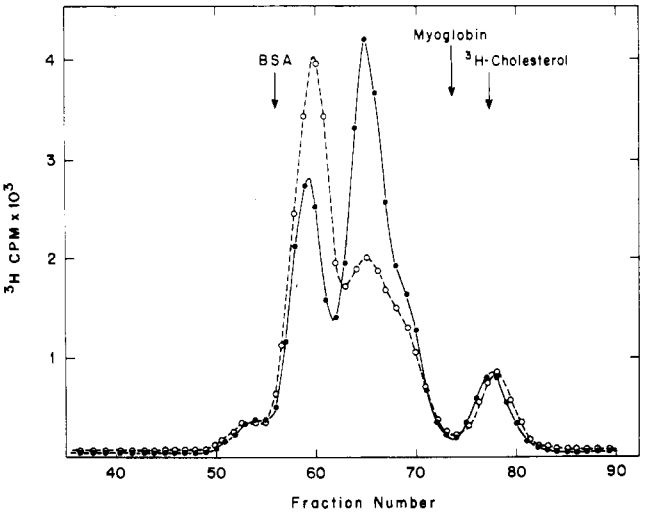

FIGURE 3: Gel filtration of labeled sialoglycopeptides in the presence of dodecyl sulfate: effects of heat pretreatment. Approximately 25 mg of ghost membranes obtained from tritiated red cells was dissolved in 5.0 ml of 1% dodecyl sulfate in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and 0.05% HSEtOH and incubated at 37 °C for 30 min and then chromatographed at 23 °C on a Sepharose 6B column (95 × 2.5 cm) equilibrated in the same buffer (○ - - ○). A comparable sample was incubated for an additional 5 min at 100 °C and chromatographed at 45 °C (● — ●). 100 μl of the fractions was mixed with 12.5 ml of scintillation liquid and counted. Only the labeled protein species are recorded. Molecular weight markers were bovine serum albumin (BSA, 67 000), horse myoglobin (17). [^{3}H]Cholesterol eluted together with the membrane lipids.

for a relative increase of ^{3}H-containing material in the PAS-3 position. All the counts always coincided with the stainable bands and the amount of stainable material or of counts lost in the PAS-1 position could be recovered entirely in PAS-2. From these data we conclude that PAS-1 and PAS-2 glycopeptides are involved in these changes and the PAS-3 and PAS-4 glycopeptides are not noticeably affected.

II. Dissociation of Glycophorin A under Conditions of Gel Filtration in Dodecyl Sulfate. When tritiated erythrocyte membrane proteins prepared by the method of Blumenfeld et al. (1972) are chromatographed on Sepharose 6B in dodecyl sulfate, the pattern shown in Figure 3 is obtained. Most of the label (approximately 90%) is recovered in the fractions containing sialic acid. Two peaks are well resolved and a third component is indicated by a shoulder around fraction 70. Analysis of peak fractions by dodecyl sulfate gels indicates that the leading peak is predominantly PAS-1, the second peak is PAS-2, and the shoulder is enriched for PAS-3. The apparent molecular weights for these three components were found to be 50 000, 31 000, and 22 500, respectively, using globular proteins as standards.

Pretreatment of the sample at 100 °C for 5 min resulted in a significant change in the elution profile. A large proportion of the leading peak shifted to the lower molecular weight form, and dodecyl sulfate gel analysis confirmed the expected band shifts.

The same change in the elution of the sialoglycopeptides could also be obtained if the leading peak of [^{3}H]glycoprotein was isolated by gel filtration before heat treatment and then rechromatographed after heat treatment at 100 °C.

