Biochimica et Biophysica Acta, 419 (1976) 51–62

BBA 77147

DISTRIBUTION OF FREE SULFHYDRYL AND DISULFIDE GROUPS AMONG PLATELET MEMBRANE PROTEINS

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(Received June 2nd, 1975)

SUMMARY

Reactive sulfhydryl and disulfide groups were identified in platelet membrane proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Platelet membranes treated with *N*-ethyl[1-^{14}C]maleimide, phenyl[^{203}Hg]mercuric acetate and *p*-chloro[^{203}Hg]mercuribenzoate showed similar patterns of distribution of sulfhydryl groups among the sodium dodecyl sulfate-solubilized membrane proteins. Four major and two minor polypeptides ranging in molecular weight from > 200 000 to 20 000 were found to have reactive SH groups. Reduction of membrane proteins by sulfite coupled with subsequent mercaptide formation of the resultant monothiols led to the identification of four polypeptides with disulfide bonds. Reaction of platelet membranes with ^{14}C-labeled 5,5′-dithio-bis(2-nitrobenzoic acid) resulted in changes in the distribution profile of the solubilized membrane proteins suggestive of a polymerization process dependent upon 5,5′-dithio-bis(2-nitrobenzoic acid)-induced intermolecular disulfide interchange.

INTRODUCTION

The nature of the proteins of cellular membranes and their role in membrane structure and function have been the subjects of innumerable investigations in recent years. Sulfhydryl (SH) and disulfide groups which proved to be of paramount importance in elucidating structure and function of soluble proteins have not yet been as thoroughly investigated in membrane proteins. Thus, although membrane SH groups have been classified according to their reactivity with certain SH reagents [1] there have been only a few attempts to relate reactive SH groups to specific membrane proteins [2, 3]. Aggregation, adhesion and platelet factor 3 activity are functions essential for normal hemostasis which depend solely or to a large extent on the plasma membrane of platelets [4–7]. Free SH groups are necessary for normal aggregation and adhesion of platelets [8–11]. By determining the distribution of free reactive SH

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groups and disulfide bonds among membrane proteins or polypeptides we eventually hope to correlate specific membrane-mediated functions with specific membrane proteins. In previous studies we have quantified free SH and disulfide groups in platelet membranes [12, 13]. This investigation deals primarily with their identification in solubilized membrane proteins.

MATERIALS

Phenyl[^{203}Hg]mercuric acetate of specific activity 18.1 mCi/g, *p*-chloro-[^{203}Hg]mercuribenzoate ($ClHgBzO^-$) of specific activity 17.7 mCi/g and 1-^{14}C-labeled 5,5′-dithio-bis(2-nitrobenzoic acid) (Nbs_2) of specific activity 20 Ci/mol were purchased from Amersham/Searle Corp., Arlington Heights, Ill. *N*-Ethyl[1-^{14}C]-maleimide of specific activity 0.5 Ci/mol and $Na^{125}I$ (carrier free) were obtained from New England Nuclear, Boston, Mass. Lactoperoxidase (EC 1.11.1.7) was obtained from Sigma, St. Louis, Mo. *N*-Carbobenzoxy-α-L-glutamyl-L-tyrosine was purchased from Cyclo Chemical, Los Angeles, Ca. and toluenesulfonyl fluoride from Eastman Chemical, Rochester, N.Y. Glucose oxidase (EC 1.1.3.4) was a product of Boehringer, New York, N.Y.

METHODS

Isolation of platelet membranes. Fresh blood was collected from male volunteers with acid/citrate/dextrose as anticoagulant. Platelets were separated, lysed and their membranes isolated as described previously [12]. Contamination with red and white cells was always less than 1 per 12 000 and 1 per 31 000 platelets, respectively. Platelets were lysed in the presence of 0.5 mM *N*-carbobenzoxy-α-L-glutamyl-L-tyrosine, a substrate inhibitor of platelet cathepsin and 0.4 mM toluene-sulfonyl fluoride, a broad spectrum protease inhibitor. For most experiments membranes were suspended in 0.154 M NaCl buffered with 10 mM Tris · HCl (Tris/NaCl), pH 7.2. The protein concentration of these membrane suspensions varied between 0.5 and 1 mg/ml. Protein was determined by the method of Lowry et al. [14] using crystalline bovine serum albumin as standard.

