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Isolation of the Fundamental Polypeptide Subunits of Biological Membranes

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Abstract. A group of peptides (some or all of them glycopeptides), of molecular weight about 5000, has been shown to be a major fraction of biological membranes. These "miniproteins" have been prepared from membranes of human and bovine red blood cells, from purified bovine liver mitochondria, and from the rhodopsin-containing membranes of the outer segments of bovine retinal rods. While the miniproteins were found in large amounts in each case, the other protein components differed markedly depending on the function of the membrane studied. This fact was particularly clear in the case of the rod membranes where the only major proteins detected were the miniproteins and rhodopsin. The large size of the miniprotein fraction in each of these membranes leads us to propose that the miniproteins play a fundamental role in the several functions which occur as common denominators in biological membranes.

Cellular membranes serve as active interfaces that govern interactions between cells and their environment and compartmentalize functions within the cell. The intermolecular organization of structural, enzymatic, and receptor molecules in particular membranes determines their specialized functions. In order to further characterize the molecular components responsible for these functions, techniques have been developed for the isolation and structural analysis of proteins found in several distinctly different biological membranes. Red blood cells were studied because they represent a naturally occurring population of cells which are devoid of all membranes except the surface membrane. Such membranes are easily prepared in gram quantities[1] and provide an excellent model system. The membranes of the subcellular organelle, the mitochondrion, were also chosen for study for similar reasons. Since the long-term goal of this project is to examine the highly specific receptor molecules of cellular membranes,[2] the rhodopsin-containing membrane derived from the outer segments of bovine retinal rods was also examined with the hope that it would serve as a simple, unifunctional model of a receptor-membrane system.

This report includes a comparison of human and bovine red blood cell membranes, bovine mitochondria, and bovine rods. While a number of protein fractions have been isolated, our primary purpose in this report is to present preliminary data relating to the miniproteins. We have not made a detailed study of the nature of the aggregation phenomenon which has led to the formation of

"structural protein" as recorded in the literature,[3,4] but we have been able to reproduce some of the preparations that have been described in the past. Some of these results are included for purposes of comparison.

Materials and Methods. Preparation of membranes: Mitochondria and mitochondrial membranes from beef liver were prepared by the method of Parsons *et al.*[5] In the limited studies of delipidated mitochondrial proteins, the membranes were treated with chloroform:methanol according to the method of Rouser and Fleisher.[4] Structural protein was prepared and purified according to the method of Allmann *et al.*[7] Bovine erythrocyte stroma were prepared according to Dodge *et al.*[1] The outer segments of rods were prepared from beef retinas by the procedure of McConnell.[8] The purity of the rod preparations was assessed by electron microscopy. If not used immediately, the membrane preparations were stored in 0.28 M sucrose and 5% glycerol and frozen at −196°C in liquid nitrogen.

Gel electrophoresis: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS)[9] was used. Gels were also examined with a Gilford scanning ultraviolet spectrophotometer at 260 and 280 nm.

Fractionation methods: The gel filtration samples were whole membranes dissolved in 5% SDS–5% β-mercaptoethanol, 0.01 M γ-amino butyric acid, and 0.05 M Tris, pH 8.5. Separation was carried out using Sepharose *6B* (Pharmacia) or Biogel P-100 (BioRad) in 0.5% SDS. The SDS detergent was removed by means of chromatography with formic acid on Amberlite CG-50.

A second fractionation procedure employed chromatography on SE-Sephadex C-50 (Pharmacia). The membrane sample was first dissolved in 100% formic acid at 4°C and then brought to 25% formic acid, 10 M urea, at pH 2.5, and applied to a column equilibrated with this buffer. A sodium chloride gradient (0–0.6 M) was used to elute the proteins. The urea and formic acid were removed after dilution by ultrafiltration on Diaflo UM-2 membranes (Amicon Corp.). The protein fractions were stored frozen.

Peptide maps: Proteins were digested overnight in 0.2 M ammonium bicarbonate (10 mg protein/ml) using subtilisin (0.03 mg enzyme/mg protein) (Nutritional Biochemicals Corp.). The digests were desalted and delipidated with a Dowex 50W-X2 resin and separated on paper by high voltage electrophoresis in 4% formic acid at 7800 V[10] (Gilson model DW Electrophorator) and by paper chromatography.

Removal of carbohydrate: Carbohydrate was removed by periodate oxidation followed by reduction with borohydride and mild hydrolysis.[11,12]

Results. Gel electrophoresis: The molecular weight distribution of proteins from three different membrane systems is shown in Figure 1.

