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ISOLATION OF HUMAN PLATELET AND RED BLOOD CELL PLASMA MEMBRANE PROTEINS BY PREPARATIVE DETERGENT ELECTROPHORESIS

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Summary

High resolution polyacrylamide gel electrophoretic techniques have been applied to the preparative isolation and analysis of plasma membrane proteins and glycoproteins from human platelets and red blood cells. The techniques presented allow relatively simple, direct, rapid and quantitative purification of a broad molecular weight range of membrane proteins, by means of continuous elution preparative gel electrophoresis of proteins solubilized with sodium dodecyl sulfate. Spectrophotometric and fluorophotometric (fluorescamine) profiling, and high resolution gel electrophoretic analysis (SDS-acrylamide gradient slab gels, and gel electrofocusing) of eluted protein species indicate that purified membrane proteins of a broad molecular weight range may be obtained in a one step procedure, and in quantities and concentrations sufficient for further analytical or experimental procedures.

Introduction

The proteins and glycoproteins of eucaryotic cellular plasma membranes are believed to have diverse roles in cellular function. These include cell-cell interaction, surface specificity, activities as message receptors and transducers, mediation of membrane transport phenomena and enzymatic specificities, and formation of dynamic structural elements of the fluid membrane [1—3]. This

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Abbreviations: SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylenediamine; bisacrylamide, *N,N'*-methylene bisacrylamide.

diversity of membrane protein function is reflected by the multitude of protein and glycoprotein species that may be observed when these macromolecules are solubilized from the lipid bilayer of the plasma membrane, and analyzed by high resolution electrophoretic techniques, using denaturing systems [4—9].

Isolation of plasma membrane proteins, in amounts sufficient for detailed study, is an essential step for elucidation of the mechanisms of membrane function. In a strategy for preparative isolation of membrane proteins, major considerations include limitations of the amount of membrane protein obtainable as starting material, the insolubility of many membrane proteins in water, and the availability of quantitative, high resolution preparative and analytical techniques suitable for separating and detecting the components of a heterogeneous membrane protein mixture. Toward these ends, we have applied preparative high resolution polyacrylamide gel electrophoresis in sodium dodecyl sulfate for the solubilization and isolation of platelet and red cell membrane proteins.

Methods

Materials

Acrylamide and *N,N'*-methylene biscarylamide were Eastman products, and were recrystallized from hot chloroform and *n*-propanol, respectively. TEMED, 2-mercaptoethanol, bromophenol blue and basic fuchsin were also from Eastman. Coomassie brilliant blue R-250, sodium deoxycholate, and Tris (Trizma base) were obtained from Sigma; sodium dodecyl sulfate was from Pierce or BDH; fluorescamine (Fluram) was from Hoffman-LaRoche; Triton X-100 was from Packard (Rohm and Haas); and ampholines were from LKB. Ultrapure urea was obtained from Heico. Human blood and blood products were obtained from the Mayo Clinic Blood Bank. All other reagents were commercial items of high quality.

Preparative procedures

Membrane isolation. Red blood cell membranes were prepared from citrate/phosphate/dextrose-anticoagulated, packed red blood cells by a modification of the method of Fairbanks et al. [4]. Packed erythrocytes (4 ml) were lysed by the addition of 40 ml of cold 5 mM sodium phosphate buffer, pH 8.0. The membranes were sedimented in 50-ml polycarbonate tubes at 4°C and 32 000 × *g* for 10 min. The pellet was resuspended and washed four times in the same buffer. When necessary, membranes were stored at —70°C before further studies were accomplished.

Platelet plasma membranes were isolated from citrate/phosphate/dextrose-anticoagulated human platelet concentrates which had been gently agitated in plastic blood bags at 22°C for 72 h following collection. The glycerol hypotonic lysis technique of Barber and Jamieson [10] was used for platelet lysis and membrane isolation, with the following modifications. Contaminating red and white blood cells were reduced to less than two cells/10 000 platelets by means of differential centrifugation. Following washing, glycerol loading, and hypotonic lysis, membranes were isolated as described from 30% (w/w) sucrose density step gradients centrifuged at 30 000 rev./min (110 000 × *g*) for 2 h at 4°C in a Beckman Spinco SW41 rotor. Membrane vesicles were washed and

sedimented twice in 5 mM sodium phosphate (pH 8) at 20 000 rev./min (48 000 × *g*) for 30 min each at 4°C using the same rotor. Prior to the second sedimentation, the membrane vesicles were resuspended uniformly in washing buffer to a known volume, from which aliquots were taken for estimation of the total membrane protein content. This estimate was about 10% larger than that of the protein content finally determined for the pelleted vesicles when they were subsequently solubilized. Transmission electron microscopy of washed membrane preparations revealed no evidence of contaminating subcellular organelles. Membrane pellets were stored at −70°C until detergent solubilization and extraction.

Protein estimations were performed by a modified Lowry-Folin technique [11], using crystalline bovine serum albumin (Sigma, 96—99%) as a protein standard, and 1% SDS (w/v) final concentration in the samples, standards, and blanks.

Membrane protein extraction studies. Washed, pelleted, and frosen aliquots of platelet membrane vesicles or erythrocyte ghosts (in amounts from 4 to 12 mg protein each) were thawed, and varying amounts of detergent solution (10% (w/v) SDS in 5 mM sodium phosphate, pH 8.0) were added on a weight basis of detergent to protein. Weight ratios of SDS to protein were 1 : 1, 2 : 1, 4 : 1, and 8 : 1. The membrane samples were then dispersed at protein concentrations of 6.5—12 mg/ml by adding appropriate volumes of 5 mM sodium phosphate, pH 8.0, and incubated at 60°C for 30 min. Aliquots for protein determination and for analytical SDS gels ('pre-extraction' aliquots) were taken from each incubation mixture. The remaining portions were centrifuged in heavy wall glass tubes at 40 000 × *g* in an angle rotor for 30 min at 20°C. From the resulting supernatant, 'post-extraction' aliquots for analysis were obtained.

