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RAPID PREPARATIVE ISOLATION OF MAJOR ERYTHROCYTE MEMBRANE PROTEINS USING POLYACRYLAMIDE GEL ELECTROPHORESIS IN SODIUM DODECYLSULFATE

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SUMMARY

1. We describe a simple method for preparative, sodium dodecylsulfate/polyacrylamide gel electrophoresis of the major proteins in human erythrocyte membranes.

2. The method is based on extraction of prestained proteins from gel slabs. Three different fluorescent dyes (*o*-phthalaldehyde, fluorescamine and 1-dimethylaminonaphthalene-5-sulfonylchloride) have been used for prestaining. The method allows separation of up to 75 mg membrane protein and isolation of mg quantities of all major erythrocyte ghost proteins, while preserving the high resolution of analytical polyacrylamide gel electrophoresis.

3. Yield depends on extraction conditions and the molecular weight of the proteins being eluted. It ranges from 43–48 % for protein 1 (apparent mol. wt approx. 310000) and 72–78 % for protein 3 (apparent mol. wt 87 000–93 000) to 87–93 % for protein 6 (apparent mol. wt 35000).

4. The labile behaviour of the high molecular "spectrin" bands (bands 1 and 2) is described. Extraction at room temperature tends to split these proteins into products of lower molecular weight. In contrast, the minor protein components 2.1 and 2.2 tend to aggregate yielding components 1 and 2.

5. N-terminal amino acid analyses have been performed on proteins 1, 2, 3, 4A, 4B, 5 and 6. Each of these bands contains several N-terminals, most of which appear constant. Some additional N-terminal amino acids vary from one donor to the next.

INTRODUCTION

One of the most potent approaches to the analysis of membrane proteins involves complete solubilization of the membranes with sodium dodecylsulfate and

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Abbreviation: TEMED: *N,N,N′,N′*-tetramethylenediamine.

subsequent electrophoretic molecular sieving in polyacrylamide gels. The high resolving power and reproducibility of this technique has been repeatedly demonstrated, e.g. in analyses of erythrocyte [1], thymocyte [2] and hepatocyte [3] plasma membranes. However, this method has not been heretofore extended to a preparative scale. Rather, preparative isolations of membrane proteins have utilized extraction procedures, more or less selective for various classes of membrane proteins [4] or glycoproteins [5]. These extractions include variations of ionic strength [4, 6] as well as application of organic solvents [7] and have generally been combined with chromatographic procedures employing ionic [8] and non-ionic [9] detergents, organic solvents [10], urea [11], guanidium · HCl [12] or chemical modification of membrane proteins [13]. Combined use of selective extraction methods with such chromatographic techniques has led to the purification of several membrane proteins, e.g. "spectrin" [4] and "glycophorin" [5] for further biochemical [14] and immunochemical [15] studies. However, none of these approaches approximates to the high separation power and versatility of sodium dodecylsulfate/polyacrylamide gel electrophoresis.

We have therefore developed a simple, one-step procedure for the preparative isolation of membrane proteins as electrophoretically pure substances in one operation, which fully preserves the analytical resolution. The method can yield mg amounts of individual erythrocyte membrane proteins for purposes of amino acid analyses, sequence studies and other characterization steps.

MATERIALS AND METHODS

(1) *Chemicals*

Unless otherwise stated, chemicals and biochemicals were obtained from Serva Feinbiochemica, Heidelberg, Boehringer, Mannheim or Merck, Darmstadt. Fresh human blood (blood group O, Rh+) was drawn into acid citrate/dextrose solution.

(2) *Preparation, solubilization and prestaining of erythrocyte ghosts*

Red blood cells were isolated as described in ref. 1; special care was taken to remove leukocytes and thrombocytes in order to avoid proteolytic degration of membrane proteins. Ghosts were prepared according to Dodge et al. [16]. Complete solubilization of erythrocyte plasma membranes in 1–3 % sodium dodecylsulfate/0.45 M mercaptoethanol or 1–3 % sodium dodecylsulfate/40 mM dithiothreitol (Calbiochem, Lucern) was as in ref. 1. The following methods were used to prestain membrane proteins.

