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RECONSTITUTION OF A PROTEIN INTO LIPID VESICLES USING NATURAL DETERGENTS

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A method is described for reconstitution of a protein into lipid vesicles using one of the natural detergents lysophosphatidylcholine or lysophosphatidic acid. The intestinal microvillus enzyme, aminopeptidase N (EC 3.4.11.2) is incorporated into lipid vesicles prepared from a total lipid extract of the microvillus membrane. The method is based on fusion of aminopeptidase-lysophospholipid micelles with liposomes prepared by sonication. The incorporation of the protein into the lipid bilayer is analyzed by gel permeation chromatography and sucrose density gradient centrifugation. The coincidence of the protein and lipid profiles is used to evaluate protein incorporation. The incorporation is visualized by electron microscopy with negative staining. The method has the advantage of using natural detergents, lysophospholipids, which are minor but natural constituents of biological membranes. The method could be of value as a tool in studies of mechanisms of insertion of newly synthesized proteins into biological membranes.

Introduction

Reconstitution of various membrane proteins into artificial lipid vesicles is a useful method for studying the structure and function of biological membranes. A number of techniques for reconstitution has been used [1]. One of these methods involves cosonication of the protein and lipid. However, this method has the disadvantage that in order to obtain unilamellar vesicles long sonication times are needed, which might lead to denaturation of the protein. Other methods for reconstitution use either detergents or organic solvents for solubilization of both protein and lipid, followed by reconstitution of the protein into lipid vesicles by removal of the detergent (or solvent). However, the ability completely to remove the detergent (or solvent) from the protein-lipid recombinant can be critical for further studies. Residual detergent (or solvent) can effect hydrocarbon and polar head-group organization which may be disturbing in physical studies [2]. An increase in permeability and conductivity is observed in transport studies [3,4] and transmembrane movement of phospholipids has been observed as a result of residual detergent molecules [5].

In this report we describe a method for reconstitution of the microvillus enzyme aminopeptidase N (EC 3.4.11.2) into lipid vesicles using either of the natural detergents lysophosphatidic acid or lysophosphatidylcholine to solubilize the microvillus membrane. Pure aminopeptidase-lysophospholipid micelles isolated by immunoadsorbent chromatography are allowed to fuse with liposomes prepared by sonication.

Materials and Methods

Chemicals

The organic solvents were of analytical reagent

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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grade and were destilled prior to use, di-[1-^{14}C]palmitoylphosphatidylcholine (spec. act. 156 μC/mg) was obtained from the Amersham International, Amersham, U.K. GB, and Aquasol from New England Nuclear, Boston, MA, U.S.A. Phospholipase A_2 (600 U/mg) from porcine pancreas was obtained from Boehringer Mannheim GmbH, F.R.G. CNBr-activated Sepharose 4B and Protein A-Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). Ultrogel AcA 34 was from LKB Products (Stockholm, Sweden).

Lysophospholipids

Egg lysophosphatidylcholine was obtained from Serdary Research Laboratories, London, Canada. Egg lysophosphatidic acid was prepared from egg phosphatidylcholine (PC) by treatment with phospholipase D and phospholipase A_2. A phospholipase D-containing preparation was obtained from white cabbage as described by Davidson and Long [6]. To 100 mg of phosphatidylcholine (PC) in 10 ml of diethyl ether was added 12.5 ml 0.1 M sodium acetate buffer, pH 6.5, and 2.5 ml 1 M $CaCl_2$ and 20 ml phospholipase D preparation and the mixture shaken for 3 h at room temperature. The reaction was stopped by addition of acetic acid to pH 2.5 and the phosphatidic acid extracted by addition of 3×40 ml chloroform/methanol 2:1 (v/v). The conversion of phosphatidylcholine to phosphatidic acid was complete as analyzed by thin-layer chromatography in the solvent system chloroform/methanol/water (65:25:4, v/v).