Samples for SDS gel analysis were made 2% (v/v) in mercaptoethanol and heated again at 60°C for 30 min. Prior to addition of reducing reagent, 'pre-extraction' samples with SDS : protein ratios of 1 : 1 and 2 : 1 were made 4 : 1 in SDS in order to serve as internal controls for the 'post-extracted' material, in which the SDS content remained the same. Two-tenths vol. of 50% (v/v) glycerol/0.2% (w/v) bromophenol blue was mixed with each sample, and aliquots of each 'pre-' and 'post-extract' were loaded on analytical slab gels in amounts of 75 and 150 μg of protein (Coomassie blue staining) or 300 μg (periodic acid-Schiff staining).

Prior to preparative electrophoresis, membrane proteins from either erythrocyte ghosts or platelet membrane vesicles were solubilized and extracted by resuspending the membrane pellets in 5 mM sodium phosphate, pH 8.0, then adding 8 mg of SDS/estimated milligram of membrane protein, and heating at 60°C for 30 min. The sample was then centrifuged to remove insoluble material. The extracted samples were made 2% (v/v) in mercaptoethanol, heated at 60°C for 30 min, then dialyzed 12—16 h at room temperature versus diluted electrophoresis buffer, using two changes of dialysate. Small-pore dialysis bags (Spectrapore No. 1 or No. 3, Spectrum Industries) were used for all sample dialysis. For the discontinuous buffer system, the sample was dialyzed against four-fold diluted upper gel buffer containing 2% (w/v) SDS. For the continuous buffer electrophoresis system, the sample was dialyzed against 0.01 M Tris/borate (pH 8.5) containing 2% (w/v) SDS. After dialysis,

samples were made 10% (v/v) in glycerol prior to application to the preparative gel.

Protein samples were sometimes labelled with fluorescamine to enhance subsequent analytical sensitivity or to enable direct monitoring of preparative separation with a long wave ultraviolet lamp. Approximately 10% of the extracted reduced membrane protein sample was dialyzed against sodium borate buffer, pH 9, of the same molarity and SDS content as the Tris buffer used for the remainder of the sample. Labelling was carried out by dissolving 1 mg of fluorescamine in 100 μl acetone, and adding this mixture to the dialyzed sample while vortex-mixing the sample tube. The labelled aliquot was then combined with the remaining unlabelled sample for application to the preparative gel.

Preparative electrophoresis. The preparative electrophoresis apparatus utilized for membrane protein isolation was designed and constructed locally [12]. The apparatus is similar to those available commercially, in which electrophoresis is performed in a gel cast between two cooled, concentric cylinders with diameters of 20 and 42 mm, respectively. Continuous elution is provided at the bottom of the gel by a buffer stream.

For continuous systems, preparative electrophoresis was conducted in 0.1 M Tris, 0.1 M H_3BO_3, and 0.3% SDS, pH 8.5. The conductance of this solution was 1090 $\mu S \cdot cm^{-1}$. Separating gels were polymerized at room temperature from a solution containing 7.31% acrylamide and either 0.188% or 0.094% bisacrylamide. The gel solution was degassed with a water aspirator vacuum source prior to the addition of SDS (0.3%), TEMED (0.05%), and ammonium persulfate (0.05%).

For the discontinuous system, four separate buffers were required. The separating gel was prepared in 0.375 M Tris, 0.06 M HCl, 0.3% SDS (pH 8.8, 5700 $\mu S \cdot cm^{-1}$). Gels were polymerized using 7.31% acrylamide and either 0.188% or 0.094% bisacrylamide. The stacking gel for the discontinuous system was prepared in 0.059 M Tris, 0.032 M H_3PO_4, 0.3% SDS (pH 7.2, 2680 $\mu S \cdot cm^{-1}$). The stacking gel concentration was 3.2% acrylamide, 0.8% bisacrylamide. Ordinarily, the stacking gel was layered at a height of 1 cm on top of the separating gel. The upper gel buffer for the discontinuous system was 0.055 M Tris, 0.051 M glycine, 0.3% SDS (pH 8.9, 900 $\mu S \cdot cm^{-1}$) while the lower reservoir and elution buffer were 0.12 M Tris, 0.06 M HCl, 0.3% SDS (pH 8.1, 5350 $\mu S \cdot cm^{-1}$).

For both systems, separating gels of 4—6 cm were used. The continuous system was pre-electrophoresed at 2—4 W overnight prior to sample loading, while for the discontinuous system, preparative electrophoresis was conducted within 1 h after completion of the gel-forming stages. Preparative electrophoresis was performed at 20 ± 2°C, using flowing tap water as a coolant. The density-stabilized sample was applied to the upper surface of the gel. The maximum protein content of preparative samples was 60 mg, in a final volume of 5.5 ml or less. Low power (approx. 2 W) was used initially until the sample had entered the gel. The power was then increased to 4—8 W. The elution buffer flow rate was 0.5—1.0 ml/min, and 5-min fractions were collected. Electrophoresis time for most separations was approximately 12 h. Protein elution was monitored spectrophotometrically by measuring absorbance at 280 nm,

and when fluorescamine labelling was used, by photofluorimetry, utilizing an Aminco Photofluorometer with an excitation at 360 nm and an emission filter peak of 485 nm.

Analytical electrophoresis. Electrophoretic analysis of eluted fractions was performed on 3-mm thick polyacrylamide slab gels in a Biorad (Hoefer) dual slab apparatus, using an SDS-disc buffer system [13] with a lower gel buffer of 0.42 M Tris, 0.031 N HCl, pH 9.18 (running pH 9.5). Separating gels were polymerized from a linear gradient prepared with equal volumes of gel solutions which contained 4.75% acrylamide, 0.25% bisacrylamide, and 14.25% acrylamide, 0.75% bisacrylamide. The stacking gel was prepared with 3.2% acrylamide, 0.8% bisacrylamide.