III. The Dissociation of Glycophorin A is Dependent on Several Parameters. The change in the distribution of PAS-1 and PAS-2 glycopeptides is influenced by the temperature and duration of heating of the sample before electrophoresis and the absolute concentration of glycoprotein in the sample (Figures 4–7). Incubation of glycoproteins in dodecyl sulfate at 65 °C for 5 min is sufficient to give a de-

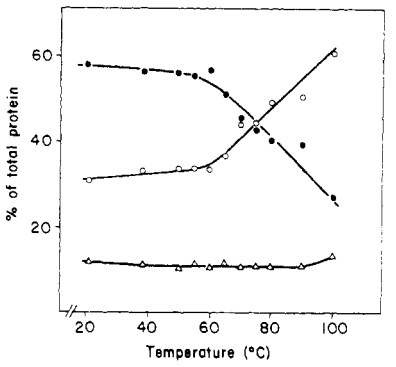

FIGURE 4: Conversion of the PAS-1 glycopeptide into the PAS-2 form as a function of temperature. Identical amounts of ghost membranes were electrophoresed on dodecyl sulfate polyacrylamide gels after incubation for 5 min in 3% dodecyl sulfate at the temperatures indicated. The gels were stained with PAS reagent and scanned at 560 nm and the peak areas were determined by triangulation. The data are expressed as relative amounts in percent of total stainable glycopeptides. PAS-1 (● — ●), PAS-2 (○ — ○), PAS-3 (△ — △).

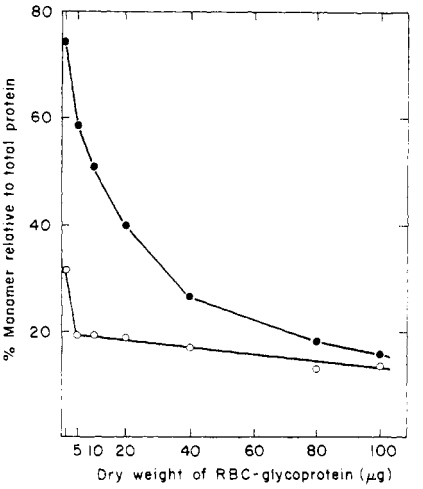

FIGURE 5: Effects of glycoprotein concentration on the conversion of the PAS-1 glycopeptide into the PAS-2 form. Varied amounts of purified sialoglycopeptides (1–100 μg/50 μl) were incubated for 30 min at 37 °C (○ — ○) and an additional 3 min at 100 °C (● — ●) and electrophoresed on dodecyl sulfate polyacrylamide gels. After staining with the PAS reagent and scanning at 560 nm, the peak areas were determined by triangulation. The data are expressed as the amount of PAS-2 relative to the total stainable glycopeptides.