Phosphatidic acid was converted to lysophosphatidic acid by treatment with phospholipase A_2. To 100 mg of phosphatidic acid was added 20 ml of diethyl ether, 5 ml of 0.1 M Tris-HCl, pH 7.5, containing 4 mM $CaCl_2$ and 500 units phospholipase A_2. The mixture was shaken at room temperature for 3 h. The lysophosphatidic acid was obtained from the reaction mixture by solvent partition as described [7]. The yield was about 20–30%. The product was pure as analyzed by chromatography on thin-layer in the solvent system chloroform/methanol/water (65:25:4, v/v).

Enzymes and other assays

Aminopeptidase N (EC 3.4.11.2) activity was determined with L-alanyl-*p*-nitroanilide as substrate [8] at 37°C by use of the Reaction Rate Analyzer LKB 8600 (LKB Products AB, Stockholm, Sweden). Alkaline phosphatase activity (EC 3.1.3.1) was measured with *p*-nitrophenyl phosphate as substrate [9], and protein by the method of Lowry et al. [10]. ^{14}C radioactivity was measured in a Nuclear Chicago scintillation counter using Aquasol as scintillation solvent.

Electron microscopy

For thin section electron microscopy the freshly prepared liposomes were mixed with an equal volume of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After 2 h at room temperature the lipsomes were pelleted by centrifugation in a Beckman Airfuge at $100\,000 \times g$ for 1 h. To the pellet was added 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer and the mixture left for 0.5 h. After rinsing in buffer, the pellet was stained at 4°C first with 1% tannic acid in buffer for 0.5 h and then with 1% uranyl acetate in water overnight. The specimen was dehydrated in series of graded ethanol solutions: 75% (3×5 min), 96% (3×5 min) and in absolute ethanol (3×15 min) before embedding in Epon. Ultrathin sections were cut on an LKB Ultratome III, stained with uranyl acetate and lead citrate [11] and examined in a Philips 201 C electron microscope.

Liposomes and the protein-lipid recombinant were negatively stained with 2% (w/v) sodium silicotungstate, pH 7.2 or 2% (w/v) sodium phosphotungstate, pH 6.8. A droplet of the sample (6 μmol phospholipid per ml) was applied to a carbon-coated Formwar film carried on a 400-mesh copper grid, glow discharged shortly before use. The grid was then flushed by ten drops of stain solution. Excess stain was drained to leave a thin film on the grid, which was left to dry at room temperature. The specimens were examined in a Philips 201 C electron microsope.

Preparation of anti-aminopeptidase-Sepharose

Aminopeptidase and antiserum against aminopeptidase was prepared as described [8]. The IgG fraction of the antiserum was isolated as in Ref. 12. High-capacity immunoadsorbent was prepared by coupling the anti-aminopeptidase fraction of the IgG, obtained by affinity chromatography on aminopeptidase-Sepharose, to CNBr-activated

Sepharose 4B. The immunoadsorbent was able to bind 25 units of aminopeptidase per ml of packed gel.

Solubilization of microvillus membrane vesicles

The standard procedure for solubilization was as follows: To 1 vol. of microvillus membrane vesicles (6 mg protein per ml) prepared as described earlier [13] were added 5 vol. of a 0.5% solution of lysophospholipid in 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl. This gave a detergent to protein ratio (w/w) of 4. The solubilization was performed at room temperature for 10 min and insoluble material was removed by filtration through a Millipore filter (poor size 0.22 μm). The degree of solubilization was determined by measuring the activity of the enzyme aminopeptidase in the filtrate. The filtrate (solubilized microvillus proteins) was used for isolation of aminopeptidase by immunoadsorbent chromatography.

Isolation of aminopeptidase

A plastic syringe (0.5×1.2 cm) was packed with 0.25 ml anti-aminopeptidase-Sepharose 4B and equilibrated with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.05% lysophospholipid. The solubilized microvillus proteins were applied to the column with a flow rate of 1 ml/h at 10°C. When the column was saturated with aminopeptidase activity, the column was washed with five volumes of the same buffer as the one used for the equilibration. The aminopeptidase was eluted from the column with 2 mM Tris-HCl, pH 8.0 [14], containing 0.05% lysophospholipid and fractions of 0.1 ml were collected and assayed. The fractions with the highest activity of aminopeptidase were used in reconstitution experiments.