Samples for analytical electrophoresis were prepared directly from eluted preparative gel fractions by adding 0.01 vol. of mercaptoethanol, 0.2 vol. of 50% (v/v) glycerol/0.2% (w/v) bromophenol blue, and heating at 90°C for 10 min. Electrophoresis was begun at a constant current of 15 mA/slab until samples had entered the stacking gel, then continued at 30 mA/slab until the tracking dye was eluted.

The molecular weight standards used for apparent molecular weights estimation were: (1) myosin heavy chain, rabbit skeletal muscle (210 000); (2) phosphorylase A, rabbit skeletal muscle (92 500); (3) serum albumin, bovine (68 000); (4) catalase, bovine liver (57 500); (5) ovalbumin, hen (43 000); (6) carbonic anhydrase, bovine RBC (30 000); (7) cytochrome *c*, horse heart (12 400). All but the first were obtained from Sigma. Myosin was a gift from Dr. J. Reynolds, Duke University.

Analytical isoelectric focusing. Isoelectric focusing of fractions eluted from preparative electrophoresis was performed by first dialyzing SDS-containing samples at room temperature against deionized 9 M urea containing 2% Triton X-100 (3—4 changes). This procedure removed most of the SDS and salts from the sample while maintaining solubility of the protein [14,15]. Focusing was carried out by polymerization of the sample in a gel prepared by mixing sample and 5.63% acrylamide, 0.625% bisacrylamide, 2% ampholine, 10 M urea, 2% Triton, $1.56 \cdot 10^{-4}$% riboflavin, $2.5 \cdot 10^{-3}$% ammonium persulfate. 1 vol. of the dialyzed sample was mixed with 4 vols. of the above solution, and the resulting mixture photopolymerized after addition to cylindrical gel tubes and overlayering with water.

Electrofocusing was performed at room temperature using 2% (v/v) phosphoric acid as anolyte, and 0.05 N sodium hydroxide as catholyte. To prevent the soft, large-pore gel from sliding out of the gel tube, the lower ends of the tubes were covered with small pieces of wet, perforated dialysis membrane, held in place with rubber bands.

Gel staining. Analytical slab gels were stained for protein using Coomassie blue. Destaining was accomplished by diffusion, or by transverse electrophoresis. Staining for glycoproteins was performed as described [16], except that the leucofuchsin stain was decolorized by agitation with activated charcoal. Isoelectric focusing gels were stained with bromophenol blue [17].

Results

SDS is an efficient and non-discriminating solubilizing agent for erythrocyte and platelet membrane proteins. For erythrocyte membranes, the yields of extracted protein were 82% for a detergent to protein weight ratio of 1 : 1, 95% for 2 : 1, and 100% for 4 : 1 and 8 : 1. For platelet membranes, the extracted protein yields were 62%, 87%, 95% and 90% for ratios of 1 : 1, 2 : 1, 4 : 1 and 8 : 1, respectively. For both cell types, all molecular weight classes of protein and glycoprotein appeared to be solubilized in equal proportions at all ratios of SDS employed, as judged by SDS gel analysis. Fig. 1 shows analytical results for platelet membrane protein extraction. Equal relative intensities are observed for the protein bands in the extracted and unextracted membrane preparations indicating the absence of selectivity for the solubilizing system. In addition, within the ranges of membrane protein concentrations studied, the effectiveness of solubilization appeared to be independent of the final protein concentration.

Relatively large amounts of SDS (2.4 mg/sample) had no adverse effects on the quality of the analytical separations achieved; nor did the presence of sodium deoxycholate (8 mg/mg protein) or of Triton X-100 (4 mg/mg protein).

Based upon these solubilization and extraction results, we employed SDS at 8 : 1 ratios of detergent to protein for membrane protein extracts that were used for preparative electrophoretic separation.

Gradient slab gel analysis of membrane proteins

The combined use of an SDS-discontinuous buffer system and linear polyacrylamide gradient slab gels, with bisacrylamide cross-linkage adjusted for maximum theoretical protein retardation [18], allowed resolution of a broad

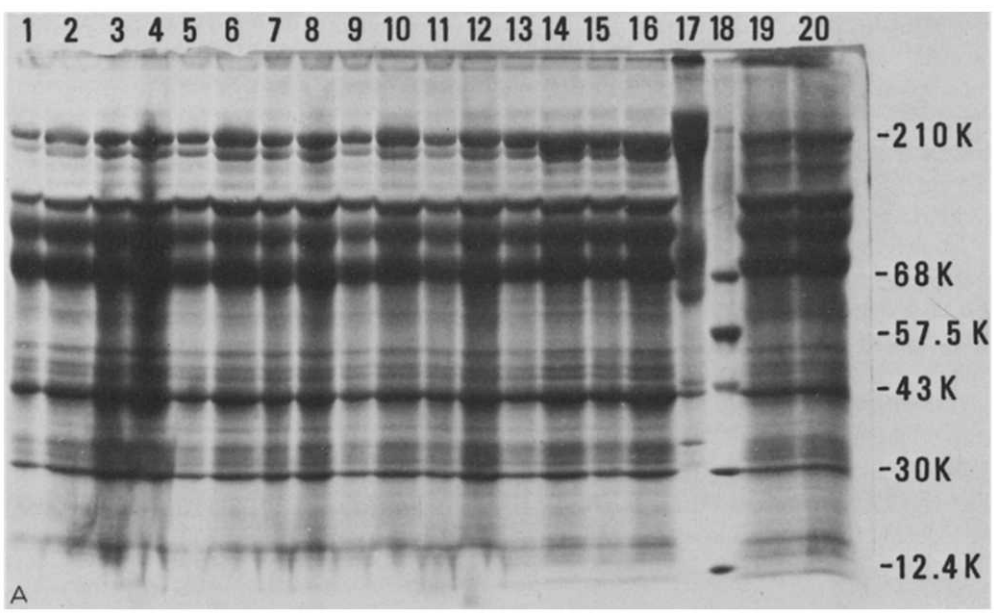

Fig. 1A.