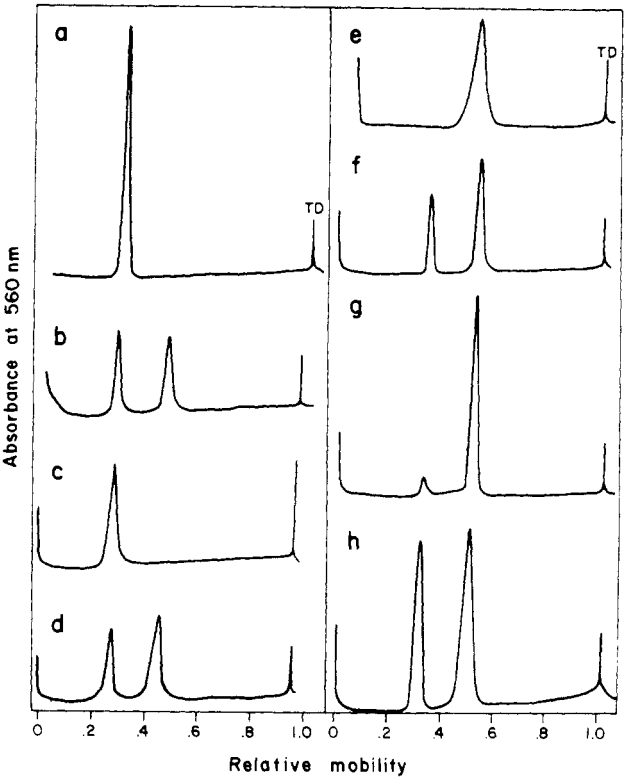

FIGURE 6: Reassociation of glycophorin A subunits. 100 μg of ghost membranes was electrophoresed as in Figure 1, and unfixed gel slices corresponding to the PAS-1 and -2 regions were incubated in 50 μl of sample buffer for 30 min at 37 °C or 3 min at 100 °C and then reelectrophoresed on fresh dodecyl sulfate gels. Material from the PAS-1 zone and incubated at 37 °C showed a single PAS-1 band upon reelectrophoresis (a), but if the same material was incubated at 100 °C both PAS-1 and PAS-2 were generated (b). Similarly, only the PAS-2 form resulted from rerunning the original PAS-2 band after incubation at 37 °C (e), but PAS-1 was also generated from this material (f) if it was heated at 100 °C. Rerunning the PAS-1 form from samples after incubation at 100 °C produced only the PAS-1 form if the material was incubated at 37 °C (c), but PAS-2 was also generated from this sample after 100 °C treatment (d). Comparable results were obtained when material from the PAS-2 zone was analyzed in the same way (g,h).

tectable shift in the mobility of PAS-1 (Figure 4) and this effect becomes more pronounced at higher temperatures. No change is observed for the PAS-3 glycopeptide.

The amount of glycoprotein in the sample is one of the most crucial factors in achieving heat-induced conversion of the PAS-1 form of the sialoglycoprotein. Heating relatively large amounts of glycoprotein (50–100 μg/sample) results in little or no conversion of PAS-1 to PAS-2 (Figure 5): however, 75% conversion is achieved if 1 μg of glycophorin (equivalent to 0.02 mg/ml) is treated in the same way.

IV. Reassociation of the Glycophorin A Subunits. So far our attempts to isolate and purify the different glycopeptides to complete electrophoretic homogeneity have not been successful. Each fraction shows more than one band when analyzed by dodecyl sulfate electrophoresis (Furthmayr et al., 1975), and it has been difficult to decide whether the multiple bands represent different aggregation states of the same glycopeptide or contaminating species of different peptides. In order to exclude possible interactions between different glycopeptides during electrophoresis, a different experimental approach was chosen. After glycoprotein fractions were electrophoresed in the usual way individual bands containing PAS-1 (or 2) were recovered from the unfixed gels, incubated under specified conditions, and then reelectrophoresed. The data obtained by this approach are illustrated in Figure 6. Several features are apparent: (a) all the sialoglycopeptides migrating in the PAS-1 position can undergo complete dissociation to the PAS-2 forms (Figure 6a–d); (b) this process is temperature and time dependent (Figure 7a and b); (c) the smaller molecular weight peptides can reassociate to form the original high molecular weight forms (Figure 6e–h); (d) the reassociation is also temperature and time dependent (Figure 7a and b) and probably is dependent also on the protein concentration (cf. Figure 6f and h), in that a higher concentration of PAS-2 favors reassociation to the PAS-1 form.

These data support the idea that the two PAS-staining bands (PAS-1 and PAS-2) represent different electrophoretic forms of the same sialoglycopeptide. According to the simplest interpretation, PAS-1 is a dimeric form of PAS-2. However, it is also conceivable that PAS-1 is an oligomeric form of more complex composition (i.e., tetramer rather than dimer) since it is difficult to determine the molecular