(1) *o*-Phthalaldehyde was employed according to Weidekamm et al. [17] with minor modifications. 1 ml packed erythrocyte ghosts in 5 mM phosphate, pH 8, was combined with 0.1 ml 0.5 M phosphate, pH 8, and 0.2 ml 20 % aqueous sodium dodecylsulfate (w/v). After heating for 1–2 min at 100 °C, 0.04 ml mercaptoethanol was added, the mixture reheated for 2 min at 100 °C and cooled to room temperature. Thereafter, 0.04 ml 1 % methanolic *o*-phthalaldehyde (freshly prepared) was added and prestaining allowed to proceed for 4–6 h at room temperature or overnight at +4 °C. The final concentrations were: 40 mM phosphate, pH 8, 2.9 % sodium dodecylsulfate, 0.44 M mercaptoethanol, 22 mM *o*-phthalaldehyde. Prior to electro-

phoresis the sample was made up to 10 % in sucrose and Pyronin Y added as tracking dye.

(2) Fluorescamine (4-phenylspiro(furan-2(2H),1'-phthalan)-3,3'-dione; Fluram, Hoffman-LaRoche and Co., Schweizerhalle, Switzerland) was employed, using a modification of the method of Udenfried et al. [18]. 1 ml packed erythrocyte ghosts in 5 mM phosphate, pH 8, was combined with 0.1 ml 0.5 M phosphate buffer, pH 8.5, and 0.2 ml 20 % aqueous sodium dodecylsulfate (w/v), the mixture heated for 1–2 min, at 100 °C and then cooled to room temperature. Thereafter, 0.1 ml fluorescamin (3 mg/ml in *N,N'*-dimethylformamide) was added with vigorous stirring (Vortex mixer). Prestaining is completed within milliseconds and can be done before or after solubilization of erythrocyte ghosts in sodium dodecylsulfate. Addition of mercaptoethanol or dithiothreitol can be omitted.

(3) Dansylation of membrane proteins followed the procedures of Gross and Labouesse [19] and Gray [20], modified for use in sodium dodecylsulfate [21].

(3) *Sodium dodecylsulfate/polyacrylamide gel electrophoresis*

Analytical electrophoresis was performed as in ref. 1. Final gel concentrations were 5 % total acrylamide, 2.5 % cross-linking with *N,N'*-methylene-bisacrylamide. Proteins were stained with Coomassie brilliant blue R-250 and glycoproteins using the periodic acid-Schiff reaction [1]. The fluorescent patterns of prestained membrane proteins were photographed using a Polaroid MP-3 camera (filter: Schott, Mainz gg 455 nm) and Polaroid Land film Type 55 P/N (50 ASA) or Type 57 (3000 ASA). A Desaga Uvis universal irradiator and Desaga intensive ultraviolet source (366 nm) were used as light sources. The exposure time was 4 s at f = 8 (Type 57). Relative mobilities and proportions of different membrane proteins were determined by densitometric scanning at 620 nm after staining with Coomassie brilliant blue [1] using a Gilford spectrophotometer (Model 240) equipped with a synchronous linear transport (Model 2410-5) and an electronic integrator (Hewlett-Packard, Model 3370-B). For molecular weight determinations gels were calibrated with the following standard proteins of known molecular weight [22]: rabbit immunoglobulin (155 000), *Escherichia coli* β-galactosidase (130 000), rabbit muscle phosphorylase A (93 000), *Canavalia ensiformis* urease (83 300); *Crotalus* ter. ter. L-amino-acid oxidase (63 000), bovine liver catalase (59 000), rabbit immunoglobulin G heavy chains (55 000), egg white albumin (45 000), rabbit muscle aldolase (40 000), bovine heart lactate dehydrogenase (36 000), bovine pancreas α-chymotrypsinogen A (25 700), rabbit immunoglobulin G light chains (23 500), horse skeletal muscle myoglobin (17 200), bovine hemoglobin (15 500), horse heart cytochrome (13 500). The molecular weights and proportions of low molecular weight proteins (< 100 000) were determined using 7 % (percent cross-linking = 2.5) and 6 % (percent cross-linking = 2.5) gels. The molecular weight and proportion of high molecular weight proteins (> 100 000) were determined using 3 % (percent cross-linking = 2.5) and 4 % (percent cross-linking = 2.5) gels (defined according to Hjerten [23]).