Preparation of mercurial membrane protein adducts. Phenyl[^{203}Hg]mercuric acetate was dissolved in 0.02 M acetic acid and $Cl^{203}HgBzO^-$ in 0.04 M NaOH. After adjusting the pH to 7.4 the solutions were centrifuged in order to remove any precipitates formed in the process, though no precipitate was observed in the concentration employed ($< 10^{-4}$ M). $ClHgBzO^-$ was then determined by measuring the absorbance at 232 nm using a molar absorption coefficient of $1.69 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ (pH 7.0). Membrane vesicles suspended in Tris/NaCl were then incubated with either mercurial reagent for 60 min at 37 °C. After incubation the membranes were washed twice with 0.154 M NaCl and once with 0.25 M sucrose buffered with 10 mM Tris · HCl, pH 7.2, and finally resuspended in 10 mM phosphate buffer, pH 7.1, containing 1 % sodium dodecyl sulfate. Solubilization of the membrane vesicles was attained within 1–2 h at room temperature. In general, however, the membrane suspensions were allowed to stand overnight at room temperature and were heated for 1 min at 60 °C prior to application onto the gels. That solubilization was complete was indicated by the absence of a precipitate after centrifugation at 105 000 × *g* for 30 min at 22 °C

and by the absence of Coomassie brilliant blue-stainable material at the cathodal end of polyacrylamide gels after electrophoresis as detailed below.

Alkylation of platelet membranes with N-ethylmaleimide. Platelet membrane vesicles suspended in 10 mM phosphate buffer, pH 7.0, containing 0.154 M NaCl were incubated for 60 min at 37 °C with ^{14}C-labeled *N*-ethylmaleimide which was added in approx. 10-fold excess over the expected number of SH groups. *N*-Ethyl-[^{14}C]maleimide dissolved in pentane was concentrated under a stream of N_2 before addition of the membrane suspension. The resultant mixture was vigorously stirred for several minutes on a Vibromixer under a stream of N_2. Excess reagent was removed by repeated washing of the membrane vesicles as described above. The alkylated membranes were then solubilized in 10 mM phosphate buffer, pH 7.1, containing 1 % sodium dodecyl sulfate.

Preservation of the correct orientation of platelet membranes and maintenance of their surface topography during the isolation procedure was determined by the lactoperoxidase-catalyzed iodination of exposed tyrosine residues [15, 16] (Fig. 1). Irrespective whether intact platelets or isolated platelet membranes were iodinated, the membrane proteins which became labeled were identical. The major membrane polypeptides are identified by P with letters A through E as subscripts while the pertinent minor polypeptides are indicated by an additional number as subscript. This code is used throughout this report.

