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ISOLATION OF PHOSPHOLIPASE A_2 FROM SHEEP ERYTHROCYTE MEMBRANES IN THE PRESENCE OF DETERGENTS

RUTH M. KRAMER *, CLAUDE WÜTHRICH, CHRISTINE BOLLIER, PETER R. ALLEGRINI and PETER ZAHLER **

Department of Biochemistry, University of Bern, Bern (Switzerland)

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Summary

Isolation of phospholipase A_2 (EC 3.1.1.4) from sheep erythrocyte membranes was carried out by a combination of (1) extraction of membranes at low ionic strength, (2) solubilization of extracted membranes with sodium dodecyl sulfate, (3) replacement of dodecyl sulfate with cholate by means of gel exclusion chromatography and (4) affinity chromatography on dialkyl-phosphatidylcholine-Sepharose in the presence of cholate. The phospholipase was prepared with good yield and purified to near homogeneity, as judged by sodium dodecyl sulfate gel electrophoresis. The protein is a minor component of the sheep erythrocyte membrane and has an apparent molecular weight of 18 500.

Introduction

Red cell membranes from sheep contain a phospholipase A_2 (EC 3.1.1.4) with the following characteristic properties: marked preference for phosphatidylcholine, specificity for the fatty acid at the 2-position, requirement for Ca^{2+}, alkaline pH optimum, activation by various detergents and stability against denaturating agents [1]. The enzyme is present in ruminant erythrocytes and is thought to play a role in maintaining the very low phosphatidylcholine content of the ruminant red cell membrane [2].

Soluble phospholipase A from different sources has been purified by conventional methods [3,4]. These procedures required a number of steps, including ammonium sulfate fractionations and a variety of column chromatographic methods. A new purification technique was recently developed by Rock and Snyder [5], who devised an affinity adsorbent to isolate soluble phospholipase

* Present address: The Biological Laboratories, Harvard University, Cambridge, Mass. 02138, U.S.A.
** To whom reprint requests should be addressed.

A_2 from snake venom, using an alkyl ether analog of lecithin as ligand. Based on the fact that this enzyme required Ca^{2+} for substrate binding [6], adsorption to the column was obtained in the presence of Ca^{2+} and subsequent elution was performed with buffer containing EDTA.

Less information is available concerning the isolation of membrane-bound phospholipases, either of the A_1 or A_2 type [4]. Whereas in recent years membrane-bound phospholipase A_1 has been purified to near homogeneity from microorganisms [7,8], membrane-bound phospholipase A_2, to our knowledge, has only been partially purified from rat liver mitochondria [9].

The erythrocyte membrane contains a variety of protein components [10]. Most of them have been characterized in terms of their arrangement in the membrane [11]. The major polypeptides of the human erythrocyte membrane have been purified and biochemically characterized [12]. Many minor proteins, however, have not yet been investigated in this respect. Phospholipase A_2 from sheep red cell membranes is considered as a minor component [1,2]. The major obstacles for the isolation of this enzyme have been (1) small quantity of enzyme present, (2) difficulties in finding suitable methods for a stepwise purification of this membrane protein and (3) the need for continued presence of detergents to maintain the enzyme in a soluble state. The current investigation presents a procedure for separation of phospholipase A_2 from the bulk of membrane proteins and describes the further purification of this enzyme to near homogeneity by affinity chromatography according to the method of Rock and Snyder [5].

Materials and Methods

Materials. Sheep blood was provided by the municipal slaughterhouse, usually within 1 h of the death of the animal. Sephadex G-75 and AH-Sepharose 4B were obtained from Pharmacia, Uppsala. Molecular weight markers for dodecyl sulfate gel calibration were bovine serum albumin (Calbiochem), pepsin (Fluka), trypsin (Sigma), myoglobin (Sigma) and hemoglobin (Fluka). Triton X-100, deoxycholate, cholate and dodecyl sulfate were purchased from Merck. L-α-Phosphatidylcholine from egg yolk was obtained from Koch-Light (grade I). *Rac*-1-(11-Carboxy) undecyl-2-hexadecylglycero-3-phosphocholine was obtained from R. Berchtold, Biochemical Laboratory, Bern. Phosphatidyl [^{14}C]choline (1.8 Ci/mmol, uniformly labelled, from *Chlorella pyrenoidosa*) and dodecyl [^{35}S]sulfate (10 Ci/mol) were products of New England Nuclear C. Inorganic salts and organic solvents were reagent grade from Fluka, Merck and Sigma.

Assay of phospholipase A_2 activity. Phospholipase activity was determined by incubating 0.3 μmol of phosphatidylcholine (made up to a specific radioactivity of $1.873 \cdot 10^5$ cpm/μmol with phosphatidyl[^{14}C]choline with appropriate amounts of enzyme in the presence of 10 mM glycylglycine (pH 8), 4 mM $CaCl_2$ and 0.5% cholate (w/v) in a total volume of 0.47 ml. After incubation at 37°C for 60 min with shaking, the reaction was stopped by adding 30 μl of 0.2 M EDTA and 2.15 ml of chloroform/methanol (5 : 8, v/v). Lipids were then extracted by the method of Renkonen et al. [13]. The chloroform layer was sampled for total radioactivity. Aliquots were chromatographed on

silica gel HR plates (Merck) with chloroform/methanol/water/acetic acid (14 : 6 : 1': 0.5, v/v) as developing solvent to separate phosphatidylcholine, lysophosphatidylcholine and free fatty acids. The chromatograms were stained with iodine vapour and individual lipid spots scraped off into counting vials. After addition of 5 ml of methanol and 10 ml of a scintillation fluid (toluene containing 7 g of 2-(4'-*tert*-butylphenyl)-5-(4''-biphenylyl-1,3,4)-oxadiazole (Ciba-Geigy) per l) the radioactivity was measured in a Packard Tri-Carb scintillation counter (Model 2450). The percentage of phosphatidyl[^{14}C]choline degraded to lysophosphatidyl[^{14}C]choline was calculated and the phospholipase activity expressed as μmol or nmol of phosphatidylcholine hydrolyzed per h.