Preparative electrophoresis was carried out in a gel-plate similar to that described by Kaltschmidt and Wittmann [24]*. In order to adapt this plexiglas apparatus for use with electrophoretic systems containing sodium dodecylsulfate, the

* Commercially available by Desaga, Heidelberg.

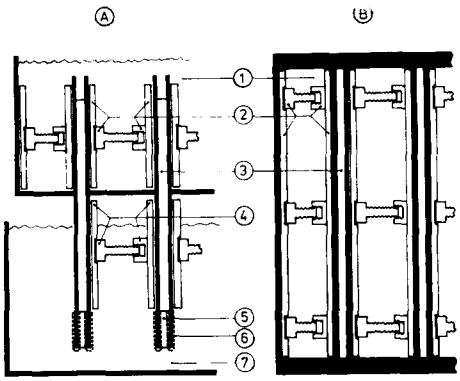

Fig. 1. Schematic drawing of the preparative electrophoretic apparatus [25] as modified for preparative polyacrylamide electrophoresis in sodium dodecylsulfate. (A) Side view. (B) Top view. 1, Upper buffer reservoir; 2, upper gel stabilizer, consisting of two plexiglass plates, one pressure screw and one bold fixed to one of the plexiglass plates; 3, separation gel; 4, lower gel stabilizer: 5, sealing gel; 6, region which is roughened with glass paper and treated with a potassium chrome alum/gelatin solution; 7, lower buffer reservoir.

following technical modifications were necessary (Fig. 1): (1) A tightening device for stabilizing the gel slabs within their moulds. (2) Roughening the plexiglas construction with fine glass paper in the region of the sealing gel (18 % polyacrylamide, 2.5 % cross-linking). (3) Repeated treatment of the sealing gel region with a potassium-chrome alum/gelatine solution*. These modifications provide a reliable sealing and proper position of the separation gel slabs within the gel mould.

The dimensions of the gel slabs are $20 \times 20 \times 0.5$ cm. The gel surface area per plate is 10 cm^2 (total surface area of five gels, 50 cm^2). The composition of buffers and gels are:

Cathodal buffer	Anodal buffer	Gel composition
40 mM Tris · HCl	40 mM Tris · HCl	40 mM Tris · HCl
20 mM sodium acetate	20 mM sodium acetate	20 mM sodium acetate
2 mM Na_2-EDTA	2 mM Na_2-EDTA	2 mM Na_2-EDTA
0.1 % Sodium dodecylsulfate		0.1 % sodium dodecylsulfate
		5 % T: total acrylamide concentration
		2.5 % C: *N,N'*-methylene-bisacrylamide proportion
		0.15 % $(NH_4)_2S_2O_8$
		0.025 % TEMED
pH 7.4	pH 7.4	pH 7.4

After polymerization, the gels were pre-electrophoresed for 30 min at 200 mA/30 V. Each gel slab was then laden with 3 ml solubilized and prestained erythrocyte ghosts (approx. 12–15 mg protein; total capacity using five gel slabs: 60–75 mg protein); Pyronin Y was used as tracking dye. Electrophoretic separation was for 7 h

* 0.5 g gelatin is dissolved in 90 ml hot water and 10 ml of a 0.5 % $KCr(SO_4)_2$ solution in water is added. The roughened plexiglas parts are wetted with this solution and dried.

at 500 mA/70 V. Cooling or buffer circulation was not necessary. After electrophoresis, the fluorescent bands were cut out and the polyacrylamide gel strips homogenized by pressing them twice through a syringe (20 ml diposable syringe, Amefa Record). To extract proteins from the homogenized gel strips, 10 vols of 5 mM phosphate, pH 8, 0. 1 % sodium dodecylsulfate was added per vol. of gel and the mixture shaken for 6–8 h at 10 °C. The extract was separated from the gel particles by use of a basket centrifuge (Ika Filtrax F 10, Janke and Kunkel, Stauffen). For maximum recoveries the extraction was done twice, for 3–4 h each. The extracts were concentrated to one tenth of their original volume by means of an Amicon pressure filtration cell using PM 10 Diaflo membranes. In order to determine the yields of the extractions, aliquots of the concentrated extracts and the extracted gel were re-electrophoresed and quantified by integrating the densitometric scans of Coomassie-stained gels.