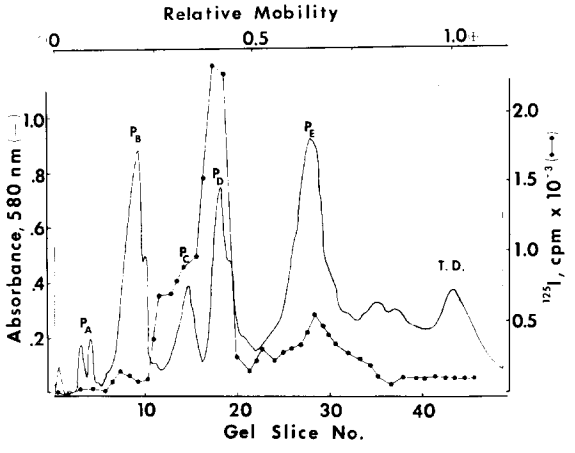

Fig. 1. Lactoperoxidase catalyzed ^{125}I iodination of platelet membrane proteins. Platelet membranes isolated by the glycerol lysis technique were suspended in 50 mM phosphate buffer, pH 7.2, containing 0.154 M NaCl and 5.5 mM β-D-glucose at a concentration of 1.5–2 mg protein per ml. Lactoperoxidase (10^{-6} M) and 80 μCi carrier-free Na^{125} I were added per ml membrane suspension. H_2O_2 was generated by glucose oxidase (2.5 μg/ml). Enzymic iodination was continued for 30 min at 30 °C. After washing the iodinated membranes twice with 10 mM Tris · HCl, pH 7.2, containing 0.25 M sucrose they were solubilized in 10 mM phosphate buffer, pH 7.1, containing 1 % sodium dodecyl sulfate and 1 % 2-mercaptoethanol and then subjected to sodium dodecyl sulfate-polyacryl amide gel electrophoresis as described in Methods. In other experiments intact platelets (15 10^9 platelets/ml) were iodinated by the same method and platelet membranes isolated after washing the iodinated platelets twice with Tris/NaCl were solubilized and subjected to electrophoresis. To each gel were applied 100 μg of protein. T.D., tracking dye.

Sulfitolysis of platelet membranes. Membrane vesicles suspended in 0.154 M-NaCl buffered with 10 mM Tris · HCl, pH 7.4, containing 0.2 M Na_2SO_3, 0.5 mM-*N*-carbobenzoxy-α-L-glutamyl-L-tyrosine, 0.4 mM toluenesulfonyl fluoride and a 10-fold excess of $Cl^{203}HgBzO^-$ over the expected number of thiols available after sulfitolysis were incubated for 60 min at 37 °C. The membranes were then washed three times as described above and solubilized for subsequent electrophoresis.

Reaction of platelet membranes with $[^{14}C]Nbs_2$. Platelet membranes suspended in Tris/NaCl at a concentration of 0.5–1 mg protein per ml were incubated with $[^{14}C]Nbs_2$ which was added in approx. 10-fold excess over the expected number of Nbs_2 reactive free SH groups. Following incubation for 60 min at 37 °C the membranes were washed and resuspended as described above for the preparation of mercurial membrane protein adducts.

In another set of experiments platelet membrane vesicles were first solubilized in 1 % sodium dodecyl sulfate buffered with 10 mM Tris · HCl ,pH 7.2. After complete solubilization the membrane proteins were incubated with $[^{14}C]Nbs_2$ for 60 min at 37 °C. Excess disulfide reagent was eliminated by dialysis against 1 mM phosphate buffer, pH 7.1, containing 0.1 % sodium dodecyl sulfate at room temperature with four changes of the dialysis buffer. The dialyzed solution was concentrated under a stream of N_2 adjusting the final concentration of protein to approx. 1 mg/ml. The solution was then ready for sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed in 5 % gels containing 0.1 % sodium dodecyl sulfate prepared essentially according to Maizel [17]. Electrophoresis was carried out in 85 mm tubes, initially with 8 mA per gel which was increased to 10 mA after 60 min. The electrode buffer was 0.1 M phosphate, pH 7.1, containing 1 % sodium dodecyl sulfate. To the sample dissolved in 10 mM phosphate buffer, pH 7.1, containing 1 % sodium dodecyl sulfate (approx. 1 mg protein per ml) were added bromophenol blue as tracking dye and sucrose to produce a final concentration of the latter of 10 %. 50–100 μg protein were applied to each gel. After electrophoresis the gels were fixed with 20 % sulfosalicylic acid for 16–24 h, stained with 0.25 % Coomassie brilliant blue for 2–3 h and finally destained with 7 % acetic acid.

Molecular weights were estimated by comparing the relative mobilities of membrane proteins with those of protein standards of known molecular weight. Bovine serum albumin, pepsin, trypsin, lactic dehydrogenase, β-chain of hemoglobin and γ-globulin were used as standards.