Preparation of affinity adsorbent. The alkyl ether anlog of lecithin (*rac*-1-(11-carboxy) undecyl-2-hexadecylglycero-3-phosphocholine) was coupled to AH-Sepharose 4B through the carboxyl group using a modified carbodiimide coupling procedure of Rock and Snyder [5]. Freeze-dried powder of AH-Sepharose 4B (5 g) was swollen and washed as recommended by the supplier to give about 20 ml of gel volume. The ligand (800 μmol) was dissolved in 10 ml tetrahydrofuran/water (pH 7) (1 : 1, v/v). The carbodiimide (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide meto-*p*-toluenesulfonate) (6 mmol) was dissolved in 30 ml tetrahydrofuran/water (pH 4.5) (1 : 1, v/v). The combined ligand and carbodiimide solution was added to 20 ml of packed gel and the pH adjusted to 5.0. The reaction was allowed to proceed for 30 h at room temperature with gently stirring and the pH of the coupling solution controlled (during the reaction the pH of the mixture decreased to 4.5). The gel was washed with 200 ml tetrahydrofuran/water (1 : 1, v/v) followed by 200 ml methanol, 400 ml NaCl (1 M), 800 ml distilled water, 200 ml 10 mM glyclyglycine (pH 8), and then equilibrated and packed in the first column buffer (10 mM glycylclycine (pH 8) containing 0.5% cholate, 10 mM $CaCl_2$, 0.2 M KCl and 0.05% NaN_3). The coupling of the ligand was examined by digesting aliquots (50 μl) of the washed gel with H_2SO_4 and determining inorganic phosphorus according to Barlett [14]. The amount of coupled ligand was 2—3 μmol per ml swollen gel.

Membrane preparation. Fresh sheep blood anticoagulated with acid citrate/dextrose was centrifuged (10 min, 600 × *g*) in a Sorvall GSA rotor. The red cells were washed three times with two volumes of 310 mosM phosphate buffer (pH 8) (10 min, 600 × *g*, at 0—4°C). After each wash the leucocytes, forming the "buffy coat", were removed as thoroughly as possible by aspiration and one-fifth of the original volume of the erythrocytes was sacrified. One flask (450 ml) of blood yielded 80—90 ml of washed erythrocytes, which were lysed in 20—30 volumes of 10 mosM phosphate buffer (pH 8) while standing at 0—4°C for 1 h. The hemolysate was centrifuged (30 min, 18 000 × *g*, at 0—4°C). The ghost pellets were washed five times with 10 mosM phosphate (pH 8) in a Sorvall SS-34 rotor (15 min, 40 000 × *g*, at 0—4°C) to yield washed ghosts which were cream-pink coloured. The sticky pellets beneath the loose ghost pellets, containing protease activity [15], were carefully removed after each wash of the ghosts.

Extraction of membranes. Sheep erythrocyte membranes were extracted with (A) 0.1 mM EDTA (pH 8), (B) 1 mM glycylglycine (pH 8), (C) 0.5 M NaCl in 5 mM phosphate (pH 8), (D) 0.75 M NaI (pH 8) and (E) 0.01 M NaOH by the procedures of Fairbanks et al. [15], Kahlenberg and Walker [16] and

Steck and Yu [17], respectively. For this purpose packed ghosts equivalent to 7.5 mg membrane protein were suspended in 7 ml of the appropriate solution. After incubation as indicated in the references (procedures A and B at 37°C for 15 min, procedures C and D on ice for 30 min and procedure E, none), the membrane suspensions were centrifuged (60 min, 150 000 × *g*, at 0—4°C) in a Beckman L-2. The sedimented membrane material of procedures A, C, D and E was washed with 7.5 mM phosphate (pH 7.5) and suspended by homogenization in the same buffer. The supernatant and pellet fractions and untreated membranes (control membranes) were dialyzed against 10 mM glycylglycine (pH 8) overnight and aliquots were assayed for protein content and phospholipase activity.

For the experiments described below ghosts were pretreated at low ionic strength according to procedure B and the resulting membranes are referred to in this report as extracted membranes.

Solubilization of membranes. Extracted membranes were suspended at a concentration of 2—4 mg protein per ml in 10 mM glycylglycine (pH 8) and mixed with equal volumes of detergent solution of various concentrations in the same buffer. After incubation at 37°C for 15 min, the mixture was centrifuged (60 min, 150 000 × *g*, at 0—4°C) in a Beckman L-2. The supernatant was then carefully removed from the pellet. Control membranes were treated in the same way without detergent. To measure protein which had been solubilized from the membranes by the detergent treatment, aliquots of the treated membranes were taken before and after centrifugation. The protein was assayed and the percentage of protein released determined. Phospholipase activity of the supernatant fractions was estimated using the standard assay. The detergent concentration of the supernatants of each detergent series was adjusted to a value suitable for measurement of enzymatic activity (0.4% for Triton X-100 and deoxycholate, 0.5% for cholate). Dodecyl sulfate was first removed by extensive dialysis as described by Razin [18] and the enzymatic activity of these preparations was subsequently determined in 0.5% cholate. The solubilization of the phospholipase by the detergent treatment was calculated by expressing the enzymatic activity of the supernatant fractions as percent of uncentrifuged control membranes (initial enzymatic activity).