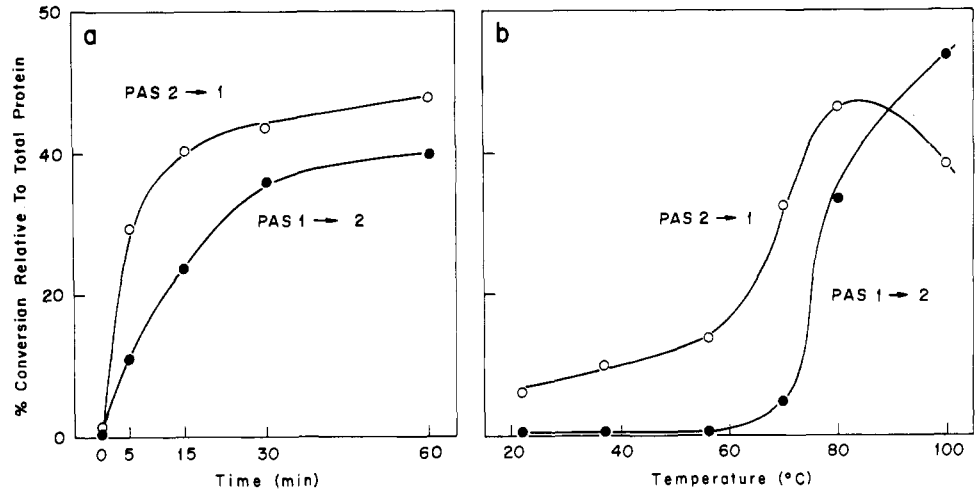

FIGURE 7: Dissociation (PAS-1 → 2), and reassociation (PAS-2 → 1) of glycophorin A subunits as a function of time and temperature. Standard amounts of membranes (25 μl) were electrophoresed on dodecyl sulfate polyacrylamide gels, and unfixed gel slices containing PAS-1 or PAS-2 glycopeptides were collected and incubated at 70 °C for various time periods (a) or for 5 min at various temperatures (b). The gel slices were then loaded onto a second set of dodecyl sulfate polyacrylamide gels and electrophoresed, stained with PAS-reagent, and scanned at 560 nm, and the peak areas were determined by triangulation. The results are expressed as relative amounts in percent of total PAS-staining protein. (●) PAS-1 → 2; (○) PAS-2 → 1.

mass of sialoglycopeptides by dodecyl sulfate gels (Segrest et al., 1971).

V. Site of Subunit Interaction: Effects of Competing Peptides. The site of association between monomeric units of the glycophorin complex was investigated by determining whether purified peptides derived from different segments of the glycophorin molecule could bind to homologous sites on intact molecules and thereby inhibit reassociation into the multimeric forms.

Heat treatment of a mixture of glycophorin A and the small hydrophobic peptide T-6, derived from glycophorin A by trypsin treatment (Tomita and Marchesi, 1975; H. Furthmayr, M. Tomita, and V. T. Marchesi, in preparation), caused a far greater amount of sialoglycopeptide to migrate in the PAS-2 region than would be achieved by a single heat treatment of glycophorin A alone (cf. Figure 8A and C). None of the other tryptic nor CNBr-derived peptides of glycophorin A were capable of producing this shift. Similarly, tryptic peptides derived from spectrin and other peptides from the erythrocyte membrane, and "hydrophobic" peptides prepared from hemoglobin and serum proteins were inactive. The simplest interpretation of these results is that the hydrophobic peptide (T-6) binds to the segment of the glycophorin molecule which normally associates with a companion molecule to form the stable complex. However, it is also conceivable that the peptide might exert its effect without binding. In order to show that the T-6 peptide was associated with the "monomeric" form of glycophorin under the conditions of electrophoresis in dodecyl sulfate, ^{125}I-labeled T-6 was prepared and used for competition experiments. The results show clearly that some of the hydrophobic peptide (T-6) is actually bound to the presumptive subunit of glycophorin A (Figure 9D), but not to the high molecular weight form PAS-1. This binding correlated well with the increase in the total amount of the sialoglycopeptide PAS-2 observed after staining of parallel gels with PAS reagent (Figure 9C).

Discussion

Early studies on the isolation and characterization of the sialoglycopeptides of the human erythrocyte membrane were consistent with the idea that these molecules represented a family of closely related molecular species (Winzler, 1970). Later studies carried out on glycoprotein isolated by the lithium 3,5-diiodosalicylate–phenol procedure suggested that the bulk of the material represented a single molecular species which was provisionally designated glycophorin (Marchesi et al., 1972). More recently it has been shown that such glycophorin preparations can be further fractionated into at least two major sialoglycopeptides which represent similar but chemically distinct entities (Furthmayr et al., 1975).