Preparation of liposomes

A total lipid extract from a microvillus vesicle preparation was prepared as described earlier [13]. To an aliquote of the total lipid extract corresponding to 9.2 mg of lipid was added 10 μl [^{14}C]PC (25 μCi/ml) and the mixture evaporated to dryness under N_2, leaving the lipid as a thin film in a flask. In order to ensure complete removal of the solvent the film was left under vacuum at least 1 h. The lipid was suspended by shaking on a Vortex mixer in 2.0 ml 10 mM Hepes, pH 7.0, containing 40 mM KCl, and the suspension left at 35°C for 10 min. The milky suspension was sonicated at 35°C with an MSE sonifier (amplitude setting of 3 at medium) using a macrotip for 2×2.5 min. The optical clarity of the solution was followed by measuring the absorbance at 300 nm. In order to remove multilamellar vesicles and iron dust the liposome preparation was centrifuged at room temperature at $16000 \times g$ for 10 min. The supernatant contained unilamellar vesicles and was used as such.

Reconstitution

The aminopeptidase was incorporated into lipid vesicles by fusion. 0.4 vol. of pure aminopeptidase-lysophospholipid micelles (about 1 unit/ml) was mixed with 1 vol. of freshly prepared liposomes (about 6 μmol/ml). The fusion was allowed to take place at room temperature for 45 min, with or without addition of acetic acid to decrease the pH. Depending on the specific activity by which the aminopeptidase was isolated by immunoabsorbent chromatography this standard condition for reconstitution gave a lipid to protein weight of about 150 and a lysophospholipid concentration in the reconstitution mixture of about 0.014%. Assuming that all the lysophospholipid is incorporated into the lipid vesicles this would give a lipid to lysophospholipid molar ratio of 15.

Sample characterization

The liposomes, the aminopeptidase-lysophospholipid complex and the protein-lipid recombinant were characterized both by gel permeation chromatography and by sucrose gradient centrifugation. Gel chromatography was performed on Sepharose 4B in 10 mM Hepes, pH 7.0, containing 40 mM KCl as stated in the legends to the figures.

Sucrose gradient centrifugation was performed using a linear sucrose gradient (0–38% (w/w)) in 2 mM Tris-HCl, pH 7.4. The gradient was overlayered with 0.2 ml of either liposomes, aminopeptidase-lysophospholipid micelles or the protein-lipid recombinant and centrifuged in a Beckman ultracentrifuge (Rotor SW 40) at $284000 \times g$ for 18 h. Fractions of 0.3 ml were collected from the bottom of the tube.

Fractions from the gel chromatography and from gradient centrifugation were analyzed for

lipid by measuring radioactivity and for protein by measuring aminopeptidase activity. The sucrose concentration of the gradients was measured by refractometry.

The mode of insertion of the protein into lipid vesicles was studied by inhibition by anti-aminopeptidase and by trypsin treatment of the protein-lipid recombination essentially as described earlier [15].

Results and Discussion

Solubilization of the microvillus membrane

A rapid separation procedure was developed, as solubilization times of only 10 min at room temperature were found to be adequate, and instead of the time-consuming centrifugation step, which normally is used to remove insoluble material, a Millipore filtration was introduced. In order to ensure that microvillus vesicles themselves did not penetrate the filter (pore size 0.22 μm), a control of microvillus vesicles treated with buffer without detergent was always included in the experiments. Solubilization of the microvillus membrane with lysophospholipid as detergent are compared to solubilization by Triton X-100 by measuring protein concentration and the activity of the two microvillus enzymes, aminopeptidase N (EC 3.4.11.2) and alkaline phosphatase (EC 3.1.3.1). The results are shown in Table I. Aminopeptidase is a protein with a large hydrophilic part protruding from the membrane and only anchored by a short peptide, while alkaline phosphatase is a protein