Isolation of phospholipase A_2. Extracted membranes were solubilized in 10 mM glycylglycine (pH 8) containing 1% dodecyl sulfate (5 mg protein/ml). An equal volume of the same buffer containing 10% cholate and 0.4 M KCl was added to give a final concentration of 2.5 mg/ml of protein, 0.5% dodecyl sulfate, 5% cholate and 0.2 M KCl. The solution was stirred at 20°C for 1 h. Upon termination of incubation solubilization of the membranes was complete, leaving no material in a sedimentable form.

The solubilized membranes were applied to a Sephadex G-75 column (size 1250 cm × 7 cm), which had been equilibrated with 10 mM glycylglycine (pH 8) containing 0.5% cholate, 0.2 M KCl and 0.05% NaN_3 (buffer A). Elution was performed with 5000 ml of buffer A at 4°C. Fractions of 30 ml were collected at a flow rate of 250 ml/h. The column chromatography was routinely followed by determination of the absorbance at 280 nm and assay of phospholipase activity. The enzymatically active fractions were combined and, after addition of $CaCl_2$ to a final concentration of 10 mM, filtered through a

dialkyl-phosphatidylcholine-Sepharose column (size 15 cm × 0.8 cm, flow rate 20 ml/h) at 4°C. The column was washed extensively with buffer A containing 10 mM $CaCl_2$. Elution of the bound phospholipase was carried out with 20 mM EDTA in buffer A. Fractions of 10 ml were collected at a flow rate of 10 ml/h. The desired fractions were dialyzed overnight against buffer A to remove the EDTA. The enzymatic activity was determined with aliquots of the fractions (0.2 ml) using the standard assay.

To determine the specific activity of the isolated phospholipase, the EDTA eluate containing the enzymatic activity was pooled and concentrated using an Amicon ultrafiltration cell equipped with a PM-10 membrane. The concentrated enzyme solution (2—3 ml) was assayed for enzymatic activity and then dialyzed against water (adjusted to pH 9 with NaOH) to remove the cholate. Aliquots of the resulting slightly turbid enzyme preparation were lyophilized. The lyophilized samples were resuspended either in distilled water (for protein determination phospholipid analysis and dodecyl sulfate gel electrophoresis) or in buffer A (for control of enzymatic activity). A similar procedure was adopted for phospholipase determination, protein and phospholipid analysis of the enzymatically active, pooled fractions from Sephadex G-75 chromatography.

Analytical procedures. Protein was estimated by the method of Lowry et al. [19] using bovine serum albumin as standard or by absorption at 280 nm. Corrections for the effect of detergents and glycylglycine on the Lowry method were carried out [20]. For protein determination in Triton X-100 containing samples dodecyl sulfate was included in the alkaline copper reagent for the Folin reaction, as indicated by Dulley and Grieve [21]. Lipids were extracted with chloroform/methanol (2 : 1, v/v) by the method of Folch et al. [22]. The phospholipids were separated by two-dimensional thin-layer chromatography using the procedure of Broekhuyse [23] and the phospholipid phosphorus determined according to Bartlett [14]. Electrophoresis in dodecyl sulfate-polyacrylamide gels was done according to Fairbanks et al. [15]. For preparative dodecyl sulfate gel electrophoresis the enzyme was solubilized in the absence of dithiothreitol, since sulfhydryl reagents were shown to destroy the lecithinase activity [1]. Solubilized lecithinase (10—20 μg protein) was layered on each of two gels. One gel was stained and destained to localize the protein band, the other gel was sliced and eluted with 0.1% dodecyl sulfate solution as described by Weber and Osborn [24]. The eluents and aliquots of the solubilized enzyme were dialyzed against 0.5% cholate buffer and then assayed for enzymatic activity.

Results

Extraction of membranes

Reagents and experimental conditions were used which are known to selectively extract various polypeptides from the erythrocyte membrane, but did not cause significant loss of enzymatic activity (see Table I).

First, sheep ghosts were placed in a low ionic strength, mildly alkaline medium containing 0.1 mM EDTA (procedure A), which is known to effect the release of weakly bound membrane proteins, such as the spectrin and actin

TABLE I

PHOSPHOLIPASE ACTIVITY OF SHEEP ERYTHROCYTE MEMBRANES AFTER VARIOUS MILD TREATMENTS

Packed ghosts equivalent to 7.5 mg of protein were suspended in 7 ml of A, 0.1 mM EDTA (pH 8); B, 1 mM glycylglycine (pH 8); C, 0.5 M NaCl in 5 mM phosphate (pH 8); D, 0.75 M NaI (pH 8) and E 0.01 M NaOH. A and B were incubated at 37°C for 15 min, C and D on ice for 30 min and E was immediately centrifuged. The supernatant and pellet fractions were isolated and assayed as described in Materials and Methods. Values are indicated as a percent of unextracted ghosts and represent the mean for three experiments.