The predominant component, now called glycophorin A, accounts for approximately 75% of the total and is composed of 131 amino acids and approximately 16 oligosaccharide chains. The complete amino acid sequence and the locations of the oligosaccharide residues have recently been determined (Tomita and Marchesi, 1975). Although glycophorin A is clearly homogeneous it still gives two electrophoretically distinct bands on dodecyl sulfate polyacrylamide gels (Furthmayr et al., 1975), but the data presented in this paper indicate that these two bands represent different but interconvertible molecular forms of the same glycopeptides.

The results reported here and from other laboratories (Marton and Garvin, 1973; Tuech and Morrison, 1974) indicate that glycophorin A forms high molecular weight complexes which are resistant to disruption by dodecyl sulfate. Such glycophorin complexes can be dissociated by heating dilute solutions of the glycoprotein in dodecyl sulfate, but if relatively high concentrations of the glycoprotein are heated under the same conditions most of the material remains in the high molecular weight form. This dependence of the dissociation on glycoprotein concentration makes it unlikely that the band shifts seen by dodecyl sulfate gels are due to differences in dodecyl sulfate binding or conformational changes, although neither possibility can be rigorously excluded. Artifacts due to different staining properties of the heat-treated protein have been excluded by analyzing labeled sialoglycoproteins, regardless of whether the label had been introduced into sialic acid (cf. Figures 2 and 3) or tyrosine.

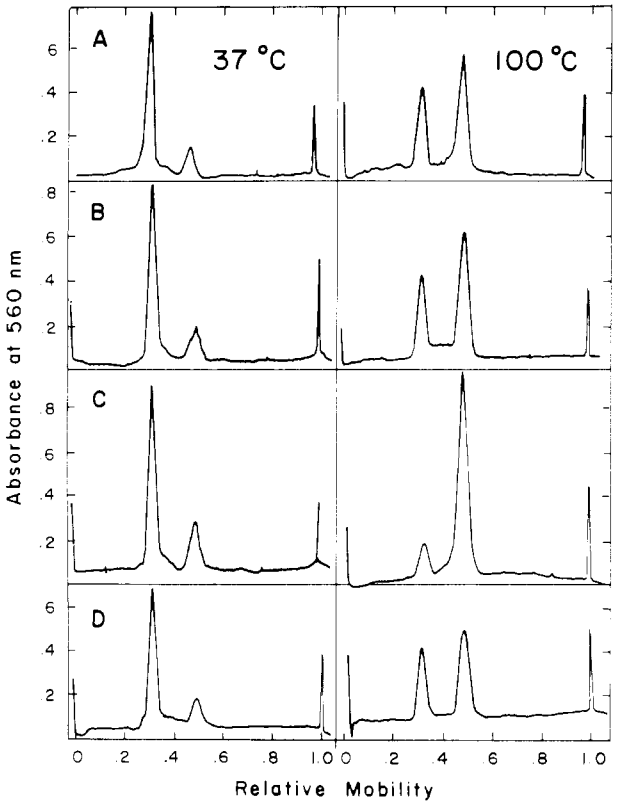

FIGURE 8: Inhibition of the reassociation of glycophorin A subunits by a hydrophobic peptide. Purified glycophorin A (12.5 μg) was dissolved in dodecyl sulfate sample buffer either alone (A) or in the presence of purified peptides (B–D) and then incubated at 37 °C for 30 min (left side) and an additional 3 min at 100 °C (right side). 25 μg of the glycopeptide (T-1) was added in B, 7 μg of the hydrophobic peptide (T-6) in C, and 25 μg of the C-terminal peptide (T-4) in D. These preparations were then analyzed by dodecyl sulfate electrophoresis, PAS-staining, and scanning at 560 nm (cf. Figure 10 and Tomita and Marchesi (1975) for the designation of the peptides). The two stained peaks represent the usual gel pattern observed for glycophorin A and correspond to PAS-1 and PAS-2 bands.