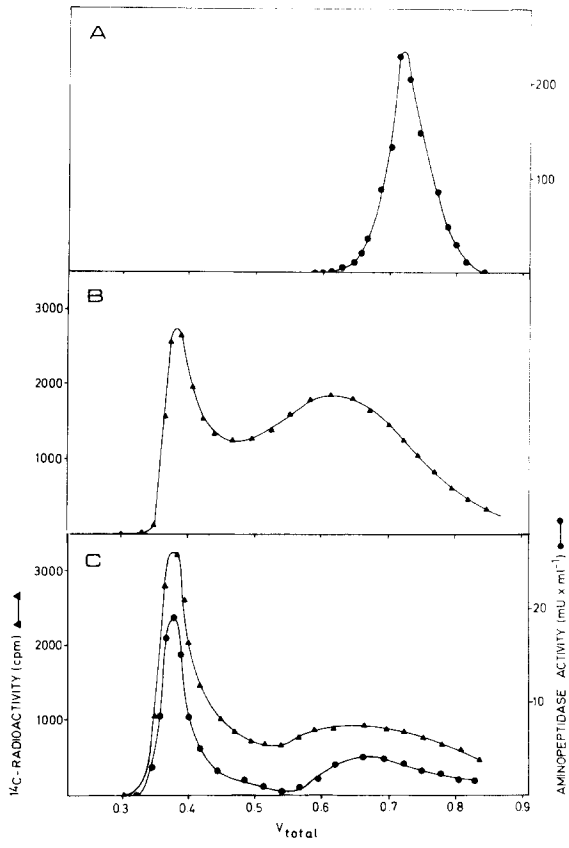

Fig. 1. Elution profile on Sepharose 4B of: aminopeptidase-lysophosphatidylcholine micelles (A), freshly prepared liposomes (B), protein-lipid recombinant obtained at pH 5.2 (C). 200 μl of either of the samples was applied to a column (0.64×55 cm, V_{total} = 35.2 ml) equilibrated and eluted with 10 mM Hepes, pH 7.0 containing 40 mM KCl at a flow rate of 2.0 ml per hour and fractions of 0.5 ml were collected and assayed for ^{14}C radioactivity and aminopeptidase activity.

TABLE I

SOLUBILIZATION OF PROTEIN AND ENZYMES FROM THE MICROVILLUS MEMBRANE OF THE PIG SMALL INTESTINE

The values are the average of double determinations, and represent the percentage solubilized protein and enzymes in the Millipore filtrate in relation to the protein concentration or enzyme activity before filtration. As a control, the microvillus membrane was treated with buffer without detergent.

Detergent	Protein	Aminopeptidase	Alkaline phosphatase
Control	10	1	0
0.5% Triton X-100	91	100	70
1.0% Triton X-100	96	98	83
0.5% Lysophosphatidylcholine	91	90	91
1.0% Lysophosphatidylcholine	97	74	100
0.5% Lysophosphatidic acid	98	70	67
1.0% Lysophosphatidic acid	96	56	59

more burried in the lipid bilayer of the membrane [16], With 0.5% solution of lysophosphatidylcholine both aminopeptidase and alkaline phosphatase were solubilized to the same degree, as when Triton X-100 was used. Lysophosphatidic acid, however, was not quite as good a detergent as lysophosphatidylcholine or Triton X-100 as the two proteins were solubilized only 50–70%. Immediately after solubilization, the filtrate was applied to the immunoadsorbent column in order to isolate pure aminopeptidase-lysophospholipid micelles. The microvillus protein-lipid micelles were only stable for about 2–3 h after which time precipitation started. The aggregation of the micelles can partly be explained as a result of hydrolysis of the lysophospholipid by lipases solubilized from the microvillus vesicles. Lysophosphatidic acid was more susceptible to hydrolysis than lysophosphatidylcholine. Hydrolysis could only partly be prevented by addition of EDTA to the solubilization buffer. When part of the lysophospholipid is hydrolyzed a mixture of free fatty acids and lysophospholipid occur and these two components will tend to adopt a bilayer configuration [17], instead of the micellar configuration adopted by the components when these are present alone [18].

Isolation and characterization of aminopeptidase lysophospholipid micelles

Pure aminopeptidase-lysophospholipid micelles were isolated by immunoadsorbent chromatography essentially as described [8,14], except that lysophospholipids were used as detergents instead of Triton X-100 throughout the entire chromatographic procedure. The aminopeptidase eluted from the immunoadsorbent column by hypotonic desorption in 0.05% lysophospholipid was analyzed by gel filtration and by sucrose gradient centrifugation. As shown in Fig. 1A the enzyme eluted from a Sepharose 4B column equilibrated in 10 mM Hepes, pH 7.0, containing 40 mM KCl and 0.05% lysophospholipid as a single peak at $0.72 \times V_{total}$, similar to aminopeptidase-Triton X-100 micelles, and corresponding to a molecular weight of approx. 400 000.