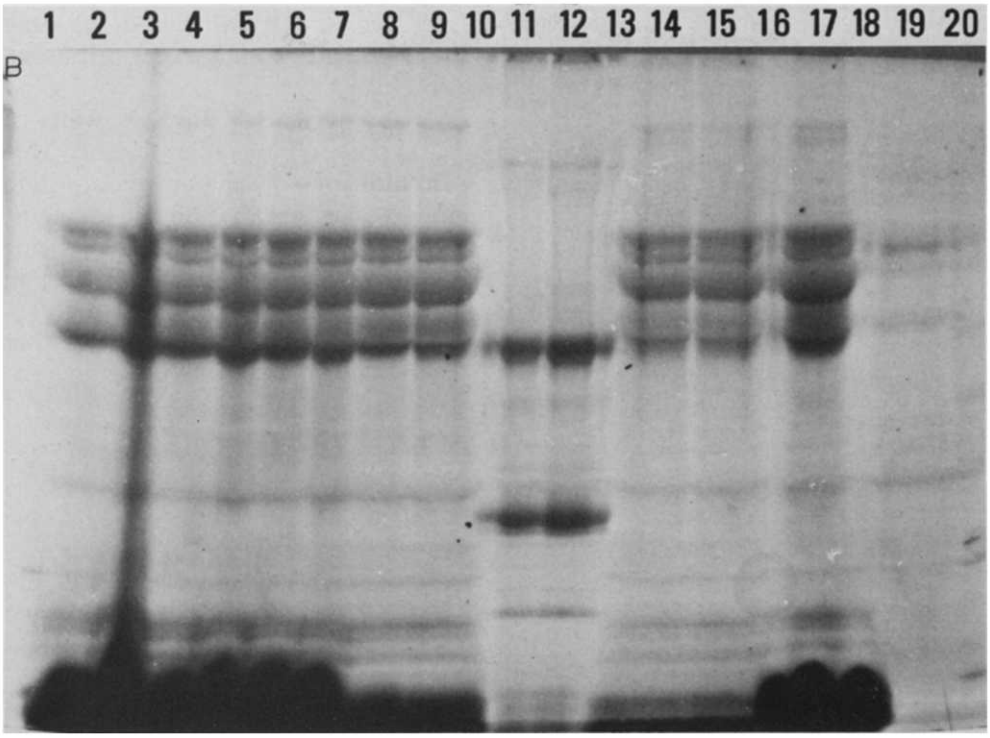

Fig. 1. SDS slab gel analysis of reduced platelet membrane protein extracts. Both slabs were run simultaneously on a dual-slab electrophoresis apparatus (see Methods). (A) Coomassie Blue R-250 stain. Wells 1—16 contained SDS 'pre-' and 'post-extracts'. The first and second wells of each set of four contained 'pre-extract' material (75 and 150 μg of protein, respectively). The third and fourth wells of each set contained 'post-extract' material in identical amounts. From left to right the sets of four represent 1 : 1, 2 : 1, 4 : 1 and 8 : 1 weight ratios, respectively, of detergent to protein. Wells 17 and 18 are protein standards. Well 17 contained 75 μg SDS-extracted (4 : 1) erythrocyte membrane proteins. Well 18 contained purified molecular weight standards whose reduced subunit sizes are indicated along the right-hand edge of the photograph. Wells 19 and 20 contained 150 μg each of deoxycholate-treated (8 mg/mg protein) platelet membrane 'pre-' and 'post-extracts', respectively. Aliquots for gel analysis were made 8 : 1 by weight in SDS with respect to protein content, 2% (by vol.) in mercaptoethanol, and heated 15 min at 90°C. (B) Periodic acid-Schiff stain. Wells 1 and 20 contained molecular weight standards in the same quantity as gel (A), well 18. Samples 2—9 contained 300 μg protein from each of the same SDS 'pre-' and 'post-extracts' in gel (A), wells 1—16. The left-hand and right-hand wells of each set of two contained 'pre-extract' and 'post-extract', respectively. Samples 11 and 12 contained 150 and 300 μg, respectively, of 4 : 1 SDS extract of erythrocyte membranes. Wells 14 and 15 contained 300-μg aliquots of the same 8 : 1 deoxycholate 'pre-' and 'post-extracts' as in gel (A), wells 19 and 20 respectively. Well 17 contained 150 μg of Triton X-100 platelet membrane 'post-extract'. Extraction was at 37°C for 1 h at 4 mg detergent/mg protein. The 'post-extracted' supernatant was made 16 : 1 by weight in SDS with respect to protein content, 2% (by vol.) in mercaptoethanol, and heated 15 min at 90°C. Sample 19 contained 250 μg of dialyzed lyophilized protein obtained from pooled, second washings of isolated platelet membrane vesicles. The lyophilizate was made 10 : 1 in SDS with respect to protein weight, 0.025 M in sodium phosphate (pH 7.1), 2% (by vol.) in mercaptoethanol, and heated 15 min at 90°C.

molecular weight range of membrane proteins on a single analytical gel. The discontinuous buffer system was additionally advantageous because of the protein-stacking feature which permitted direct analysis of large volume samples.

Graphical analysis of R_F versus log M_r of protein standards yielded sigmoidal plots with relatively linear mid-portions. These results appear similar to those

obtained by Neville [13] and Glossmann and Neville [19] who used the same buffer system and separating gels of uniform polyacrylamide composition to characterize membrane proteins and glycoproteins.

Erythrocyte membrane glycoproteins (PAS-1 and PAS-2; Fig. 1B, wells 11 and 12) migrated in these gradient gels at apparent molecular weights 15% greater than expected from the literature. In addition, the major glycoprotein bands of human platelet membranes migrated at somewhat lower apparent molecular weight (M_r 70 000—145 000) than those previously reported using SDS zone electrophoresis [20—24] or SDS disc electrophoresis [8,25,26] in uniform polyacrylamide gels. By analogy with erythrocyte membrane glycoproteins and with soluble glycoproteins [19], it is reasonable to suppose that the actual molecular weights of some of these major platelet membrane glycoproteins may be even less than the apparent values for SDS gels.