Mitochondrial structural protein and delipidated mitochondrial membranes, along with standards, are shown in Figure 1*a;* electrophoresis was run according to the standard procedure.[9] Electrophoresis of proteins from three different membrane systems, and purified miniprotein are shown in Figure 1*b*. In the experiment of Figure 1*b*, the electrophoresis time was shortened and entire membranes were dissolved in SDS–β-mercaptoethanol without prior delipidation or precipitation. Miniprotein and insulin run faster than marker dye and are not normally seen unless the period of electrophoresis is shortened. All three membranes share a major miniprotein component which is present at about the same position as insulin (mol wt 5700) while the larger protein components seem to differ in each membrane. Furthermore, apart from rhodopsin, the miniprotein is the only prominent polypeptide component seen in the rod membrane.

We found that several factors were important in obtaining the electrophoretic pattern shown in Figure 1*b*. The membrane samples were either fresh or stored frozen at −196°C in sucrose and glycerol. Lyophilized or delipidated mem-

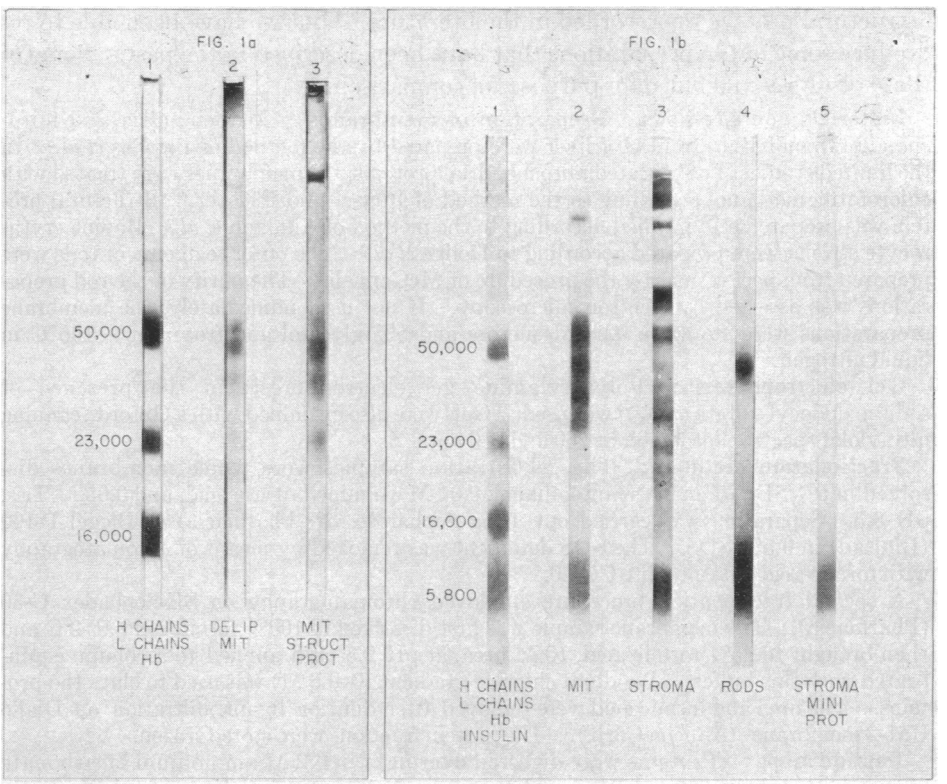

FIG. 1.—Molecular weight analysis of proteins and peptides in various membrane fractions. (*a*) 8 hr gel electrophoresis at 50 V. (1) Standard mixture of heavy and light chains of γ-globulin and hemoglobin; (2) delipidated membranes of bovine liver mitochondria; (3) purified structural protein prepared from bovine liver mitochondria. (*b*) 6 hr gel electrophoresis at 40 V. (1) Standard mixture of heavy and light chains of γ-globulin, hemoglobin, and insulin; (2) bovine liver mitochondria; (3) bovine red blood cell stroma; (4) bovine retinal rods; (5) miniprotein prepared from bovine red blood cell stroma. Coomassie blue stain was used to visualize proteins.

brane samples, if soluble at all, gave a pattern after electrophoresis showing mainly high molecular weight bands on the gels, as seen in Figure 1*a*. It was also necessary to wash the gels thoroughly with 20% sulfosalicylic acid after electrophoresis to remove all SDS and to obtain proper fixation of proteins and peptides. Further, we found that the appearance of the miniprotein monomers on the gels required the use of an adequate concentration of SDS when initially dissolving the samples. With these membranes, 2.5% (w/v) SDS–2.5% β-mercaptoethanol (v/v) solutions were dialyzed into the standard application buffer. Adequate dialysis is important, since high concentrations of SDS or lipid in the sample create an artifact band. Gels were also scanned at 260 and 280 nm to confirm the presence of polypeptide chains in the areas that were later stained.