When labeled membrane proteins were electrophoresed the gels were fixed and stained as described above. The destained gels were scanned at 580 nm in a UA-5 Optical Monitor (Isco Corp., Lincoln, Nebr.) and then divided into slices of 1.5 mm thickness which were dried at 50–60 °C. After complete drying (usually 12–14 h) 0.1–0.2 ml of 30 % H_2O_2 were added, the vials were tightly capped and heated at 55 °C. In general complete dissolution of the gel slices was obtained after 3–6 h. To each sample 1.5 ml solubilizer, Biosolv BBS-3 (Beckman Instr., Fullerton, Ca.), were added and 10 ml of scintillation fluid consisting of toluene with 0.4 % 2,5-diphenyl-oxazole and 0.05 % 1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene. The radioactivity was determined in a liquid scintillation spectrometer with automatic external standardization. Quenching was monitored but counts were not corrected.

RESULTS

Distribution of reactive monothiols among polypeptides of platelet membranes

Platelet membrane vesicles were incubated with different radioactive sulfhydryl reagents. The conditions of incubation used in these experiments including time, pH, temperature and concentration of SH reagent have been previously shown to be optimal for this system [12]. The labeling pattern of the solubilized membrane polypeptides was then assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The mercurial adducts resulting from the reaction of phenyl[^{203}Hg]mercuric acetate or Cl^{203}HgBzO$^-$ with platelet membranes were identically distributed among the solubilized membrane polypeptides (Fig. 2). Four major membrane proteins and two other polypeptides present in low concentration were able to form mercurial adducts. The absolute radioactivity was always greatest in the polypeptide of molecular weight 32 000 (P_E). A similar distribution of monothiols among membrane proteins was found when platelet membrane vesicles were alkylated with *N*-ethylmaleimide (Fig. 2). Irrespective of the thiol reagent used the polypeptide of molecular weight 120 000 (P_C) failed to show reactive sulfhydryl groups.

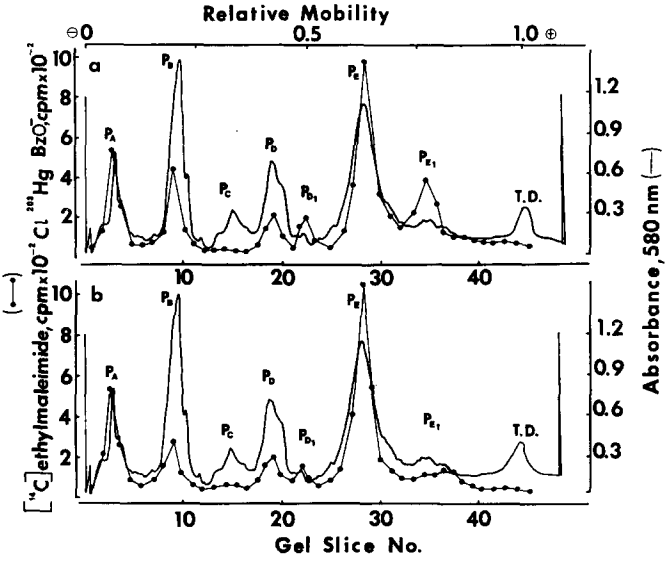

Fig. 2. Labeling patterns of platelet membrane proteins with Cl^{203}HgBzO$^-$ (a) and *N*-ethyl[^{14}C]-maleimide (b). Isolated membrane vesicles were labeled with either radioactive reagent, solubilized and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Methods. To each gel were applied 75 μg platelet membrane protein.

Incubation of platelet membrane vesicles with organic mercurials did not alter appreciably the electrophoretic distribution profile of the sodium dodecyl sulfate-solubilized membrane proteins as measured by densitometric scanning of the Coomassie brilliant blue-stained gels.