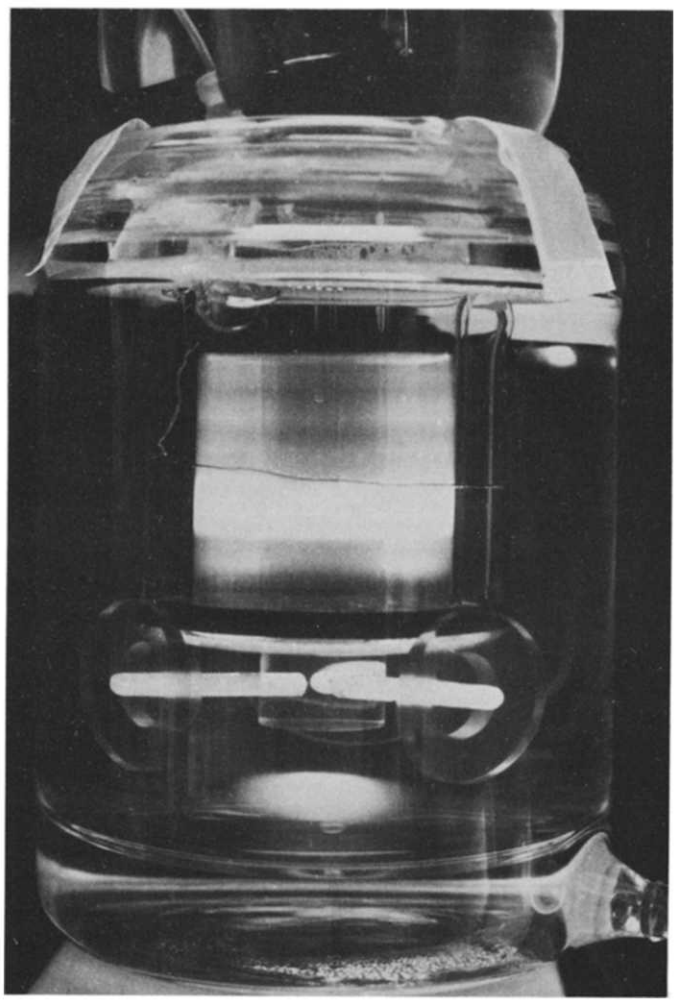

Fig. 2. Preparative gel electrophoresis of fluorescamine-labelled platelet membrane proteins. Gel height 5 cm; Tris/borate/SDS electrophoresis system.

The gradient gel procedure permitted resolution and detection of several minor, low molecular weight ($M_r < 30\,000$) platelet membrane glycoproteins (Fig. 1B), as well as periodic acid-Schiff-positive minor species of higher apparent molecular weight ($M_r > 250\,000$).

Preparative electrophoresis

With use of fluorescamine labelling, the movement of membrane protein bands through the separating gel could be directly monitored. As indicated in Fig. 2, the bands of protein maintained a horizontal front during preparative electrophoresis, indicating uniform heat dissipation and electrical field in the preparative separation gel. Avoidance of band skewing or curvature is of major importance for optimal resolution of protein species. By using the fluorescamine tracking procedure, we were able to apply sufficient power to accomplish separation in a relatively short time period, while maintaining uniform mobility of protein bands.

The elution profile for a typical preparative electrophoretic separation of erythrocyte membrane proteins is shown in Fig. 3. Sharp resolutions and relatively concentrated elution fractions were obtained with the protein loading (20—60 mg), power and elution buffer flow rate used. Fluorescamine labelling of proteins was found to be particularly helpful for detection of smaller amounts of protein.

Optimal resolution was obtained by appropriate selection of gel concentration and height, based on estimates from analytical gels [12,27]. Moderate power of 4—8 W was applied for lower molecular weight proteins, while 8—

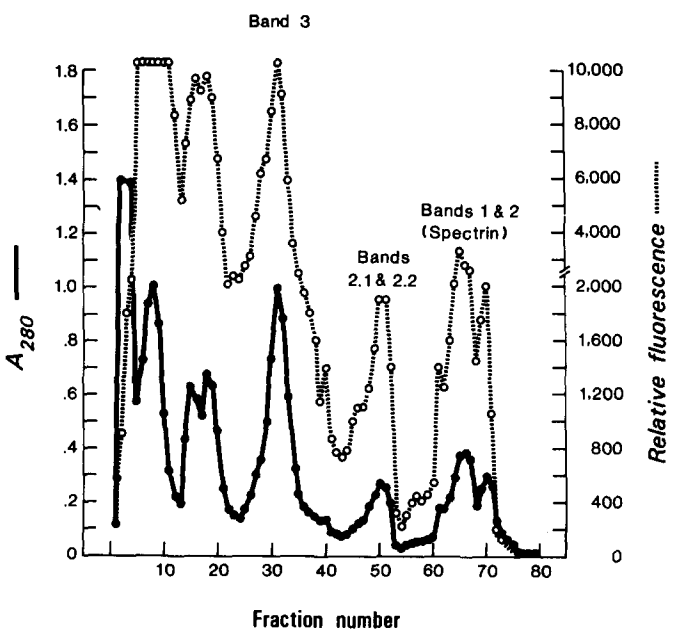

Fig. 3. Elution profile of preparative electrophoretic separation of erythrocyte membrane proteins. Sample: 50 mg SDS-extracted, reduced membrane protein, fluorescamine labelled. Separating gel: 5 cm height, 7.5% T, 2.5% C. Buffer system SDS-disc. ●———●, A_{280}; ○- - - - -○, fluorescence. Major protein isolates identified by the convention of Steck [2].