Treatment	Protein (percent of initial)		Phospholipase activity (percent of initial)	
	Pellet	Supernatant	Pellet	Supernatant
(A) 0.1 mM EDTA (pH 8)	68	27	92	4
(B) 1 mM glycylglycine (pH 8)	66	25	95	4
(C) 0.5 M NaCl in 5 mM phosphate (pH 8)	86	17	96	8
(D) 0.75 M NaI (pH 8)	61	31	52	55
(E) 0.01 M NaOH	49	46	17	85

components [15]. The results show that the eluted protein fraction, comprising 27% of the total membrane protein, contained almost no phospholipase activity. A similar extraction of sheep ghosts without EDTA (procedure B) resulted in a slightly higher recovery of enzymatic activity, about the same amount of protein being eluted from the membranes. Second, sheep erythrocyte membranes were exposed to elevated ionic strength (procedure C). This treatment was used for solubilization of glyceraldehyde-3-*P* dehydrogenase [15] and the Rh(D) blood group receptor component [25] from human red cell membranes. As demonstrated in Table I, it was found that upon extraction of sheep ghosts with buffered 0.5 M NaCl about 96% of the phospholipase activity remained associated with the membrane residue. Third, sheep ghosts were treated with iodide (procedure D), an inorganic chaotrope, which was shown to increase water solubility of membrane proteins by favoring the transfer of apolar groups to water [26]. As recently demonstrated by Kahlenberg and Walker [16], NaI selectively removes various proteins from human erythrocyte ghosts. The remaining membrane residue, which consisted of about one-half of the membrane proteins and all the membrane lipids, fully retained the glucose transport capacity. Extraction of sheep red cell membranes with 0.75 M NaI removed about one-third of the thost protein (Table I). In this case 55% of the initial phospholipase activity was obtained in a soluble form and 52% was recovered in the membrane pellet.

Finally, the effect of elevated pH was investigated (procedure E). It has been reported that almost all proteins associated with the cytoplasmic surface of the human red cell membrane can be selectively released in alkaline media (pH 12) leaving a residual membrane, which contains all of the lipids and one-half of the membrane proteins, namely the glycoproteins [17] (nomenclature is according to Steck [11]). As can be seen in Table I, about 50% of the membrane proteins were removed from sheep ghosts upon short exposure to 0.01 M NaOH. Inter-

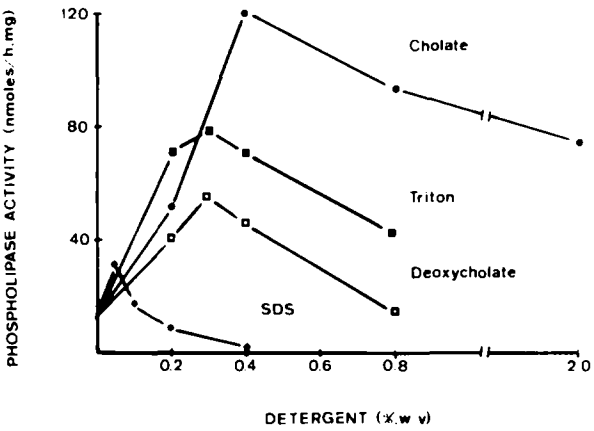

Fig. 1. Phospholipase activity in the presence of detergents. Phospholipase activity of sheep erythrocyte membranes was determined using the standard assay conditions as described in Materials and Methods, with the exception that the detergent concentration was varied as indicated. ●———●, cholate; ■———■, Triton X-100; □———□, deoxycholate; ⋆———⋆, sodium dodecyl sulfate (SDS).

estingly, the NaOH eluate contained 85% of the phospholipase activity, compared to 17% remaining in the membrane residue.

Solubilization of membranes with detergents

Solubilization of the phospholipase was studied by disrupting the membrane with detergents. Previous studies [1] concerning the catalytic activity of phospholipase showed that in general addition of detergents to the enzyme assay caused stimulation of the original activity (Fig. 1).

For the solubilization experiments low ionic strength extracted sheep ghosts were treated with several types of detergent at various concentrations as indicated in Fig. 2. The unsolubilized material was removed by centrifugation and the supernatant assayed for lecithinase activity. In this context it is important to note that the enzymatic activity of the Triton, deoxycholate and cholate series was determined in the presence of appropriate equal amounts of the corresponding detergent (0.4% for Triton X-100 and deoxycholate, 0.5% for cholate). For the dodecyl sulfate series, the detergent was first removed by extensive dialyses and the phospholipase activity of the corresponding supernatants determined in the presence of cholate (0.5%).

As demonstrated in Fig. 2, the degree of solubilization of total membrane proteins depended on the type of detergent studied. Dodecyl sulfate solubilized the membrane proteins completely while deoxycholate solubilized about 80% of the protein. The solubilizing capacity of deoxycholate could be somewhat increased when the ionic strength of the medium was elevated by adding salt (data not shown). Triton X-100 almost completely solubilized extracted membranes, whereas with cholate less than 20% of the membrane proteins were solubilized (Fig. 2). This is in agreement with the reported selectivity of these detergents with respect to solubilization of erythrocyte membrane proteins [27–29].