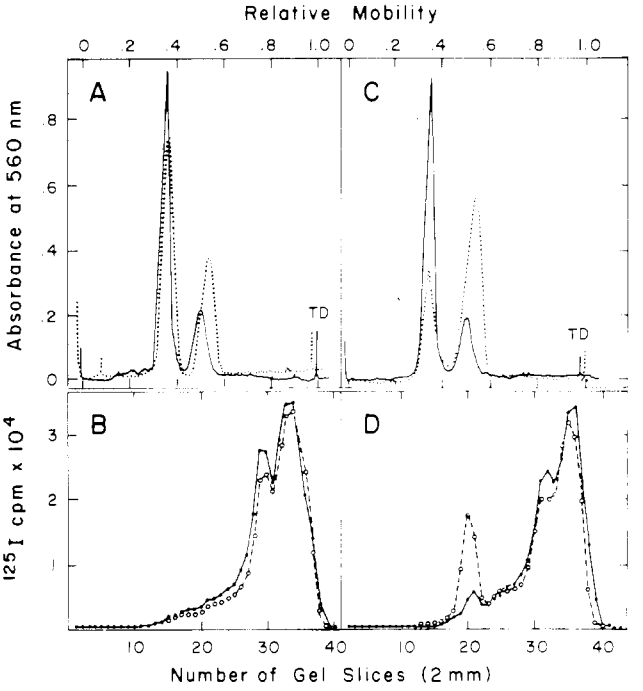

FIGURE 9: Interaction of the hydrophobic peptide T-6 with the subunit of glycophorin A. Purified glycophorin A was incubated either alone (A) or with ^{125}I-labeled T-6 peptide (C,D) in dodecyl sulfate buffer for 30 min at 37 °C (—) or an additional 3 min at 100 °C (··· or - - -). Iodinated T-6 peptide was also incubated alone in dodecyl sulfate buffer under the same conditions (B). All preparations were then electrophoresed in the usual way, and parallel gels were stained with the PAS reagent and scanned at 560 nm (A,C) or sliced and counted in a gamma counter (B,D).

Our gel filtration experiments are also in agreement with the interpretation that two subunits of glycophorin A are in an association–dissociation equilibrium (Figure 3). The lower molecular weight form, which corresponded to the PAS-2 band on polyacrylamide gels, was stable after initial heating of the protein solution only when the column was operated above room temperature (45 °C vs. 23 °C) and when small amounts of isolated sialoglycoproteins were separated. We were unable to obtain preparative amounts of the small molecular weight form probably due to reaggregation during rechromatography.

Glycophorin A thus seems to be a protein which takes up dodecyl sulfate slowly and forms stable aggregates similar to the behavior of catalase and other enzymes (Nelson, 1970), or *Ricin* (M. Tomita, unpublished; Nicolson et al., 1974). Recently it has been observed that ferritin subunits can also be further dissociated into polypeptide chains of 7000–8000 and 10000–11000 daltons after heating in dodecyl sulfate solutions (Niitsu et al., 1973).

The problem of determining the molecular weights of the glycophorin A monomer and its various aggregation states is still unsettled. Since the original description by Kathan et al. (1961) a variety of different molecular weight estimates have been reported, ranging from 22000 to 500000 (Springer et al., 1966; Zvilichovsky et al., 1971; Fukuda and Osawa, 1973; Janado et al., 1973). It was not clear in most of these studies whether the glycoprotein was present in an aggregated or monomeric form. Some of the earlier data obtained by sedimentation equilibrium of various glycoprotein preparations in the presence of detergents have been criticized recently by Grefrath and Reynolds (1974), who reported a monomeric molecular weight of 29000 after a more careful determination of dodecyl sulfate binding. It is not clear how their result correlates with our finding that glycophorin A may be a stable dimer in dodecyl sulfate, since we do not know whether the procedure they used for isolation of the sialoglycoprotein resulted in monomer or dimer forms. The value of 29000 they calculated is essentially identical to the value of 31000 calculated from sequence data (Tomita and Marchesi, 1975).