Preparative electrophoresis with continuous electrophoretic elution. In a series of experiments, we used a continuous preparative electrophoresis apparatus (Buchler, Polyprep 200). Gel dimensions were: height, 6 cm; surface, 17.5 cm^2. The gel composition was as in the sodium dodecylsulfate/polyacrylamide gel electrophoresis of gel slabs and the buffer compositions were:

Cathodal buffer	Anodal buffer	Elution buffer
40 mM Tris · HCl	40 mM Tris · HCl	20 mM Tris · HCl
20 mM sodium acetate	20 mM sodium acetate	10 mM sodium acetate
2 mM Na_2-EDTA	2 mM Na_2-EDTA	1 mM Na_2-EDTA
0.2 % sodium dodecylsulfate	0.2 % sodium dodecylsulfate	0.2 % sodium dodecylsulfate
pH 7.4	pH 7.4	pH 7.4

5 ml of human erythrocyte membrane proteins, prestained with *o*-phthalaldehyde were applied and electrophoresed at 45 mA/45 V for 60 h at 16 °C. The elution buffer was then pumped through the elution chamber at 35 ml/h. The eluted proteins were concentrated with an Amicon column eluate concentrator (Model CEC 1), using a Diaflo PM 10 membrane for low molecular weight proteins and a Diaflo XM 50 membrane for the high molecular weight proteins. The absorption of the elution buffer was monitored at 280 nm (absorbance monitor model UA-4 with a dual beam, dual flow cell optical unit model Type 4, ISCO).

N-terminal amino acid analyses. Isolated and concentrated preparations of dansylated membrane proteins were precipitated and hydrolysed for 4 and 16 h as in ref. 19. Both hydrolysates were lyophilized over NaOH and extracted twice with water-saturated ethyl acetate. The extracts were dried and dissolved in pyridine/water (1 : 1, v/v). The soluble fractions (1a and 1b) were retained for chromatography. The residues, which contain dansylated N-terminal histidine and arginine were dissolved in pyridine/water. This solution usually contained large amounts of salts and dansyl hydroxide. It was chromatographed in one dimension on 5 × 5 cm thin-layer micropolyamide plates (Schleicher and Schüll, Kassel) using water/90 % formic acid (200 : 3, v/v) as solvent in order to remove these substances. The fluorescent spots at the solvent front were scraped from the plate and extracted twice with pyridine/water (sample

TABLE I

Protein band	Glycoprotein band	Apparent molecular weight	Percentage of total staining		Comments
			Coomassie brilliant blue*	Periodic acid-Schiff stain**	
1		310 000	15.4 %		
2		290 000	13.0 %		"Spectrin" bands
2.1		265 000	4.9 %		
2.2		248 000	2.2 %		
2.3		230 000	0.6 %		Inconstant
2.4		215 000	1.2 %		Region
2.5		190 000	0.5 %		
2.6		170 000	0.3 %		
2.7		148 000	0.8 %		
	Periodic acid-Schiff stain 1			40.2 %	"Glycophorin"
3		93 000–87 000	29.0 %		
4.A		79 000	4.3 %		
4.B		72 000	4.8 %		
	Periodic acid-Schiff stain 2			11.8 %	
4.1		61 000	3.2 %		
4.2		54 000	2.1 %		
	Periodic acid-Schiff stain 3			38.4 %	
4.3		49 000	2.7 %		
5		42 000	4.5 %		
6		35 000	4.7 %		
	Periodic acid-Schiff stain 4			9.6 %	
7		29 000	2.9 %		
7.1		25 000	1.0 %		
8		21 000	2.0 %		

* According to quantification of Coomassie blue staining.
** According to quantification of periodic acid-Schiff staining.