Fig. 2 also shows that phospholipase activity was solubilized in parallel with

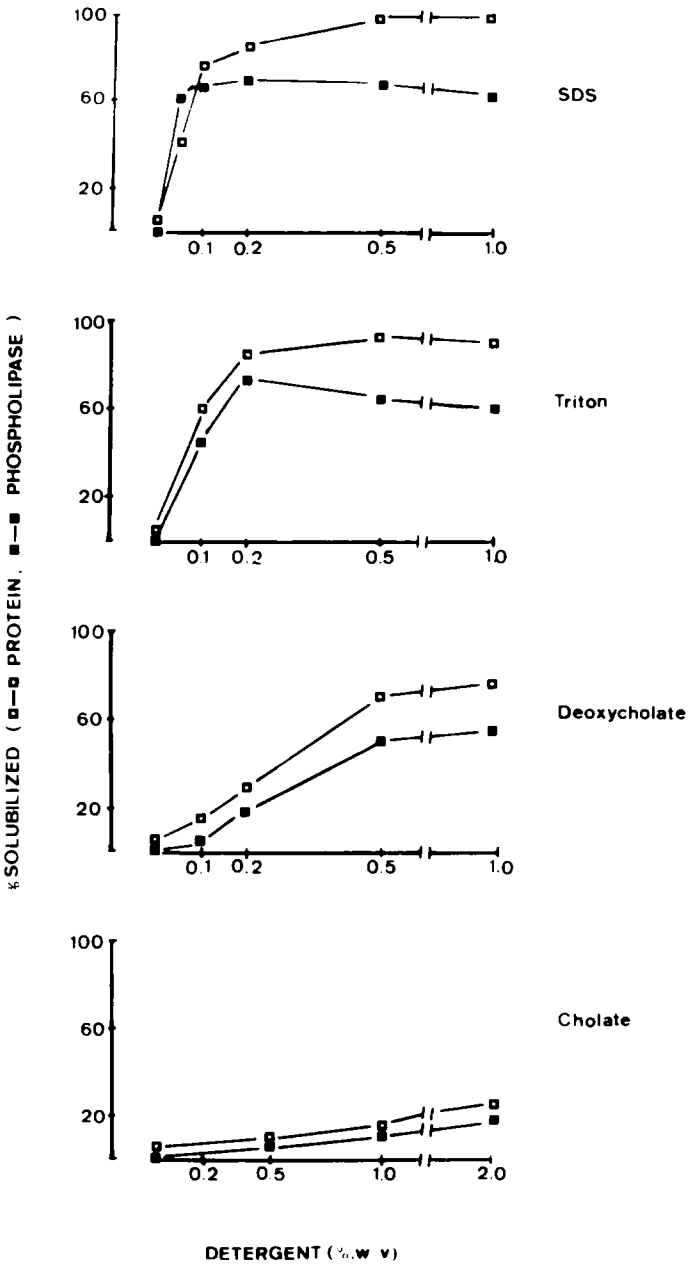

Fig. 2. Solubilization of membrane proteins (□———□) and phospholipase (■———■) from extracted sheep erythrocyte membranes. Sheep red cell membranes, extracted at low ionic strength, were suspended at a concentration of 2 mg/ml protein in 10 mM glycylglycine (pH 8) containing increasing concentrations of various detergents as indicated. SDS, sodium dodecyl sulfate. Details are given in Materials and Methods.

the total membrane proteins. No differential release was obtained with Triton X-100, deoxycholate or cholate. However, some selective solubilization was observed with dodecyl sulfate at concentrations below 0.1%. The maximal solu-

bilized enzymatic activity which could be obtained with the detergent treatments amounted to about 60—70% of the initial activity. As determined with appropriate controls (data not shown) treatment of membranes with deoxycholate and Triton X-100 at concentrations above 0.5% affected the enzyme functioning whereas with cholate no inactivation was observed up to concentrations of 5%. After dodecyl sulfate treatment phospholipase activity was reduced to the same extent in all concentrations tested. Since dodecyl sulfate has proved to be strongly inhibitory for enzyme function above 0.05%, this partial inactivation is thought to be the result of incomplete removal of the detergent by the dialysis procedure.

Purification of phospholipase A_2

Low ionic strength extraction. Membranes were extracted using procedure B as described in Materials and Methods (see also Table I). As demonstrated in Table II this was accompanied by a 95% recovery of total phospholipase activity and a 1.4-fold increase in specific enzyme activity.

Gel exclusion chromatography (replacement of dodecyl sulfate by cholate). Extracted membranes, solubilized in buffer containing 0.5% dodecyl sulfate and 5% cholate, were applied to a Sephadex G-75 column (equilibrated with 0.5% cholate buffer) and eluted with 0.5% cholate buffer (details are given in Materials and Methods). A typical chromatographic profile is shown in Fig. 3. More than 80% of the applied membrane protein appeared in the void volume (Fractions 30—50). Dodecyl sulfate was considerably retarded (Fractions 110—140), and was clearly separated from the bulk of membranes protein. The phospholipase was eluted in two peaks, a minor one coinciding with the total membrane protein, and a major enzymatically active peak which was retarded (Fractions 85—110). Dodecyl sulfate began to emerge only with the very last fractions of this peak. The elution profile for the membrane phospholipids (Fig. 3) shows a broad peak appearing towards the end of the elution, which was somewhat retarded with respect to the major phospholipase peak. Table II summarises the partial purification of the enzyme obtained by this procedure. More than 80% of the enzymatic activity which was applied to the Sephadex column was recovered in the retarded phospholipase peak (overall yield 78%). A purification of 58-fold was obtained. The fractions containing

TABLE II

PURIFICATION OF SHEEP ERYTHROCYTE MEMBRANE PHOSPHOLIPASE A_2

Protein content and enzymatic activity of the fractions were determined as described in Materials and Methods.

Fraction	Protein (mg)	Total activity (μmol/h)	Specific activity (μmol/h per mg)	Overall yield (%)	Purification (-fold)
I Membranes	300	36.0	0.12	100	—
II Extracted membranes	200	34.2	0.17	95	1.4
III Sephadex G-75 filtrate	4	27.9	6.98	78	58
IV Affinity column eluate	0.085	24.1	283.5	67	2835