Liposomes

The liposomes were prepared by sonication of a total lipid extract of the microvillus membrane. The preparation appeared in electron micrographs of thin sections (Fig. 2A) as a rather uniform preparation of vesicles surrounded by a single membrane, which appeared triple-layered (Fig. 2B). Vesicles with multi-layered membranes were never seen. The size of the vesicles varied between 50 and 100 nm with a few vesicles around 200–300 nm. Freshly prepared lipsomes eluted with an elution profile on Sepharose 4B as seen in Fig. 1B. The liposome preparation gave a bimodal distribution, corresponding to a peak at $0.38 \times V_{total}$, and a broad peak at $0.62 \times V_{total}$. By standing or by decreasing the pH to 5.2, the amount of the smaller liposomes, corresponding to the peak at $0.62 \times V_{total}$ decreased, as a result of fusion. Fusion of small liposomes is a known phenomena and has

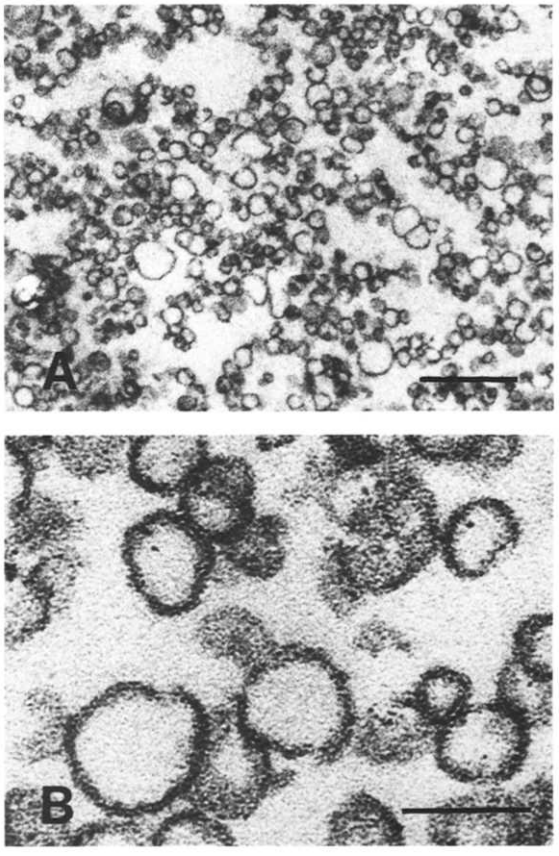

Fig. 2. Electron micrographs of thin sections of liposomes prepared by sonication of a total lipid extract of the microvillus membrane of the pig small intestine. (A) Bar 0.5 μm. (B) The same preparation at higher magnification. Bar 100 nm.