2a and 2b). Two-dimensional separation of dansylated amino acids of the four samples (1a, 1b, 2a, 2b) was done as in refs 20 and 25, using four successive solvent systems.

RESULTS

(1) *Results of the preparative electrophoresis*

Fig. 2 shows the normal protein and glycoprotein pattern of the human erythrocyte membrane in a preparative gel slab (A) and an analytical gel (B). The corresponding molecular weights, the percent distribution and nomenclature of the individual bands used below are given in Table I. The bands in the region between 2.1 and 3 are variable in proportion. The optimal gel concentration giving a good separation of the proteins, a consistence suitable for slicing and an optimal recovery upon extraction is 5 % total acrylamide with 2.5 % cross-linking. Other gel concentrations may be suitable for specialized separation problems.

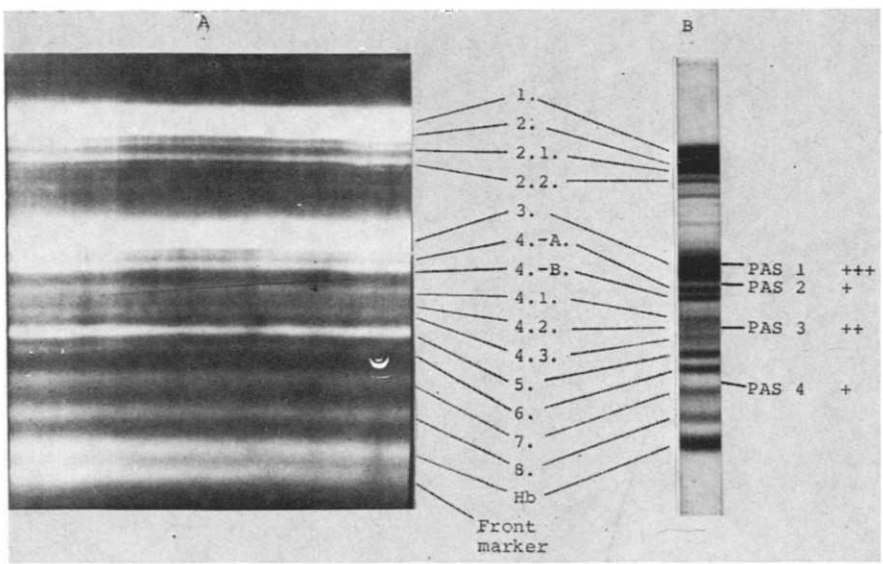

Fig. 2. Photograph of the fluorescence distribution in preparative gel slab after separation of membrane proteins prestained with *o*-phthalaldehyde (A) and an analytical gel of the same material stained with Coomassie blue (B). Protein numbering as in ref. 1 and Table I. The staining of the periodate-Schiff stain (PAS)-positive bands is marked by (+): ±, slight staining; ++, easily visible staining; +++, strong staining. The reproduction of the fluorescent protein pattern does not show the separation of bands 1 and 2 in the gel slab, although this is easily detectable on the original slab. This is due to the fact that one cannot resolve closely spaced highly fluorescent and weakly fluorescent bands using one photograph.

The distribution of fluorescent staining (*o*-phthalaldehyde) on the 5% preparative gel slab (A) clearly corresponds precisely to that on the analytical gel and is such as to allow one to cut out even bands migrating closely together. The separation of the samples that had been prestained with fluorescent dyes corresponds exactly to

that of proteins that had not been prestained. Analytical re-electrophoresis of the individual bands (Fig. 3) demonstrates the separation power of this preparative procedure.