Molecular weight estimates for glycophorin A by dodecyl sulfate polyacrylamide gel electrophoresis have given results ranging from 90000 to 53000 (Lenard, 1970; Segrest et al., 1971; Bretscher, 1971; Banker and Cotman, 1972). The migration rate was found to depend on the acrylamide concentration (Bretscher, 1971; Segrest et al., 1971), but the free electrophoretic mobility was lower when compared to other proteins and this was attributed to decreased binding of dodecyl sulfate (Banker and Cotman, 1972). Although dodecyl sulfate gels have proven to be a valuable and simple way to analyze the molecular sizes of many common proteins, the anomalous behavior of this sialoglycoprotein has been demonstrated repeatedly, and it seems clear that this technique must be used cautiously until well-characterized glycoprotein standards can be analyzed.

The data in this paper also indicate that glycophorin A subunits may interact via hydrophobic segments of their polypeptide chains. This conclusion is based on the capacity

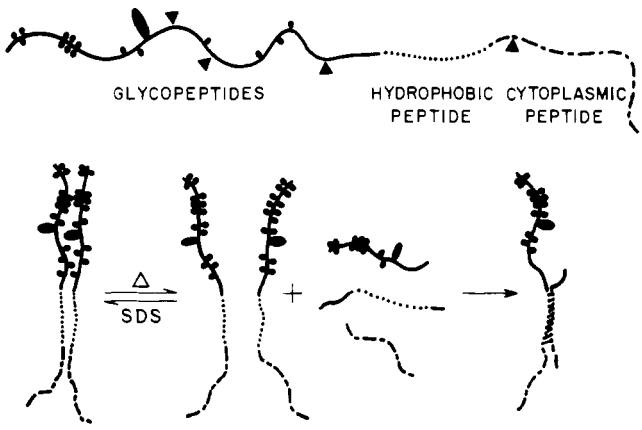

FIGURE 10: Model of the glycophorin A subunit and its interaction with the hydrophobic peptide T-6. Heat-induced separation of the subunits in the presence of dodecyl sulfate allows the hydrophobic peptide T-6 to interact with the hydrophobic segment of the glycophorin A subunit. (▲) Tryptic cleavage sites (cf. Tomita and Marchesi, 1975); (● ●) location of oligosaccharide units.

of purified T-6 peptides, derived from the hydrophobic region of glycophorin A, to interact with and bind to homologous regions of the monomer forms (Figure 10). Since these experiments were carried out in the presence of dodecyl sulfate, we cannot exclude the possibility that native glycophorin A monomers might also interact at other segments of the polypeptide chains. The amino acid sequence of the hydrophobic region has been reinvestigated and corrected recently (H. Furthmayr, M. Tomita, and V. T. Marchesi, in preparation) and synthetic peptides are being made to determine the length of amino acid sequence required to form stable complexes.

We also have some preliminary data that methionine at position 81 of the polypeptide chain (Tomita and Marchesi, 1975) may play some role in the interaction between glycophorin A monomers. Peptides derived from the hydrophobic region by cyanogen bromide cleavage which converts methionine to homoserine do not influence the interactions between glycoprotein subunits (cf. Figure 8), and the binding of the T-6 peptide to glycophorin A monomer is inhibited by alkylation of methionine-81 (Silverberg et al., 1976). Such data support the notion that there are specific structural requirements for the formation of stable glycophorin complexes under the experimental conditions we have used. We do not know the conformation these peptides assume in detergent solutions or within the environment of the lipid bilayer, or indeed what role conformation plays in such interactions. Predictions of conformation based on sequence data may not be applicable to this membrane glycoprotein since the rules governing these predictions have been empirically determined from information derived from soluble proteins (Chou and Fasman, 1974).