Distribution of disulfide bonds in platelet membrane proteins

In previous experiments we were able to show that sulfitolysis followed by reaction with organic mercurials was an effective method for the determination of membrane disulfide bonds [13]. This technique was utilized in the present study to determine the location of the existing membrane disulfides (Fig. 3). Free monothiols

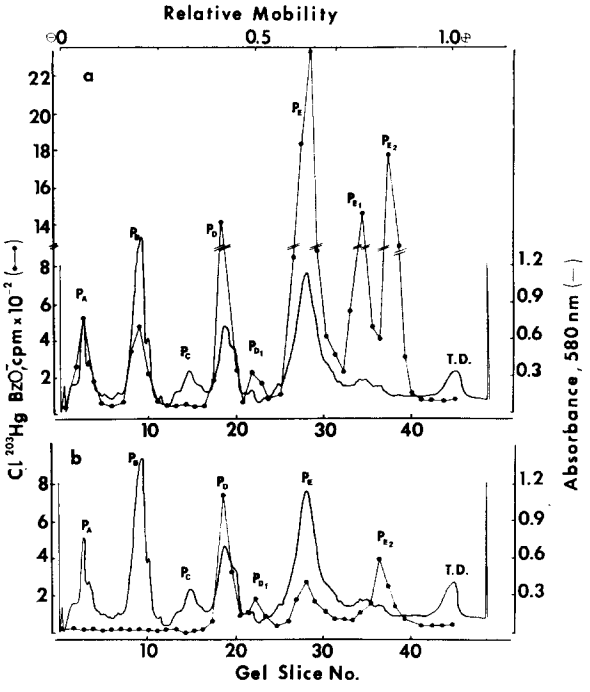

Fig. 3. Distribution of mercurial adducts among platelet membrane proteins labeled with $Cl^{203}HgBzO^-$. (a) Platelet membranes were reduced with 0.2 M Na_2SO_3. The resultant thiols were reacted with $Cl^{203}HgBzO^-$. Labeled platelet membranes were processed and membrane proteins fractionated as described in Methods. (b) Platelet membranes pretreated with *N*-ethylmaleimide were sulfitolyzed and then labeled with $Cl^{203}HgBzO^-$. To each gel were applied 75 μg platelet membrane protein.

were initially alkylated with *N*-ethylmaleimide. The effectiveness of this procedure could be demonstrated by incubating *N*-ethylmaleimide-treated platelet membranes with $Cl^{203}HgBzO^-$. There were no significant radioactive peaks in the sodium dodecyl sulfate-solubilized membrane polypeptides. The omission of *N*-ethylmaleimide and addition of sulfite led to the appearance of a number of distinct zones of radioactivity signifying the formation of mercurial adducts of membrane polypeptides varying in molecular weight between 14 000 and > 200 000. A comparison of this pattern (Fig. 3a) with that of membranes reacted with the organic mercurial without inclusion of sulfite (Fig. 2a) showed a new peak of radioactivity at the anodal end of the gel but did not differ noticeably in the remainder of its profile. Alkylation of free SH groups with *N*-ethylmaleimide followed by sulfitolysis and $Cl^{203}HgBzO^-$ adduct formation allowed us to determine the location of disulfides (Fig. 3b). Three zones of radioactivity could be seen in the solubilized membrane polypeptides, the highest number

of counts occurring in the polypeptide of molecular weight 70 000 (P_D) followed in decreasing order of activity by the area near the anodal end of the gel (P_{E_2}) and by the polypeptide of molecular weight 32 000 (P_E).

Sulfitolysis of platelet membranes coupled with subsequent mercaptide formation of the resultant monothiols did not produce significant changes in the band pattern of the solubilized membrane polypeptides.

Modification of platelet membrane proteins by Nbs_2

Reaction of platelet membranes with Nbs_2 resulted in the disappearance of one of the major polypeptides of platelet membranes, a protein of molecular weight 150 000 (P_B). At the same time one or two new high molecular weight proteins made their appearance at or near the cathodal end of the gel (Fig. 4). When sodium dodecyl

