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The solubilization and fractionation of human erythrocyte membrane proteins

R.L. JULIANO

Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, N.Y. (U.S.A.)

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SUMMARY

Hexafluoroacetone and 3% sodium laurylsulfate completely solubilized all classes of erythrocyte membrane proteins. Other solvents, including 6 M guanidine hydrochloride, were less effective in this respect. The erythrocyte membrane proteins were dissolved in hexafluoroacetone, dialysed into acidic 8 M urea and fractionated on an agarose column.

Many attempts have been made to fractionate, in preparative quantities, the proteins of the erythrocyte membrane (see refs 1 and 2 for references). The most successful approach thus far has been to dissolve the membrane proteins in a sodium laurylsulfate-containing solution and to fractionate them on Sephadex columns[3,4]. Unfortunately, sodium laurylsulfate binds strongly to proteins, and thus the fractions prepared in this manner cannot be used for biophysical studies such as the interaction of membrane proteins with artificial phospholipid membranes[5]. Lenard[6] has shown that most protein bound sodium laurylsulfate may be removed by ion exchange. However, even traces of sodium laurylsulfate may be sufficient to perturb sensitive lipid–protein interactions.

Trayer *et al.*[7] have fractionated reduced erythrocyte membrane proteins on agarose columns using 6 M guanidine hydrochloride as a solvent. However, Maddy and Kelly[8] have pointed out that there seem to be intermolecular forces between membrane proteins and lipids which are resistant to the solvating effects of 6 M guanidine hydrochloride. This would render guanidine hydrochloride an inadequate solvent for some types of membrane proteins.

Previous work has indicated that the erythrocyte membrane proteins may be grouped into several classes as a function of the solvents required to extract the proteins from the membrane[1]. Approximately 60–80% of total membrane protein may be extracted by successive use of aqueous solvents such as 1 M NaCl, 1 mM EDTA at pH 9, and 8 M urea. This hydrophilic fraction contains a pair of high ($2 \cdot 10^5$) molecular weight polypeptides

which account for about 20% of total membrane protein and which are probably identical with the protein called "spectrin" originally isolated by Marchesi *et al.*[1,9,10]. The hydrophilic fraction also contains several smaller proteins. The remaining membrane protein, which is tightly bound to membrane lipid, consists largely of a single polypeptide species of $1 \cdot 10^5$ molecular weight[11]. This species and its associated lipid have been named "PIV".(The PIV fraction may also contain some of the major sialoprotein of the erythrocyte[12].) Thus while much of the protein of the erythrocyte membrane can be dissolved in aqueous solvents, a substantial portion (30%) seems to be lipophilic, and is resistant to solubilization under these conditions.

The present report concerns the solubility of de-lipidated proteins from the intact erythrocyte membrane and from the PIV fraction. It also describes a simple method for fractionation of all of the erythrocyte membrane proteins, without recourse to detergents.

Ghosts (erythrocyte membranes) and PIV were prepared as previously described[1,11] Lipids were removed by an ethanol–ether extraction procedure adapted from Rosenberg and Guidotti[11,3]. Solubility studies were performed by incubating 2 mg of dried de-lipidated ghost protein or PIV protein with 2 ml of solvent for 4 days at room temperature in a closed tube. The tubes were then centrifuged, and the supernatant was removed. The absorbance of the supernatant in the range 350–240 nm was measured. The degree of solubilization achieved by 3% sodium laurylsulfate was taken as 100%[3], and the solubilization achieved by other solvents was computed by taking the ratio of the absorbance peak height to that of the sodium laurylsulfate sample.

Total de-lipidated ghost protein was fractionated in the following manner. About 20–40 mg of protein was dissolved overnight in 2 ml of hexafluoroacetone sesquihydrate (Aldrich Chemical Co.) whose pH had been adjusted to 3 with triethylamine. This sample was then dialysed for 24 h *versus* 8 M urea *plus* 50 mM formic acid (pH 3). The sample was then applied to a 32 cm x 1.6 cm column packed with agarose (Biogel, Bio-Rad Laboratories) which had been pre-equilibrated with acidified 8 M urea solution. The column was eluted with the same solvent and fractions were collected and analysed for protein by the Folin reaction[13]. The column was calibrated with known marker proteins (cytochrome *c*, lactoglobulin, ovalbumin and albumin) whose elution volumes were directly proportional to the logarithm of molecular weight. The exclusion limit for the column was approximately 10^6.