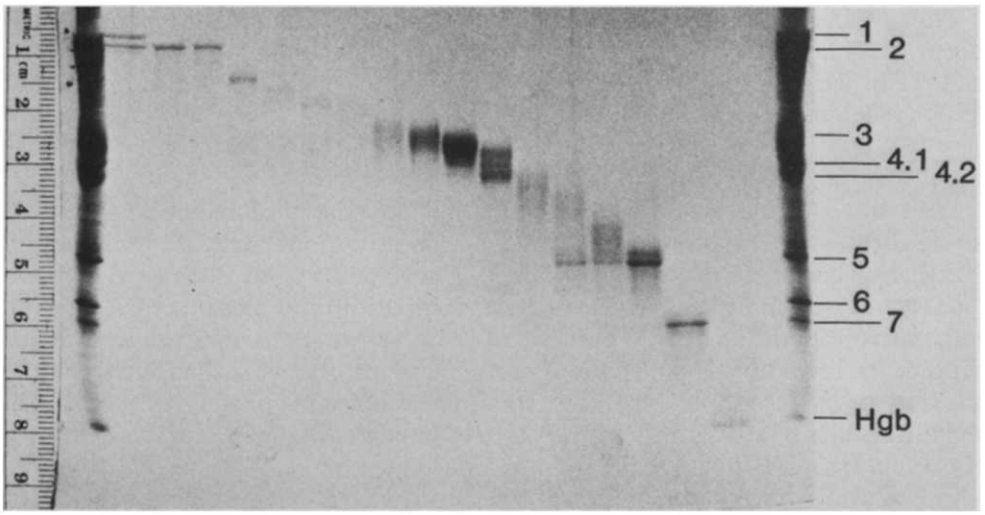

Fig. 4. SDS slab gel analysis of preparative elution profile of erythrocyte membrane proteins (Coomassie blue staining). Preparative sample: 30 mg SDS-extracted, reduced membrane protein. Preparative separation gel: 4.5 cm height, 7.5% T, 1.25% C. Preparative buffer system: Tris/borate/SDS. Analytical samples on the left and right edges contain whole membrane extract, with band numbering according to Steck [2]. Aliquots of 100 μl from selected elution fractions were applied to the remaining sample wells for identification of the protein content of the fractions and for assessment of purity (see Methods).

15 W were applied for the higher molecular weight proteins with slower mobility.

The eluted fractions were sufficiently concentrated to allow direct analysis of the protein content by analytical gel electrophoretic techniques. Fig. 4 is an illustration of the molecular weight range of separations achievable from a single preparative electrophoresis experiment. Aliquots of eluted fractions from a preparative electrophoresis of erythrocyte membrane proteins were applied to the slab gel for direct analysis, as described in Methods. The bands are identified by the convention of Steck [2]. Molecular weights of resolved species range from approximately 240 000 and 220 000 for the spectrin bands on the left, to 15 100 for the globin band (HGB) on the right. Bands 1—5 and 7 and HGB are well resolved.

By proper selection of electrophoretic conditions, it is possible using a single preparative separation to isolate many of the major protein bands representing the broad molecular weight range of the major erythrocyte or platelet membrane proteins, in yields and concentrations sufficient for further analytical or experimental procedures. Fig. 5A shows an analytical electrophoretogram of selected, unconcentrated fractions. Coomassie blue staining demonstrates near homogeneity of the erythrocyte spectrin species (well 3), erythrocyte band 3 (wells 5 and 6) and platelet myosin heavy chain species (well 10). Shown greatly purified are three major platelet membrane protein bands (samples 11—13), and platelet 'actin' (sample 16).

Fig. 5B shows the same quantities of membrane protein isolates stained for carbohydrate, and demonstrates the degree of purification achievable in one step for glycoproteins of a broad size range. Of particular interest is the elution