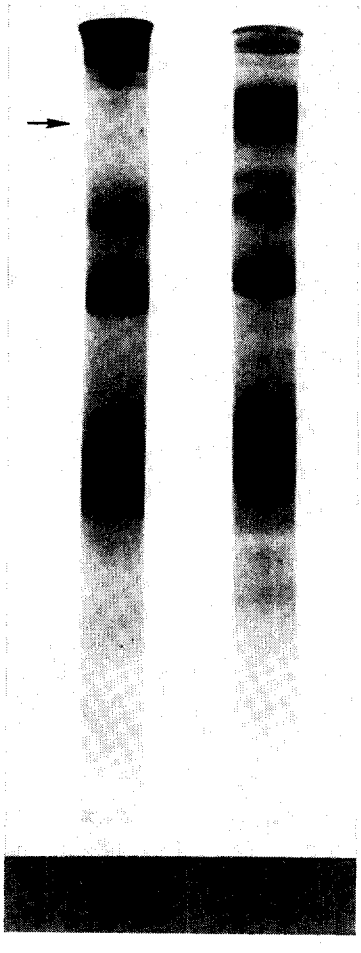

Fig. 4. Platelet membrane protein pattern of control (−) and Nbs_2-treated (+) platelet membrane vesicles solubilized by sodium dodecyl sulfate and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For details see Methods. The arrow indicates the area from which a major polypeptide band P_B has disappeared after membranes were treated with Nbs_2.

sulfate-solubilized platelet membranes were incubated with Nbs_2 this change in the electrophoretic pattern was not observed. Formation of mixed disulfides between [^{14}C]Nbs_2 and the thiols of sodium dodecyl sulfate-solubilized membrane proteins were recognized in all but one of the major polypeptides (Fig. 5). The changes recorded in the distribution profile of polypeptides derived from Nbs_2-treated intact membranes were also reflected in the pattern of mixed disulfides. Complete absence of reactive thiols in the zone occupied in control platelets by polypeptide P_B and a marked increase in ^{14}C-labeled mixed disulfides near the cathodal end of the gels were consistent findings in Nbs_2-reacted platelet membranes. These changes could be completely reversed by incubation of the platelet membranes with dithiothreitol in a concentration equal or greater than that of Nbs_2 present. Alkylation of membrane proteins with *N*-ethylmaleimide prior to reaction with Nbs_2 resulted in a band pattern of the solubilized membrane polypeptides essentially identical to that of untreated controls.

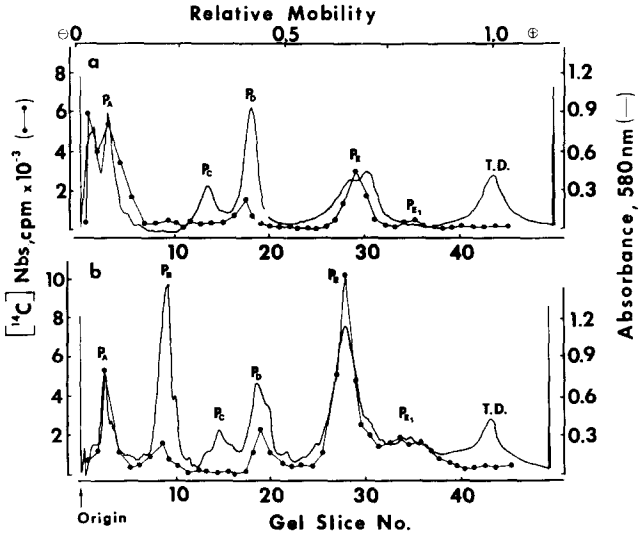

Fig. 5. Labeling patterns of platelet membrane proteins treated with [^{14}C]Nbs_2. (a) Platelet membranes were reacted with [^{14}C]Nbs_2, washed, solubilized and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Methods. (b) Platelet membranes were solubilized with 1 % sodium dodecyl sulfate, reacted with [^{14}C]Nbs_2, dialyzed and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To each gel were applied 75 μg membrane protein.

Stoichiometry of mercurial adduct formation

The results of the correlation of the densitometrically estimated amounts of individual membrane polypeptides with their reactive sulfhydryl groups before and after sulfitolysis are shown in Table I. The polypeptides of molecular weight 52 000 and > 200 000 formed the greatest number of mercurial adducts. While all but one of the major membrane proteins have reactive thiols only two were found to possess disulfide bonds. A small molecular weight polypeptide P_{E_2} (approximate molecular weight 14 000) appeared to have only disulfide bonds and no free reactive sulfhydryl groups.