Cross-linking studies carried out by incubating intact red cells with a variety of different bifunctional reagents have not provided a clear answer as to whether glycophorin A exists as a specific complex in the cell membrane. Aggregated forms of glycophorin A have been generated by some agents (Ji, 1974) but not by others (Steck, 1972; Wang and Richards, 1974). These studies should be repeated since they were carried out before the interconvertible nature of the sialoglycopeptides was realized; perhaps cross-linked dimers passed unnoticed.

Circumstantial evidence also favors the existence of glycoprotein complexes in membranes. Proteolytic release of cell surface glycopeptides commonly results in a loss of activity of receptors known to be formed by carbohydrate moieties. This result is somewhat inexplicable, but could be explained by assuming that some ligands require multivalent associations with receptors for appropriate binding or stimulation to occur. A "receptor" according to this model would be a complex of glycopeptides composed of at least two or more subunits with each subunit contributing to binding. Ligand binding might require the presence of preexisting glycoprotein complexes on the cell surface, or ligands might be able to generate such complexes by recruiting monomer units. Either mechanism could be controlled by the factors which regulate glycoprotein interactions within the membrane. Further work along these lines using model glycoprotein systems should provide some insight into the molecular biology of cell surface receptors and a better understanding of the recognition functions of these molecules.

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Methionine Sulfoxide Cytochrome *c*†

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ABSTRACT: Cytochrome *c* has been chemically modified by methylene blue mediated photooxidation. It is established that the methionine residues of the protein have been specifically converted to methionine sulfoxide residues. No oxidation of any other amino acid residues or the cysteine thioether bridges of the molecule occurs during the photooxidation reaction. The absorbance spectrum of methionine sulfoxide ferricytochrome *c* at neutrality is similar to that of the unmodified protein except for an increase in the extinction coefficient of the Soret absorbance band and for the complete loss of the ligand sensitive 695 nm absorbance band in the spectrum of the derivative. The protein remains in the low spin configuration which implies the retention of two strong field ligands. Spin state sensitive spectral titrations and model studies of heme peptides indicate that the sixth ligand is definitely not provided by a lysine residue but may be methionine-80 sulfoxide coordinated via its sulfur atom. Circular dichroism spectra indicate that the heme crevice of methionine sulfoxide ferri- and ferrocytochrome *c* is weakened relative to native cytochrome *c*. The redox potential of methionine sulfoxide cytochrome *c* is 184 mV which is markedly diminished from the 260 mV redox potential of native cytochrome *c*. The modified protein is equivalent to native cytochrome *c* as a substrate for cytochrome oxidase and is not autoxidizable at neutral pH but is virtually inactive with succinate-cytochrome *c* reductase. These results indicate that the major role of the methionine-80 in cytochrome *c* is to preserve a closed hydrophobic heme crevice which is essential for the maintainance of the necessary redox potential.

Cytochrome *c* is a heme protein which transfers single electrons from cytochrome c_1 to cytochrome oxidase in the mitochondrial respiratory chain. The protein is composed of a polypeptide of approximately 100 amino acid residues and an iron protoporphyrin IX (heme) prosthetic group (Margoliash and Schejter, 1966; Dayhoff and Eck, 1973) which is capable of undergoing alternate oxidation and reduction of its central iron ion. The heme moiety of cytochrome *c* is covalently bonded to the protein via two cysteine thioether bridges and via coordination of histidine residue 18 and methionine residue 80 of the protein as the fifth and sixth (axial) ligands to the heme iron (for a review see Lemberg and Barrett, 1973).

The mechanism of action of cytochrome *c* in the mitochondrial electron transfer chain remains to be elucidated. Electron transfer to the heme iron of cytochrome *c* has been proposed to occur via peripheral attack on the solvent exposed edge of the heme (Dickerson et al., 1971; Sutin and Yandell, 1972) or via transfer through a portion of the pro-

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