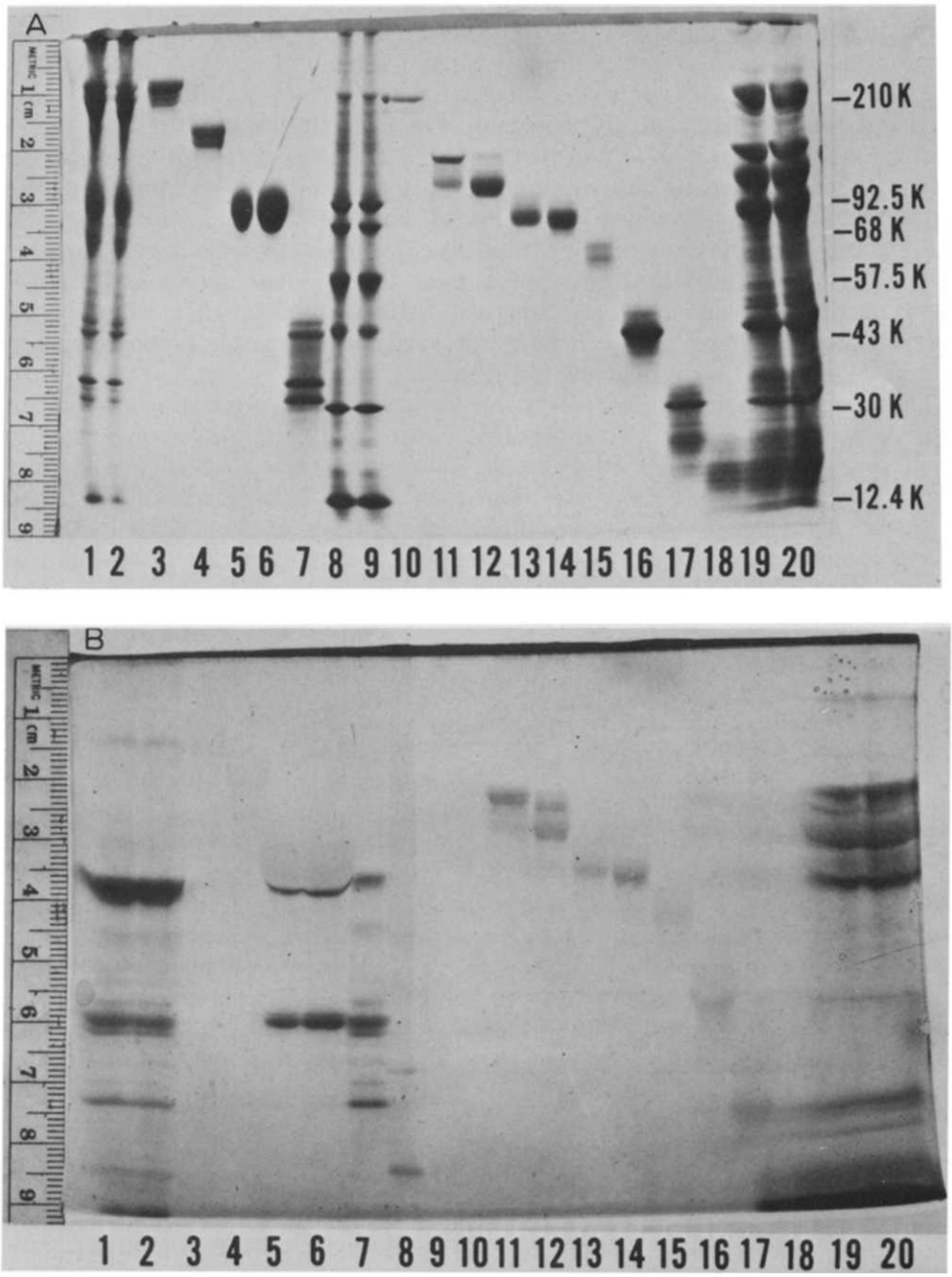

Fig. 5. SDS slab gel analysis of selected erythrocyte and platelet membrane protein isolates obtained from one step preparative electrophoresis. (A) Coomassie blue staining. Wells 1 and 2, 8 and 9, and 19 and 20 contained protein standards which are, respectively: erythrocyte membrane proteins (SDS extracted, reduced, approximately 75 μg each); purified protein molecular weight standards (the approximate molecular weights of which are indicated along the right-hand edge of the figure); platelet membrane proteins (SDS extracted, reduced, approximately 100 μg each). The remaining samples contained 100-μl aliquots (or smaller) from selected elution fractions from one step preparative electrophoretic separations of erythrocyte membrane proteins (samples 3—7) and platelet membrane proteins (samples 10—18). (B) Periodic acid-Schiff staining. This gel contains samples identical to those in gel (A) with the exception that larger quantities (approximately 300 μg each) of membrane protein standards (wells 1 and 2, 19 and 20) were applied, and protein standard well 9 was omitted. Both gels were run simultaneously under identical conditions in a dual-slab electrophoresis apparatus

fraction containing erythrocyte band 3, which eluted during preparative electrophoresis as a single, broad, Coomassie-staining band of apparent M_r 75 000—105 000 (Fig. 5A, wells 5 and 6). Periodic acid-Schiff staining revealed unexpected heterogeneity of glycoprotein (Fig. 5B, samples 5 and 6), in that a periodic acid-Schiff-positive band of low molecular weight ('PAS-2', apparent M_r 36 000) is present in this fraction in addition to the 'PAS-1' band (apparent M_r 75 000). The appearance of both 'PAS-1' and 'PAS-2' bands in this preparative fraction suggests that both of these glycoproteins migrated during preparative electrophoresis as molecules of similar hydrodynamic size and charge/mass ratios. Subsequently, partial dissociation to a smaller subunit size (36 000 daltons) seems likely to have occurred during sample preparation for, or in the process of analytical electrophoresis.

These observations suggest reversible association of glycophorin subunits, and are in accord with previous studies describing apparent interconvertability of 'PAS-1', 'PAS-2' and 'PAS-4' bands. The phenomenon appears to be concentration, temperature and ion dependent, in the presence of SDS, but independent of the presence or absence of sulfhydryl-reducing agents [28—30]. The glycophorin A subunit ('PAS-2') has been assigned a molecular weight of approximately 29 000 by sedimentation equilibrium and velocity studies [31], and approximately 31 000 by compositional and sequence studies [32]. It seems likely that the observed glycophorin interconversions occurred during reheating of the elution fractions in preparation for analytical electrophoresis, and not during preparative electrophoresis.

Examination of periodic acid-Schiff stained elution fractions of platelet membrane proteins (Fig. 5B, wells 10—18) reveals no evidence of the interconvertible subunit phenomenon that was noted for erythrocyte membrane glycoproteins. It is clear, however, that for both cell types, some glycoprotein species migrate with the same or similar relative mobility as do some major, Coomassie-staining proteins. This is evident, for example, on inspection of Fig. 5A and B, wells 5 and 6, which contain both erythrocyte Coomassie band 3 (average M_r 95 000) [33], and the 'PAS-1' band. In this case, the protein and the glycoprotein are known to be different molecular species [2,3,33], the former displaying broad-band SDS gel electrophoretic mobility presumably on the basis of variable glycosylation of the carbohydrate-containing (7% by weight) carboxyl terminal portion of the molecule, and/or variable SDS binding to the molecule, and/or unknown heterogeneity.

Identity or lack of identity among comigrating or closely migrating platelet membrane proteins and glycoproteins (e.g. Fig. 5A and B, wells 11—14) has not yet been well defined. Our analytical SDS gel data, and data from others [23,24,26,34,35], would suggest that some of these closely migrating major proteins and glycoproteins are probably non-identical, as judged by non-identical mobility and differential staining or radiolabelling. Other glycoprotein species (e.g. Fig. 5A, well 17) appear to be clearly non-identical with the most closely migrating, densely staining Coomassie-staining band. Therefore, while achieving high resolution separation of protein-SDS species of differing mobility, these preparative techniques will not allow one step separation of non-identical species which comigrate in SDS gels.