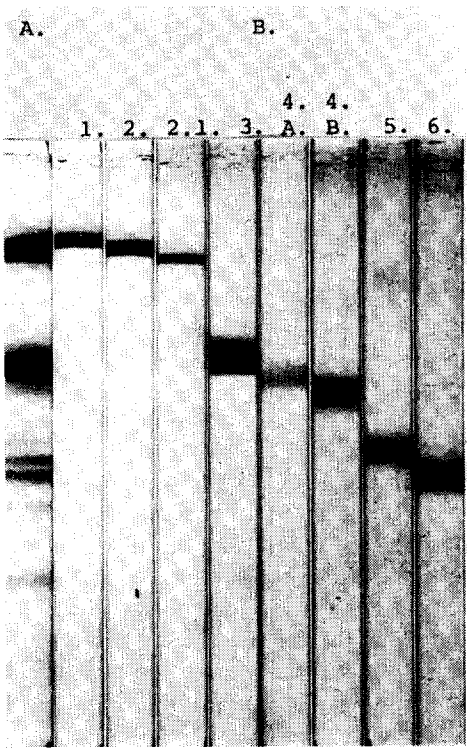

Fig. 3. Re-electrophoresis of the isolated bands 1–6. (B) together with an aliquot of the original material (A) applied to the preparative gel: this sample was stored at +4 °C during the period of isolation.

In order to obtain reproducible results for the high molecular weight bands 1 and 2, it is necessary to adhere to the stated extraction times, extraction temperatures and homogenization procedure; otherwise, bands of lower molecular weight appear (about 100 000–200 000). These new bands lie in the "inconstant" range between 2.1 and 3. Proteolysis of bands 1 and 2 during the isolation procedure can be excluded, because unelectrophoresed samples yield stable patterns for at least 1 week.

When the specified conditions of extraction are not maintained, bands 1 and 2 may reappear after re-electrophoresis of bands 2.1 and 2.2, although contamination of bands 2.1 and 2.2 by bands 1 and 2 can be excluded with certainty by re-electrophoresis of the homogenate directly after the homogenization procedure.

The recovery of the individual bands depends on the acrylamide concentration, the molecular weight of the proteins to be eluted, extraction time and extraction volume. Using two successive extractions with two times ten volumes of the gel homogenate and using 5 % gels we find the following yields: band 1, 43–48 %; band 3, 72–78 %; band 6, 87–93 %.

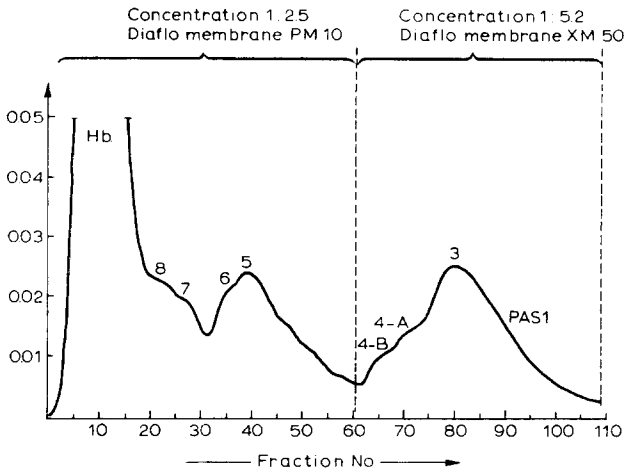

Fig. 4. Typical elution profile obtained by "continuous" preparative electrophoresis. PAS = periodate-Schiff stain.

Preparative sodium dodecylsulfate/polyacrylamide gel electrophoresis with continuous elution. We have succeeded in getting an elution profile up to band 3 (Fig. 4). Longer runs, designed to elute bands 1 and 2 were unsuccessful because air bubbles invariably accumulate beneath the lower surface of the elution chamber. This distorts the electrical field and leads to a deformation of the migrating bands. Also, during prolonged electrophoresis, the gel column swells into the elution chamber. Then small gel particles tear off into the elution buffer and block the column eluate concentrator or the detection unit. The elution profile also shows incomplete separation of adjacent bands. Thus, we do not consider this method suitable for preparative isolation of membrane proteins.