Pooled fractions were electrophoresed in a sodium laurylsulfate-containing solvent as previously described[9]. Gels were stained for carbohydrate and counter stained for protein using methods adapted from Fairbanks *et al.*[14]. The gels were scanned in a Gilford densitometer.

Table I shows that sodium laurylsulfate and hexafluoroacetone completely solvated ghost proteins. Acidic urea and guanidine hydrochloride solutions were less effective, each solubilizing about 60% of total membrane protein. Buffered salt solution was a poor solvent. The protein of the PIV fraction could also be solubilized by sodium laurylsulfate and hexafluoroacetone, but was almost completely resistant to solubilization by acidic urea, guanidine hydrochloride or buffer. The supernatants of the sodium laurylsulfate and hexafluoroacetone samples contained protein which was in solution rather than in

TABLE I

THE SOLUBILITY OF ERYTHROCYTE MEMBRANE PROTEINS

Solvent	*% Solubilization*★	
	Total ghost protein	*PIV protein*
3% sodium laurylsulfate	100	100
Hexafluoroacetone (pH 3)	109	98
6 M guanidine hydrochloride	58	12
8 M urea (pH 3)	64	16
Phosphate-buffered saline	19	11

★The value given is the mean of duplicates. The duplicates agreed within 10%.

suspension since these samples did not scatter light appreciably and had distinct absorbance maxima in the 280–270-nm region (see Fig. 1). The above results suggest that attempts to dissolve ghosts with aqueous solvents containing denaturing agents result in fractionation of the total membrane proteins rather than solubilization of a cross section of these proteins. The more hydrophilic proteins such as "spectrin" are solubilized, while the lipophilic proteins (such as the PIV protein) are resistant to solvation.

Hexafluoroacetone seems to be a useful, non-detergent, medium for the solubilization of membrane proteins. The use of polar organic liquids in the solubilization of membrane proteins was pioneered by Zahler and Wallach[15]. Mitochondrial membrane

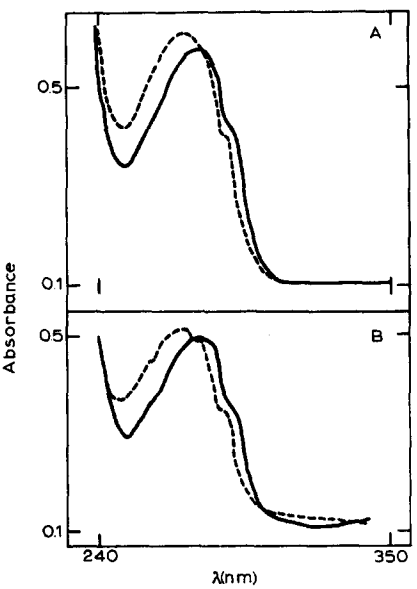

Fig. 1. Absorbance of erythrocyte membrane proteins. A. Total ghost protein dissolved in 3% sodium laurylsulfate (——), or hexafluoroacetone (- - -). B. Protein of the PIV fraction dissolved in 3% sodium laurylsulfate (——), or hexafluoroacetone (- - -).

proteins have been fractionated on methylated Sephadex columns using acidified chloroform–methanol as a solvent[16]. Undiluted hexafluoroacetone will not swell unmodified column packing materials such as Sephadex or Biogel. A 50:50 mixture of hexafluoroacetone and water will swell Sephadex, and this solvent has been used in the fractionation of certain peptides[17]. Unfortunately, a solution of ghost proteins in hexafluoroacetone will be precipitated upon dilution with water. Hexafluoroacetone may prove to be a useful solvent for the fractionation of membrane proteins on novel chromatographic supports such as methylated Sephadex or glass beads of controlled porosity[18]. However, these materials are not readily available at present.

An alternative approach is to use a polar organic solvent to solvate membrane proteins which may then be dialysed into an aqueous medium and fractionated by more conventional techniques. This approach was originally suggested by Rosenberg and Guidotti[3], but no results were reported. We have achieved a partial fractionation of total ghost protein using this approach. The protein was fractionated on an agarose column equilibrated with acidic urea. The result is shown in Fig. 2. About 30% of the protein eluted just behind the void volume and represents aggregated material (see below). The remaining protein eluted as several broad peaks corresponding to molecular weights of $1 \cdot 10^4$–$2 \cdot 10^4$ to $2 \cdot 10^5$–$3 \cdot 10^5$.