Column fractionation: Fractionation of the red cell, mitochondria, or rod outer membranes on a calibrated gel filtration system of Sepharose-SDS yielded a miniprotein peak at a position corresponding to a molecular weight well below

10,000 (Fig. 2). This fraction contained a small portion of the total amount of protein in the sample. Gel electrophoresis showed, however, that the higher molecular weight fractions also had miniprotein present as reversible polymers of the subunit. While this fractionation procedure gave a low yield of miniprotein in the form of monomers, it served as one measure of the size of the monomers. The molecular weight distribution shifted to higher values when the membranes were delipidated or lyophilized and the aggregates could not be dissociated by means of the techniques used.

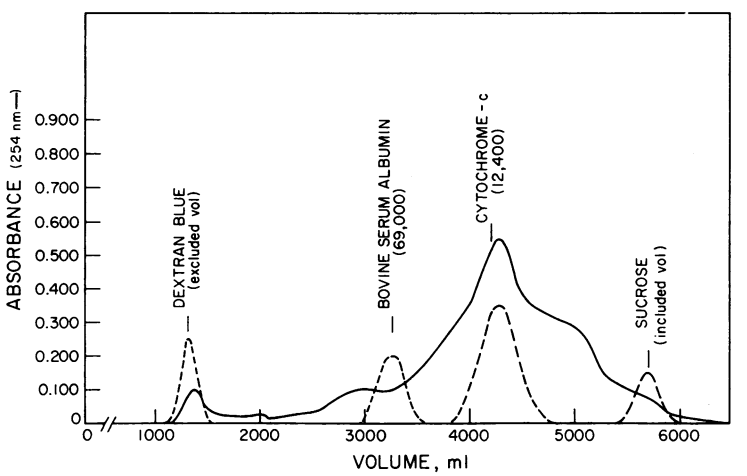

FIG. 2.—Separation of the proteins of bovine liver mitochondria by gel filtration on Sepharose 6B. The buffer used was 0.5% SDS, 0.5% β-mercaptoethanol, and 0.05 M Tris-HCl, pH 8. The column was calibrated using bovine serum albumin and cytochrome *c* as standards. Sucrose was added to the sample as an internal standard.

Chromatography on SE-Sephadex, in the presence of 25% formic acid and 10 M urea, divided the proteins of mitochondria and red cell membranes into two major fractions. The first fraction, appearing in the runoff peak, contained the miniprotein and lipids; the second fraction seemed to contain the remainder of the proteins present in the membranes. The fact that miniprotein was the only protein not bound to the ion exchanger made it possible to use a simple batch method for purification. The sample was mixed with the ion exchanger and the miniprotein was collected from the supernatant. Large quantities of the stromal miniprotein were prepared using this system. More than 50% of the total protein of the red cell membrane was found (by amino acid analysis) in the miniprotein fractions. The appearance of stained gels suggests that retinal rods contain a comparable or larger percentage of miniprotein.

A stromal miniprotein fraction obtained by SE-Sephadex fractionation was freed of carbohydrate and fractionated by gel filtration on Biogel P-100 in SDS to determine the molecular weight of the polypeptide chains alone (Fig. 3). Part of this fraction behaved as low molecular weight material, giving a wide peak between the molecular weight regions 4000–6000, a result in agreement with