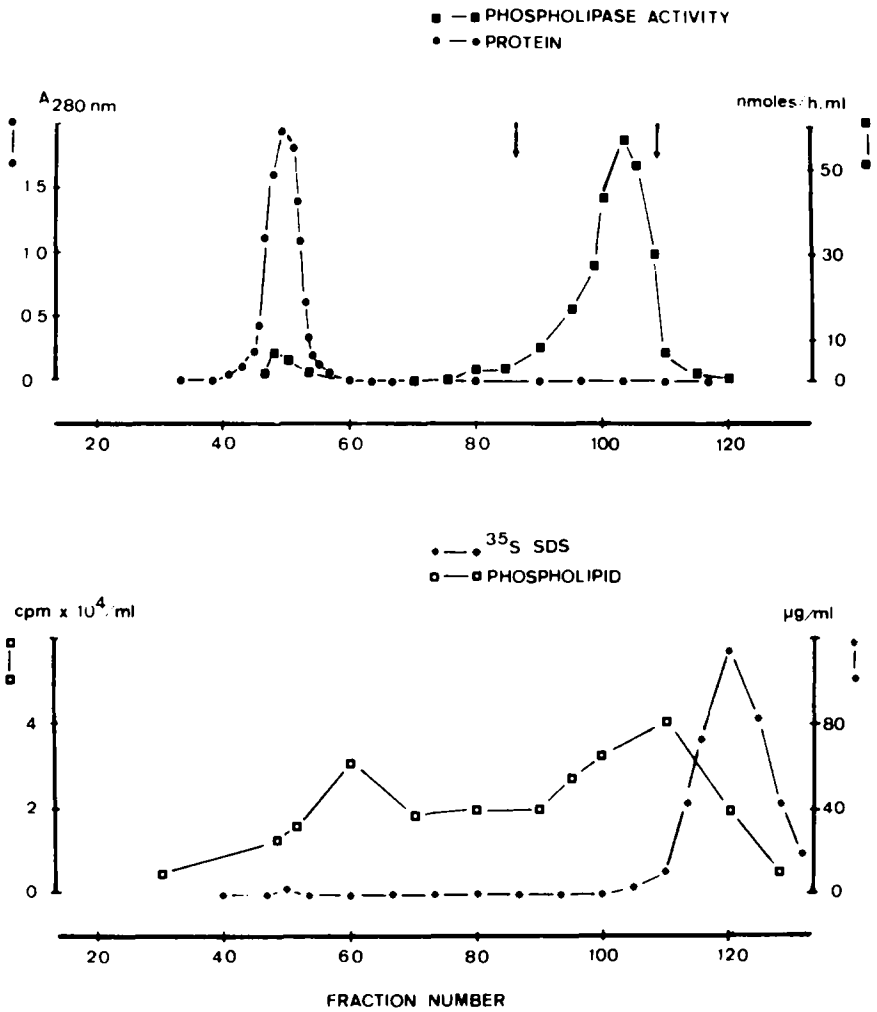

Fig. 3. Sephadex G-75 chromatography of dodecyl sulfate-solubilized sheep red cell membranes in the presence of cholate. The membrane solution containing 0.5% dodecyl sulfate (SDS) was applied to a Sephadex G-75 column and eluted with buffer containing 0.5% cholate and 0.2 M KCl. Phospholipase activity was determined using the standard assay with 0.5 ml of the fractions as indicated. For the dodecyl sulfate elution profile, the solubilization was accomplished using dodecyl[^{35}S]sulfate ($6.8 \cdot 10^4$ cpm/mg) and the eluate fractions analyzed for ^{35}S radioactivity. The fractions containing the enzyme activity were pooled as indicated by arrows. For details see Materials and Methods.

the phospholipase were pooled and, after addition of $CaCl_2$ to a final concentration of 10 mM, used for affinity chromatography.

Affinity chromatography. The partially purified phospholipase, in 0.5% cholate buffer containing 10 mM $CaCl_2$, was filtered through a dialkyl-phosphatidylcholine-Sepharose column equilibrated with the same buffer. After extensive washing the enzyme was eluted wity 0.5% cholate buffer containing 20 mM EDTA. The applied enzymatic activity was followed during adsorption, washing and elution steps (for details see Materials and Methods). As shown in Fig. 4, the phospholipase began to emerge with the leading edge of the EDTA-

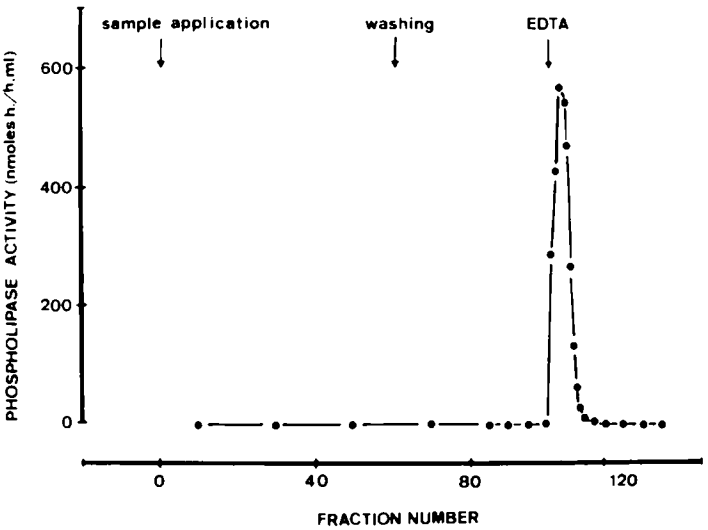

Fig. 4. Affinity chromatography of purified sheep red cell membrane phospholipase on dialkyl-phosphatidylcholine-Sepharose in the presence of cholate. After addition of $CaCl_2$ to a concentration of 10 mM the phospholipase pool from Sephadex G-75 chromatography was filtered through a column packed with dialkyl-phosphatidylcholine-Sepharose. Elution of the bound enzyme was performed with buffer containing 20 mM EDTA. Details are described in Materials and Methods.

containing buffer, the elution being completed 50—80 ml later. Table II indicates that the procedure outlined above yielded a phospholipase preparation (67% recovery) which was purified more than 2800-fold in comparison to erythrocyte membranes. Fraction IV was concentrated to 1—2 ml by ultrafiltration without loss of enzymatic activity and was stable for several weeks at this stage when stored in the presence of 0.05% NaN_3 at 0—4°C. Upon removal of cholate by extensive dialysis against water (pH 9) the enzyme tended to aggregate and adsorb to glass. Moreover, an approximate decrease of 50% in the specific activity was observed. When the enzyme preparation was stored at this stage, high molecular aggregates were detected on dodecyl sulfate gels.