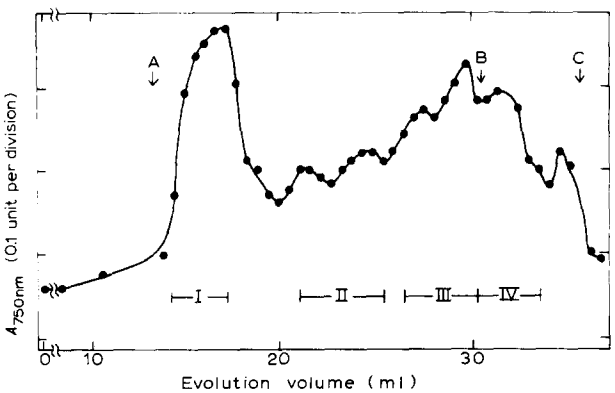

Fig. 2. Elution profile of erythrocyte membrane proteins. The column packing was a 50:50 mixture by weight of Biogel A-5M (6% agarose) and A-15M (4% agarose) which had been equilibrated with 8 M urea (pH 3). The molecular weight markers are (A) blue dextran 2000, (B) albumin, (C) cytochrome *c*.

The column fractions were pooled into four (I–IV) samples as indicated in Fig. 2. These samples were analysed by sodium laurylsulfate gel electrophoresis as described above. Scans of gels containing Samples I–IV and also whole ghosts are seen in Fig. 3. Gels of ghosts stained for protein revealed two major peaks, X ($2 \cdot 10^5$ molecular weight) which is "spectrin" and Y ($1 \cdot 10^5$ molecular weight) which is the PIV protein[9,11]; in addition there were numerous minor peaks of lower molecular weight and some aggregated material at the top of the gel. Carbohydrate staining revealed a peak (presumably the ghost sialoprotein) in the region of Peak Y. However, the sialoprotein is probably not identical to Y[12,14]. All of

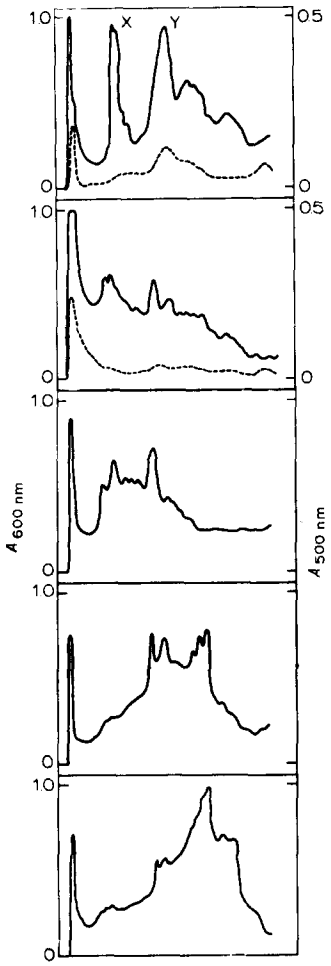

Fig. 3. Gel patterns of erythrocyte membrane protein fractions. Samples containing about 20 μg of protein were electrophoresed on 6% gels in a solvent which contained 1% sodium laurylsulfate and a reducing agent. The gels were run at 8 mA/tube for 45 min at room temperature. Gels were stained and scanned as described in the text. Gels stained for carbohydrate were scanned at 500 nm (- - -) and then restained for protein and scanned at 600 nm (——).

the components of the intact ghost seem to have been recovered (at least partially) in the various column fractions. Fraction I (void-volume fraction) was apparently composed of an aggregate of Proteins X, Y and smaller components. The aggregated material was only partly dissociated by the sodium dodecylsulfate-containing electrophoresis solvent and much remained at the top of the gel. Fraction I was the only fraction which contained appreciable carbohydrate as judged by the staining reaction, and this was associated with the aggregated material. Fractions II to IV contained ghost proteins of decreasing molecular size including Proteins X and Y. Thus a fractionation according to size was achieved by the technique of gel filtration in acidic urea. The results suggest that the membrane sialoprotein

may be more susceptible to aggregation than the other proteins, but this is yet to be documented.

The procedure described above may become a useful addition to the repertoire of techniques available for dealing with membrane proteins, and should complement the technique of fractionation in sodium laurylsulfate. The sodium laurylsulfate technique, which is simple, can provide fractions for chemical analyses. The procedure described here may be useful in obtaining fractions for biophysical studies where the presence of detergent is a liability.

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