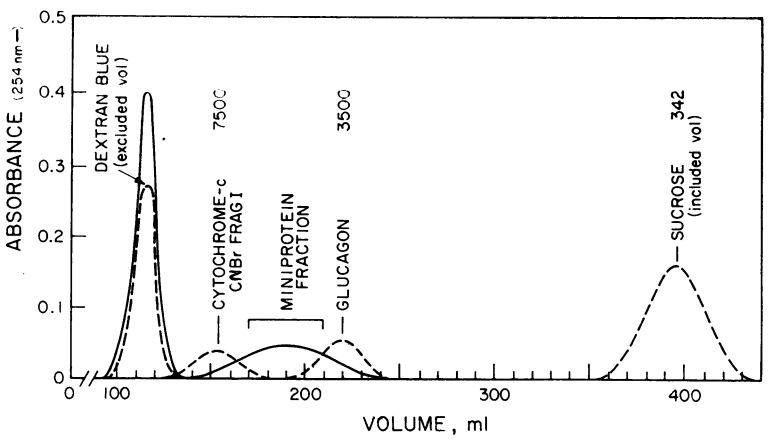

FIG. 3.—Separation of carbohydrate-free miniprotein of bovine red cell stroma by gel filtration on Biogel P-100. The buffer used was 0.5% SDS, 0.1% β-mercaptoethanol, and 0.05 M Tris-HCl, pH 8.0. The column was calibrated using cytochrome *c*, cleaved at a methionine residue with cyanogen bromide (*CNBr fragment I*), and glucagon as standards.

the data from gel electrophoresis in SDS (Fig. 1) and from chromatography on Sepharose *6B* (Fig. 2). This value must, nevertheless, be considered tentative until complete purification and structural analysis of these molecules is accomplished.

Regardless of the purification system used, dialysis was not used for removal of solvents because of the possible loss of peptide through the dialysis tubing. The Diaflo membranes proved satisfactory for removal of urea from the samples.

Characterization of the miniproteins: Peptide maps from subtilisin digests of freshly prepared membranes from whole red cells, as well as purified miniprotein from red cell membranes and mitochondria, are shown in Figure 4. We chose subtilisin for the preparation of peptide maps because it gave reproducible results and digested even the most insoluble protein preparations. Peptide maps of the purified miniprotein fraction seem to have all the peptides that are present in high molar amounts in the peptide map of the whole membrane, indicating that the miniprotein fraction contains the major polypeptide components of the intact membrane. The peptide maps of purified miniproteins from mitochondria and red cell membranes are extremely similar, indicating that the miniproteins from these sources may be products of similar, if not identical, genes. These peptide maps are too complex to result from a single polypeptide sequence. This was shown by comparison of fingerprints of a variety of other peptides and proteins, digested under identical conditions, run as controls. It remains to be seen whether miniproteins from different membranes within the same organism are strictly identical in amino acid sequence.

The amino acid composition of the purified miniprotein fraction from red blood cell stroma resembles the composition of structural protein, previously reported by Criddle *et al.*[3] The amino acid analysis also showed the presence of amino

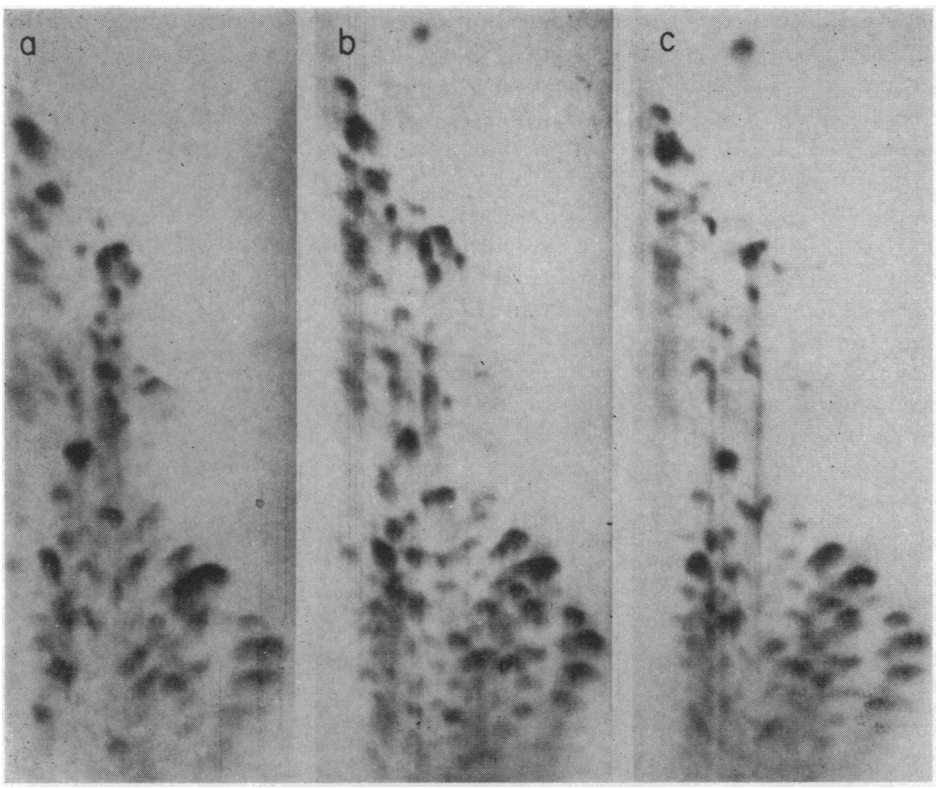

FIG. 4.—Peptide maps of subtilisin digests of: (*a*) freshly prepared bovine red blood cell membranes; (*b*) miniprotein purified from bovine red blood cell membranes; (*c*) miniprotein from bovine liver mitochondria. Chromatography was with butanol–acetic acid–water 27:8:40, horizontal direction, and electrophoresis in formic acid, pH 1.7, at 7800 V for 90 min, vertical direction.