TABLE I

STOICHIOMETRY OF MERCAPTIDE FORMATION OF PLATELET MEMBRANE PROTEINS

Platelet membranes with or without sulfite pretreatment were incubated with $Cl^{203}HgBzO^-$ under standard conditions described in Methods. After solubilization by sodium dodecyl sulfate the membrane proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The relative amounts of protein in the individual bands were estimated by densitometry of the Coomassie brilliant blue-stained gels. The radioactivity was assayed as described in Methods. Each value represents the mean of five determinations.

Protein	Approximate molecular weight	Without sulfite		With sulfite	
		Mercurial adducts formed (pmol)	Molar ratio $ClHgBzO^-$/protein	Mercurial adducts formed (pmol)	Molar ratio $ClHgBzO^-$/protein
P_A	> 200 000*	435	11.0	410	10.5
P_B	150 000	409	3.3	404	3.2
P_D	70 000	338	2.6	667	4.6
P_{D_1}	52 000	173	6.1	251	8.2
P_E	32 000	1170	1.8	1635	2.3
P_{E_1}	21 000	684	4.1	1071	6.5
P_{E_2}	14 000	–	–	1203	6.4

* The calibration curve of molecular weight markers was extrapolated to yield a rough estimate of 200 000 or slightly more for this polypeptide band.

DISCUSSION

Platelet membranes solubilized by the anionic detergent sodium dodecyl sulfate have been shown to contain a spectrum of polypeptide chains ranging in molecular weight from 14 000 to > 200 000. A similar distribution of platelet membrane proteins has been obtained by Nachman and Ferris [18] using mechanical homogenization to disrupt thrombocytes rather than the glycerol lysis technique of Barber and Jamieson [19] employed by us. The electrophoretic distribution of the major polypeptides and of most of the minor bands was constant and did not vary significantly on comparing platelets from different donors. The presence of protease inhibitors during lysis of platelets and separation of their membranes was important for obtaining reproducible profiles of solubilized membrane polypeptides. Evidence for the preservation of the membrane structure during the isolation procedure was provided by the results of the enzymic iodination of membrane proteins. All three major glycopeptides of platelet membranes became iodinated by this method.

The mapping of sulfhydryl groups in membrane proteins appeared to be influenced only to a very limited degree by the nature of the SH reagent used. Of the five major polypeptides four formed mercaptides and were alkylated by *N*-ethylmaleimide. While alkylation of red cell membranes seems to be relatively non-specific in that the extent of the reaction with *N*-ethylmaleimide paralleled size and concentration of the respective proteins [3], this was not observed in platelet membranes.

The stoichiometry of mercaptide formation (Table I) clearly indicated that the apparent heterogeneity in molecular weight of membrane proteins is the result of a heterogeneity in type. Considering the molecular weights of the major membrane polypeptides only, polymerization of the relatively small polypeptide P_E might have been a possible explanation for some of the other prominent electrophoretically separated protein bands. This hypothesis, however, stands clearly refuted by the results of the stoichiometric evaluation of the mercurial adducts of platelet membranes.

In view of the reported selective solubilization of relatively polar polypeptides from red cell membranes by a variety of "protein perturbants" including organic mercurials [20], the possibility that incubation of platelet membranes with $ClHgBzO^-$ or phenylmercuric acetate might lead to similar selective losses of membrane components during the subsequent washing procedure was investigated. Although in our experiments quantitative and qualitative evaluation of the electrophoretically separated membrane polypeptides revealed no change after treatment with organic mercurials, this does not exclude the possible occurrence of this phenomenon in platelet membranes at higher concentrations of these reagents than used by us.