(2) *N-terminal amino acid analyses*

N-terminal amino acids were determined for the major components (i.e. bands 1, 2, 3, 4A, 4B, 5 and 6), which together comprise approx. 75 % of the membrane proteins. The proteins were dansylated before electrophoresis. The dansylated bands were cut from the gels after electrophoresis and hydrolysed after extraction.

TABLE II

CONSTANT AND VARIABLE N-TERMINAL AMINO ACIDS OF THE MAJOR MEMBRANE PROTEINS

Band	Constant	Variable
1	Pro-Val-Leu-Phe	Gly-Thr-Met
2	Pro-Val-Leu-Phe	Gly-Thr-Met
3	Pro-Val-Leu-Phe-Met	Gly-Thr-Glu-Ala
4A	Leu-Glu-Ile	Phe
4B	Leu-Val-Glu	Ala-Gly
5	Pro-Val-Leu-Phe-Ile-Gly	Glu-Met
6	Glu-Gly-Leu	Lys

This excludes artefacts due to proteolysis. Analytic re-electrophoresis of the extracts shows pure bands 1, 2, 3, 4A, 4B, 5 and 6. All four bands have multiple N-termini. In all cases, one must distinguish between constantly appearing N-termini and variable N-termini according to different blood samples. Bands 1 and 2 share constant as well as variable N-termini in all cases analysed. Band 5 shows all the constant N-termini of bands 1 and 2, as well as other constant and variable N-termini. Table II summarises the data for the bands examined.

DISCUSSION

Preparative system

Our method of preparative isolation of membrane proteins on large gel slabs constitutes a direct extension to large scale of analytical sodium dodecylsulfate/polyacrylamide gel electrophoresis which preserves the resolution power of the analytical method. This procedure has the following advantages over preparative system relying on continuous elution.

(1) The simple design of the apparatus allows rapid and easy operation and maintenance.

(2) In this procedure, identical or different samples can be run concurrently.

(3) The total gel surface area of 50 cm^2 is much greater than that offered by the commercially available preparative polyacrylamide gel electrophoresis apparatuses (between 10 and 20 cm^2) with continuous elution. This allows separation of correlation capacity considerably higher.

(4) The time necessary for electrophoresis and further processing (extraction and concentration) is about 36 h, less than half that required for electrophoresis alone using the continuous approach.

(5) In contrast with "continuous" systems which require gels of 2–4 cm thickness, our apparatus uses gel slabs only 0.5 cm thick, i.e. as in analytical systems. This yields excellent heat dissipation and provides high resolution without requiring a cooling system.

(6) Our prestaining procedures yield fluorescent protein complexes that migrate as do the unstained proteins, but allow much greater detection sensitivity than is achieved in continuous systems. This and avoidance of elution artefacts permits the same level or resolution as in analytical electrophoresis.

(7) The proteins are obtained in high concentrations, whereas in continuous systems they are highly diluted, creating problems in detection methods and requiring time-consuming concentration. A similar method using thick gel rods (2.5 cm in diameter) was used by Langdon [26], who also separated dansylated erythrocyte membrane proteins. His device, however, has a much lower separation capacity (4 mg protein) and he does not quantitatively extract the proteins from the gel.

Our system has the following disadvantages. (1) Prestaining with covalently reacting fluorescent dyes yields modified proteins (free NH_2-groups: danslylation, fluorescamine and *o*-phthalaldehyde and/or the SH groups: *o*-phthalaldehyde). This might be disadvantageous for the reconstitution of biological activity. However, the high sensitivity of fluorescence and the identical migration of stained and unstained proteins allows one to bypass this difficulty using mixtures of prestained and unstained

proteins. Denaturation by sodium dodecylsulfate is a more serious problem, but this is inherent in the basic separation principle*.

High molecular weight bands

The degradation of the so-called "spectrin"-bands (bands 1 and 2) as well as the occasional aggregation bands 2.1 and 2.2 deserve comment. While reproducible conditions were found for isolation without degradation or aggregation, it was not possible to find consistent conditions for the generation of these effects. However, we can state that both phenomena tend to occur with extended extraction time (above 8 h), high extraction temperatures (above 15 °C) and large extraction volume (protein concentration below 0.04 mg/ml). Proteolysis influence can be ruled out for the following reasons:

(1) Once samples are boiled after solubilization, they reveal no evidence of proteolysis even after weeks of storage at 4 °C or room temperature.