sugars. There are differences in the amino sugar composition of red cell and mitochondrial miniprotein preparations. This is compatible with our observation that the antigenic properties of these fractions differed with respect to the blood group antigens (unpublished results).

We were unable to obtain an *N*-terminal group by any method tested, or by degradation of the miniproteins with an automatic sequential degradation instrument.[13] This result indicates that the terminal amino group is chemically blocked or that the miniproteins are cyclic structures. Analyses of new terminal groups that appear after specific cleavage of the miniprotein fraction from red blood cells showed that this fraction is in fact a mixture of several peptides (Ruoslahti *et al.*, in preparation).

Upon electrophoresis the miniprotein band from all three membranes gave a positive periodic acid–Schiff reaction, again indicating that the miniprotein fraction contains glycopeptides.

Discussion. A fraction that contains large glycopeptides, or miniproteins, has been found to represent a major component in each of three different mem-

brane systems: mitochondrial membranes, red blood cell membranes, and the outer segments of the retinal rods. These miniproteins appear as a common denominator of each of the membranes studied; the other proteins differ depending on the function of each membrane. We have shown that more than 50% of the total amino acid content of the red blood cell membrane is in the miniprotein fraction. A considerable proportion of the protein of the retinal rod outer segments is also accounted for by the miniprotein fraction. In fact, the only other polypeptide component detected in major amounts in these rod membranes had the molecular weight of the visual receptor protein, rhodopsin. This observation is of particular importance in relation to the proposed function of the miniproteins.

There seem to be several reasons why the miniproteins have escaped notice for the many years in which work on membrane proteins has been carried out in numerous laboratories. Such frequently used techniques as delipidation and lyophilization[3,4,14,15] appear to cause aggregation of the membrane proteins. Once this type of aggregation has occurred, various combinations of the most powerful dissociating methods known to us failed to disaggregate these proteins and peptides to their original states. We have reproduced the preparation of structural protein reported in the earlier literature.[3,4] Gel electrophoresis, together with peptide mapping after subtilisin digestion, showed that structural protein contains a very high molecular weight aggregate of miniprotein, together with some of the other protein components of membranes. No similar aggregates are found in parallel analyses of fresh membranes. Dialysis, too, leads to loss of peptides with molecular weights lower than 10,000. This would eliminate most of the miniprotein which is not present in an aggregate. Furthermore, peptides diffuse out of the gels used in SDS electrophoresis during fixation and destaining unless they are carefully precipitated with sulfosalicylic acid. The high electrophoretic mobility of these peptides causes them to move more rapidly than the customary tracking dye, thus leading to loss of peptides unless the normal time of electrophoretic separation is shortened.

The abundance and similarity of the miniprotein fraction in different membranes suggests an important role for these glycopeptide subunits of biological membranes. This work also shows that no higher molecular weight protein exists as a major component in common.

Evidence is lacking concerning the detailed structure and mode of assembly of natural biological membranes. We believe, however, that the ability of the miniproteins to undergo reversible polymerization *in vitro* may reflect a self-assembling system which is responsible for the formation and growth of the biological membranes *in vivo*. A honeycomb type of structure might easily be formed by such a mechanism, with lipid layers filling in the openings. Recent x-ray crystallographic data indicate that mycoplasma membranes contain a 10.4 Å repeating unit caused by the presence of protein.[16] The 4.2 Å repeating unit suggests the presence of the stretched hydrocarbon chains of a lipid bilayer. Such results seem compatible with the honeycomb type of structure proposed here.

According to this model the membranes of retinal rod outer segments consist

primarily of such a honeycomb lattice with a superimposed laminate of the visual pigment, rhodopsin. We feel that the miniprotein lattice may undergo a coordinated configurational change[17] as a result of absorption of a photon by rhodopsin. A transient alteration in ion permeability[18] is assumed to occur as an integral part of the energy amplification process.

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Abbreviation: SDS, sodium dodecyl sulfate.

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