Both the continuous and the discontinuous preparative buffer systems

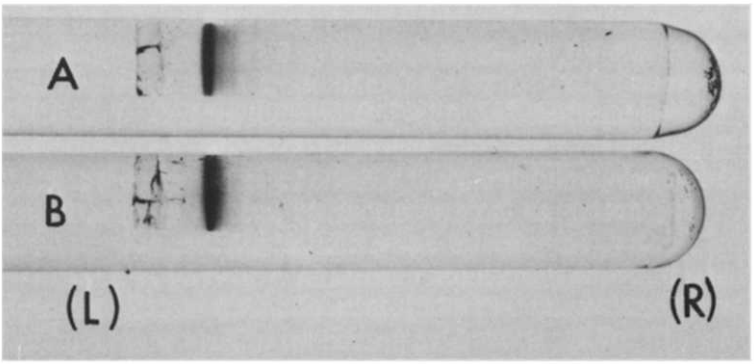

Fig. 6. Analytical gel isoelectric focusing of membrane proteins. (A) Isolated platelet 'actin' from the same unconcentrated elution fraction shown in Fig. 5A, sample 16. (B) Isolated erythrocyte band 3 from the same unconcentrated elution fraction shown in Fig. 3, tube 31. Final ampholine concentration of these gels was 1.6%, of which 2/3 was pH 3.5—10 and 1/3 was pH 4—6. Anode is to the left, and the pH gradient extended from 3.0 to 9.3.

allowed high resolution separation of membrane proteins. Within a restricted molecular weight range, the discontinuous system yielded discrete resolution, but did not resolve well throughout the broad molecular weight range of membrane proteins. For example, excellent resolution of higher molecular weight erythrocyte membrane protein was achieved in an experiment with the disc buffer system (Fig. 3 and Fig. 5A and B, samples 3—6), but resolution of proteins of less than M_r 60 000 was impaired. In contrast, fractionation using a continuous buffer system yielded nearly equivalent resolution over a broader molecular weight range (Fig. 4). The increased complexities of the disc buffer system make it less attractive for general fractionation purposes, although it appears advantageous for increased resolution capability within a restricted molecular weight range.

The yield of purified membrane proteins recovered from preparative gel electrophoresis was determined by comparing estimates of relative membrane protein abundance (obtained from the literature or from analytical gel scanning) with the Lowry-Folin protein determinations for total sample and for eluted protein fractions which were judged to be essentially homogeneous. The recovery for selected membrane proteins was: erythrocyte spectrin 1 and 2 (Fig. 5A, well 3), 90%; erythrocyte band 3 and comigrating 'PAS band' 1 (Fig. 5A and B, wells 5 and 6), 95%; platelet actin (Fig. 5A, well 16), 92%; platelet '75 K' Coomassie band (Fig. 5A and B, wells 13 and 14), 85%.

Fig. 6 shows analytical gel electrofocusing patterns of erythrocyte Coomassie band 3 and platelet membrane-associated 'actin', isolated by preparative gel electrophoresis. The individual protein isolates electrofocus at acidic pH, as do most of the platelet membrane proteins [9]. The isolated membrane proteins appear to be relatively homogeneous on electrofocusing in a broad-range pH gradient, using a single method of protein detection. This analytical technique suggests also that a high degree of protein purification was achieved from a single step preparative procedure.

Discussion

The protein content of plasma membrane preparations is most often defined by studies of enzymatic activities and/or by gel electrophoretic resolution of its protein species. Isolation of the proteins may then be pursued by methods varying in their specificity, separative capacity and resolution ability. We have employed an extension of a commonly used high resolution analytical technique, SDS-polyacrylamide gel electrophoresis, for the direct, high resolution preparative scale purification of the membrane proteins defined by the micro-analytical technique.

Knüfferman et al. [36] reported successful preparative scale slab SDS-polyacylamide gel electrophoresis of 60—75-mg samples of erythrocyte membrane proteins using fluorescent protein labelling, multiple gel slabs, and gel homogenization and extraction techniques for protein recovery. Yields of recovered protein ranged from 45% to 90%, decreasing as the molecular weight of the protein increased. Compared to results we obtained with continuous elution, the gel extraction preparative technique offers the potential advantage of obtaining major protein bands under direct visualization. Its disadvantages are that protein labelling or staining probably are obligatory, the yields may be smaller, and several recovery operations are required for each band in each gel slab. Additionally, modification of some proteins was reported to occur during the extractive process. The gel extraction technique has more often been used on a smaller scale to obtain microgram amounts of protein, suitable for use as antigens [37,38], or for limited analyses [39].

A variety of isolation procedures have been devised for purification of specific, membrane-associated platelet and erythrocyte proteins. These procedures generally have employed differentical solubilization or extraction combined with one or several chromatographic or solubility separations for further purification [40]. Extrinsic or loosely associated membrane proteins and enzymes have proved to be more readily obtainable, and have been more frequently purified and characterized [41—51]. Although the goal of our studies was not the isolation of specific extrinsic membrane proteins, this was achieved in several broad range fractionations of erythrocytes or platelets (Figs. 3—6).

Specific techniques have been devised and successfully applied to the preparative isolation of a few integral or intrinsic membrane proteins of erythrocytes and platelets [33,34,39,52—62]. The more successful of these approaches, in terms of yield and electrophoretically defined purity, have relied upon specific macromolecular activities (e.g. lectin binding) or specific physical properties (e.g. protein sulfhydryl content) to achieve high resolution separation. Preparative electrophoretic results presented here indicate that this approach, using continuous elution techniques, is useful for the direct purification of intrinsic membrane proteins.

Implicit in the preparative electrophoresis method employed is the exposure of proteins to SDS, and the risk of significant denaturation. This may be of consequence only if desired biological activity is compromised. Additional limitations of the method include moderate load capacity, and potential comigration of non-identical protein species. These limitations may be of less relative importance for many preparative applications compared to the several positive attributes.

This preparative electrophoretic approach is relatively rapid and simple, is quantitatively adequate, is applicable to and specific for a wide range of molecular size, and its employment and expected results can be based upon assessment of the analytical scale electrophoretic mobility of membrane protein-SDS complexes. A degree of resolution comparable to that of microanalytical methods is obtainable. These features recommend this technique as a useful adjunct to current approaches to preparative scale isolation of membrane proteins and glycoproteins.

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