Lipid and detergent content of enzyme fractions. As demonstrated in Table III, lipids co-chromatograph with the phospholipase through all steps of the purification. Fraction III (Sephadex G-75 filtrate) contained about 8.5 mg phospholipid per mg protein, accounting for 30% of the phospholipids present in Fraction IIa (solubilized membranes). The composition of the phospholipids extracted from Fraction III expressed as percent of total was: sphingomyelin 48%, phosphatidylethanolamine 30%, phosphatidylserine 17%, phosphatidylinositol 3%. This composition approximately reflects the phospholipid composition of sheep erythrocyte membranes [30]. There was no enrichment for specific phospholipid components in Fraction III. It is noteworthy that no lyso compounds were detected which could be the consequence of phospholipid hydrolysis during the column chromatography. As can be seen in Table III, Fraction III still contained small amounts of dodecyl sulfate (less than 0.002%). Whereas phospholipids were also present in Fraction IV (affinity column

TABLE III

LIPID AND DETERGENT CONTENT OF ENZYME FRACTIONS

Sheep erythrocyte membrane phospholipase was isolated as described in Materials and Methods. Aliquots of the enzyme fractions were dialyzed against water (pH 9) and the phospholipids determined as indicated in Materials and Methods. For determination of the sodium dodecyl sulfate (SDS) content, phospholipase was purified using dodecyl [^{35}]sulfate ($6.8 \cdot 10^4$ cpm/mg).

Fraction		Protein (mg)	Phospholipid (mg)	SDS (%)	Cholate (%)
I	Membranes	300	126	—	—
II	Extracted membranes	200	114	—	—
IIa	Solubilized membranes	200	114	0.5	5
III	Sephadex G-75 filtrate	4	34	<0.002	0.5
IV	Affinity column elutate	0.085	0.2	0	0.5

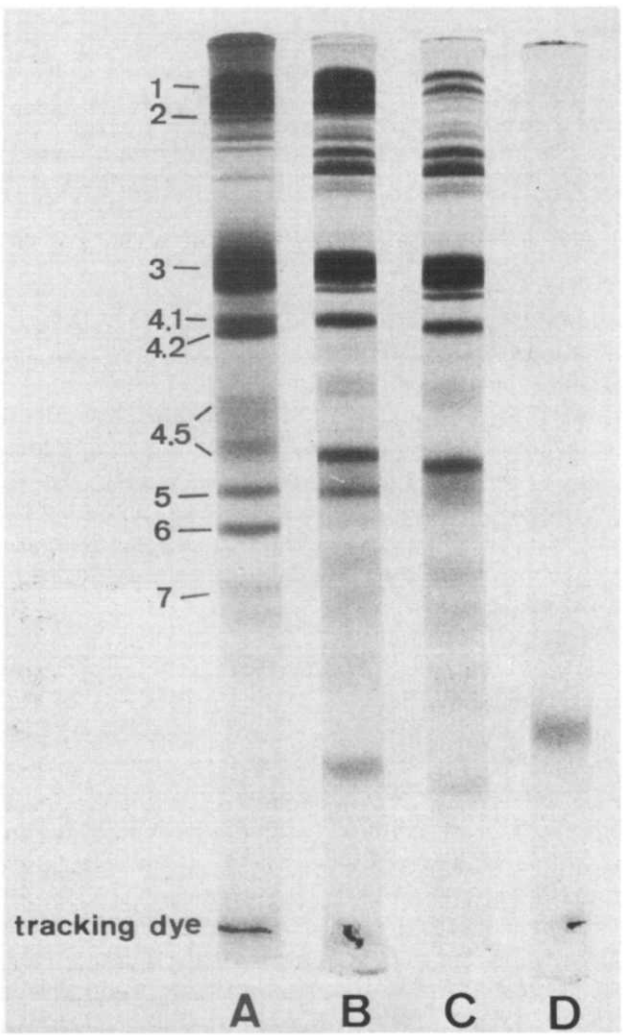

Fig. 5. Dodecyl sulfate gel electrophoresis of erythrocyte membranes and purified phospholipase. A, human erythrocyte membranes; B, sheep erythrocyte membranes; C, extracted sheep erythrocyte membranes and D, phospholipase. Gels (5.8%) were loaded with 50 μg (A—C) and 10 μg (D) of protein and stained with Coomassie Blue. Nomenclature for human erythrocyte proteins according to Steck [11]. TD, tracking dye.

eluate) with a ratio of lipid to protein of approx. 2, dodecyl sulfate could not be detected after affinity chromatography.

Enzyme purity. The purity of the isolated phospholipase was investigated by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate. As shown in Fig. 5, the protein was present as a single band indicating that the polypeptide was electrophoretically pure. Moreover, as determined by preparative gel electrophoresis, the phospholipase activity was recovered in the region corresponding to the protein band of the stained gel. Recovery of enzymatic activity was 71% of applied activity.