The reduction of disulfide groups by sulfite has been shown to depend on a number of factors including pH, concentration of the reagent, presence of $ClHgBzO^-$ or other agents allowing sulfitolysis to go to completion and the steric hindrance by neighboring chemical groups. The significance of these variables has been pointed out by a number of authors [21–23]. In order to exclude the possible activation of proteolytic enzymes by sulfite treatment of membranes, sulfitolysis was performed in the presence of protease inhibitors. In general the reduction of disulfide bonds in proteins whether soluble or structural will result either in smaller subunits when the disulfide groups link different polypeptide chains or will leave the protein unchanged in appar-

ent size when the disulfide bridge is intramolecular. In platelet membrane proteins we found definite evidence only for the latter. The appearance of a new zone of reactive thiols near the anodal end of the gel in sulfitolyzed, sodium dodecyl sulfate-solubilized platelet membrane proteins probably indicates the result of reductive cleavage of a platelet membrane protein. Proteolysis as the cause of this phenomenon appears to be improbable as broad spectrum protease inhibitors were present during sulfitolysis.

The disulfide reagent Nbs_2 has been widely used to determine free sulfhydryl groups in proteins and in low molecular weight thiol compounds [24, 25]. The formation of mixed disulfides between Nbs_2 and reactive thiols is associated with the release of 5-thio-2-nitrobenzoate. Evidence has recently been presented for the occurrence of intramolecular disulfide interchanges in a protein in which free SH groups were reacted with Nbs_2 [26]. Concomitantly with the release of 5-thio-2-nitrobenzoate from the thio-nitrobenzoate-protein disulfide bonds were formed.

The results obtained with platelet membrane proteins suggest that at neutral pH and low Nbs_2 concentration in the medium the thionitrobenzoate polypeptides were able to undergo a disulfide interchange resulting in the polymerization of sodium dodecyl sulfate-solubilized membrane polypeptides. Evidence for the establishment of new disulfide bridges under these conditions was the complete reversal of the electrophoretic distribution pattern of polypeptides by dithiothreitol. Its effectiveness in reducing existing mixed disulfides was also demonstrated by the release of all radioactive counts from the solubilized polypeptides.

All our results with Nbs_2 appear to confirm the hypothesis of a disulfide interchange which may be presented in accord with the following reactions:

(a) $(\text{P-SH})_{2n} + n\ \text{Nbs}_2 \rightarrow (\text{P-S-S-Nbs})_n + (\text{P-SH})_n + n\ \text{Nbs}$

(b) $(\text{P-S-S-Nbs})_n + (\text{P-SH})_n \rightarrow (\text{P-S-S-P})_n + n\ \text{Nbs}$

(Sum) $(\text{P-SH})_{2n} + n\ \text{Nbs}_2 \rightarrow (\text{P-S-S-P})_n + 2n\ \text{Nbs}$

Nbs in this reaction scheme denotes thionitrobenzoate, and P represents a membrane polypeptide which in non-solubilized membranes may exist as a polymer of undefined size. In Reaction a the native protein undergoes a primary disulfide interchange with Nbs_2 with the liberation of free thionitrobenzoate. In Reaction b the modified protein undergoes a secondary intermolecular disulfide interchange resulting in a molecular species in which individual subunits are linked by disulfide bridges. The possibility that the disulfide interchange occurred between individual membrane proteins and not between subunits of a single protein appears to us unlikely because it would necessitate extremely close proximity of the interacting proteins. While there exists ample evidence that proteins embedded in membranes can migrate in the plane of these membranes [25–28], it is highly improbable that all molecules of a specific protein species would be involved in an intermolecular linkup as the virtually complete disappearance of polypeptide P_B implies. The essential role of steric proximity for the establishment of interchain disulfides is also suggested by the failure of membranes solubilized by sodium dodecyl sulfate prior to reaction with Nbs_2 to display the electrophoretic changes observed when intact membranes were treated with the disulfide reagent. These results indicate that Nbs_2 is not only valuable in determining SH groups but also represents a useful reagent for probing membrane structure.

ACKNOWLEDGEMENT

This study was supported by contract No. 71-2252 from the National Heart and Lung Institute.

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