(2) Two-dimensional polyacrylamide electrophoresis of membrane proteins separated in the first dimension using sodium dodecylsulfate, after a weak of storage at room temperature, shows no degradation of bands 1 and 2 upon electrophoresis in the second dimension.

We do not know what process leads to the degradation of bands 1 and 2. Unstable isopeptides might be involved [27]. It is also possible that bands 1 and 2 consist of subunits which are dissociated by sodium dodecylsulfate only after these proteins are separated and extracted from the gel (higher dilution of the proteins). However, the preparation of non-degraded proteins 1 and 2 by the method given here allows further investigation on the nature of the processes which are involved in this phenomenon.

The aggregation of the bands in the inconstant range (2.1, 2.2) may represent the reverse phenomenon. It cannot be prevented by incorporation of disulfide splitting reagents into the polyacrylamide gel. Aggregation of low molecular weight membrane proteins into high molecular weight proteins have been reported by others [28–31].

Peptide heterogeneity

Two-dimensional separation of membrane proteins [32] and crossed immune-electrophoresis of membrane proteins [33] show that proteins which migrate as individual bands in sodium dodecylsulfate/polyacrylamide gel electrophoresis represent heterogeneous mixtures. Our N-terminal amino acid analyses on bands 1, 2, 3, 4A, 4B, 5 and 6 indicate the same. To avoid proteolytic artefacts or degradation of the high molecular bands during either the preparative procedure or dansylation, dansylation was performed prior to electrophoresis to remove free dansyl chloride. Nevertheless, all of the proteins examined showed multiple N-termini. Some of them occur constantly (constant N-termini), others vary with the blood donor (variable N-termini). The latter may reflect genetic differences between the various blood donors, as

* Staining of a "reference strip" as a means for detecting bands in gel slabs is not suitable for precise localization. Sensitive staining takes too long and the protein bands in preparative gel slabs diffuse; quick staining is not sensitive enough. Also, in the case of dense band pattern, mutual contamination among neighbouring proteins cannot be avoided because of slight band irregularities not apparent in the reference strip. This is more so because the gels swell during staining and the stained strips can no longer be exactly compared with the unstained segment.

has been demonstrated by Segrest et al. [14] for the major membrane glycoprotein. Bands 1 and 2 always yield the same constant and inconstant N-termini. These results are in accord with those of Maddy and Dunn [30] for the "spectrin" bands from ox erythrocytes. These workers also found several N-termini. On the other hand, our data do not fit the report by Rosenberg and Guidotti [34] that "spectrin" has only one N-terminus. This might be due to the fact that the method used by these authors is less sensitive than dansylation. In the case of protein 6, we find glutamic acid, leucine and glycine as constantly appearing N-terminal amino acids: glycine is the N-terminal of erythrocyte D-glyceraldehyde-3-phosphate dehydrogenase [35].

Our data are in accordance with those of Langdon [26] in that we find multiple N-termini for all major erythrocyte membrane proteins separable by polyacrylamide gel electrophoresis in sodium dodecylsulfate. However, we do not find the identical N-termini reported by Langdon [26]. The probable reason for the discrepancies are as follows. (a) Langdon's separation pattern [Fig. 6 of ref. 26] indicates extensive degradation of bands 1 and 2 with appearance of new components between bands 2 and 3 as well as between 4B and 5: if this degradation occurred by proteolysis prior to dansylation, new N-termini would appear. (b) Langdon [26] does not use three-dimensional separation of dansylated amino acids in his analyses of the separated components. Therefore, he cannot have separated serine from threonine and glutamic acid from aspartic acid as claimed by him [Fig. 6 of ref. 26]. (c) Proline and valine account for 22 % of the total N-termini, but only valine is recovered in the separated proteins. (d) The N-terminal analyses of whole ghosts do not fit those in ref. 36: these differences might again reflect proteolysis.

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