Molecular weight. As judged by the Sephadex G-75 chromatography, the phospholipase has a molecular weight of approx. 12 000. Based on migration relative to marker proteins, an apparent molecular weight of 18 500 was estimated by dodecyl sulfate electrophoresis.

Discussion

The affinity system described by Rock and Snyder [5] appeared to be an elegant approach for isolation of the erythrocyte phospholipase A_2. However, many different aspects had to be taken into consideration. First of all, the reactions had to be carried out in a media capable of solubilizing the membrane-bound enzyme with retention of a native-like structure and maintaining the solubilized membrane components in a dissociated state in the presence of Ca^{2+}. Moreover, specific ionic conditions were required for obtaining a selective adsorption of the enzyme without non-specific binding of other proteins to the fixed charged lipid sites of the column. Therefore, membrane extracts obtained at elevated pH for selective release of phospholipase A_2 could not be used, because the eluted proteins tended to aggregate after introduction of Ca^{2+} [31]. Also, the above-mentioned criteria could not be satisfied using membranes solubilized with various types of detergent. Therefore, dodecyl sulfate was used for initial solubilization of the enzyme and was then replaced by cholate, using gel exclusion chromatography [32]. By this, more than 90% of the lecithinase activity could be recovered in the fraction of the Sephadex G-75 column and about 80% of the eluted lecithinase activity was clearly separated from the bulk of membrane proteins, which were eluted with the void volume in an aggregated form.

Our results indicate that although cholate cannot accomplish release of the phospholipase A_2 from the membrane, it is capable of maintaining the enzyme in a soluble state once monomerization of the membrane components has occurred. The procedure yielded a partially purified, fully active lecithinase which was dissolved in an ideal medium for affinity chromatography. Thus, the phospholipase A_2 was specifically bound to the lipid-Sepharose in the presence of Ca^{2+} and could be eluted from the gel with EDTA in a highly purified, enzymatically active form, as judged from dodecyl sulfate gel electrophoresis and values of specific activity.

Phospholipase A_2 from rat liver mitochondrial membranes was purified 160-fold in the absence of detergents and was isolated in an enzymatically unstable, aggregated form [9]. The sheep erythrocyte membrane phospholipase A_2, in contrast, was purified 2800-fold and showed a remarkable stability against

aggregation and loss of enzymatic activity when stored in the presence of cholate.

The current study is the first attempt to isolate membrane-bound phospholipase A_2 in a stable and active form. Whereas the major functional role of the lecithinase may be the maintenance of the very low lecithin content in the sheep erythrocyte membrane [2], phospholipase A_2 from other plasmamembranes is thought to be responsible for initiation of prostaglandin formation by release of polyunsaturated free fatty acids from the membrane phospholipids [33]. The feasibility to purify membrane-bound phospholipase A_2 will enable further investigations on the characteristic properties of this enzyme with regard to its possible regulatory function for biosynthesis of prostaglandins and related substances.

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References

1 Kramer, R., Jungi, B. and Zahler, P. (1974) Biochim. Biophys. Acta 373, 404—415
2 Zwaal, R.F.A., Flückiger, R., Moser, S. and Zahler, P. (1974) Biochim. Biophys. Acta 373, 416—424
3 Gatt, S. and Barenholz, Y. (1973) Annu. Rev. Biochem. 42, 61—90
4 van den Bosch, H. (1974) Annu. Rev. Biochem. 43, 243—277
5 Rock, C.O. and Snyder, F. (1975) J. Biol. Chem. 250, 6564—6566
6 Wells, M.A. (1972) Biochemistry 11, 1030—1041
7 Scandella, C.J. and Kornberg, A. (1971) Biochemistry 10, 4447—4456
8 Nishijma, M., Akamatsu, Y. and Nojima, S. (1974) J. Biol. Chem. 249, 5658—5667
9 Waite, M. and Sisson, P. (1971) Biochemistry 12, 2377—2383
10 Juliano, R.L. (1973) Biochim. Biophys. Acta 300, 341—378
11 Steck, T.L. (1974) J. Cell Biol. 62, 1—19
12 Marchesi, V.T., Furthmayr, H. and Tomita, M. (1976) Annu. Rev. Biochem. 45, 667—698
13 Renkonen, O., Kosunen, T.U. and Renkonen, O.V. (1963) Ann. Med. Exp. Biol. Fenniae 41, 375—381
14 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
15 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
16 Kahlenberg, A. and Walker, C. (1976) J. Biol. Chem. 251, 1582—1590
17 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220—232
18 Razin, S. (1972) Biochim. Biophys. Acta 265, 241—296
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Ji, T.H. (1973) Anal. Biochem. 52, 517—521
21 Dulley, J.R. and Grieve, P.A. (1975) Anal. Biochem. 64, 136—141
22 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
23 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457—461
24 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
25 Abraham, C.V. and Bakerman, S. (1975) Clin. Chim. Acta. 60, 33—43
26 Hatefi, Y. and Hanstein, W.G. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1129—1136
27 Kirkpatrick, F.H., Gordesky, S.E. and Marinetti, G.V. (1974) Biochim. Biophys. Acta 345, 154—161
28 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233—247
29 Coleman, R., Holdsworth, G. and Finean, J.B. (1976) Biochim. Biophys. Acta 436, 38—44
30 Nelson, G.J. (1967) Biochim. Biophys. Acta 144, 221—232
31 Carraway, K.L., Triplett, R.B. and Anderson D.R. (1975) Biochim. Biophys. Acta 379, 571—581
32 Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457, 133—170
33 Symposium on Phospholipases and Prostaglandins (1977) in Advances in Prostaglandin and Thromboxane Research (Samuelsson, B. and Paoletti, R., eds.), Raven press, New York, in the press