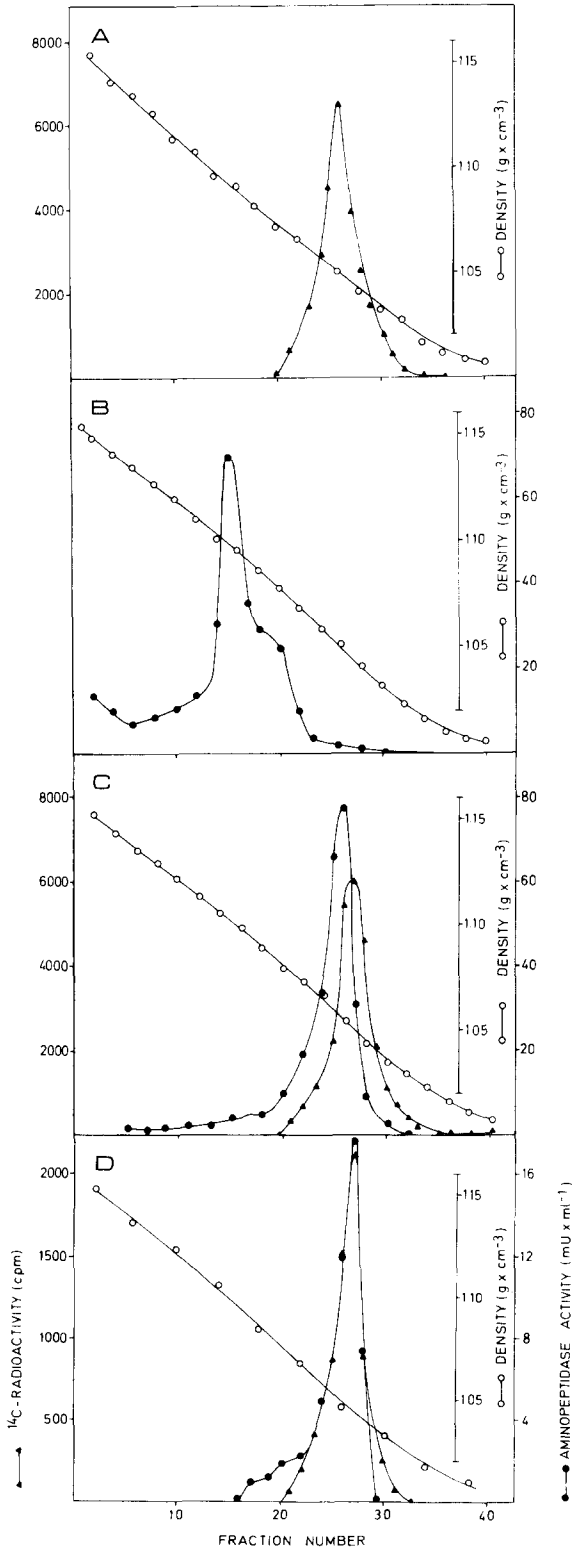

Fig. 3. Sucrose gradient pattern of: liposomes (A), aminopeptidase-lysophosphatidylcholine micelles (B), protein-lipid-recombinant formed at pH 5.2 using lysophosphatidylcholine as detergent (C), and protein-lipid recombinant formed at pH 5.2 using lysophosphatidic acid as detergent (D). ▲———▲, ^{14}C radioactivity; ●———●, aminopeptidase activity, ○———○, density (sucrose concentration).

recently been examined by Wong et al. [19]. In sucrose gradient centrifugation the liposomes appeared as one peak at a density of 1.048 ± 0.006 g/cm^3 (mean $\pm$S.D. of 10 experiments) (Fig.3A).

Reconstitution and characterization of the protein-lipid recombinant

Reconstitution was performed by mixing of liposomes with aminopeptidase-lysophospholipid micelles with or without addition of acetic acid in order to decrease the pH. The lipid to lysophospholipid molar ratio in the reconstitute was 15. This lysophospholipid concentration did not cause solubilization of the liposomal membrane as judged from centrifugation experiments, nor did it cause any morphological changes judged from electron micrographs.

Incorporation of protein into lipid vesicles was assessed by comparison of protein and lipid elution profiles both in gel chromatography and in sucrose gradient centrifugation. The protein-lysophospholipid micelles eluted from the Sepharose column at a significantly higher volume than most of the liposomes. As seeen in Fig. 1 the elution volume of protein-lysophospholipid micelles is $0.72 \times V_{total}$, while the liposomes eluted at $0.37 \times V_{total}$ and $0.62 \times V_{total}$. Protein aggregates, which were formed when no incorporation occurred and the column was equilibrated with detergent free buffer, eluted as a broad band which coincided with the small liposomes. Thus coincidence of protein and lipid profiles can only for the larger liposomes be an indication of incorporation (Fig. 1). In a sucrose gradient when centrifuged alone, the aminopeptidase activity was found at densities between 1.08 and 1.15 g/cm^3 (Fig. 3B), while the liposomes banded with a peak at a density of 1.05 g/cm^3 (Fig. 3A). Thus overlap of protein profiles with lipid profiles gives a good indication of protein incorporation (Fig. 3C and D). However, a small shift in the lipid and the protein profile is

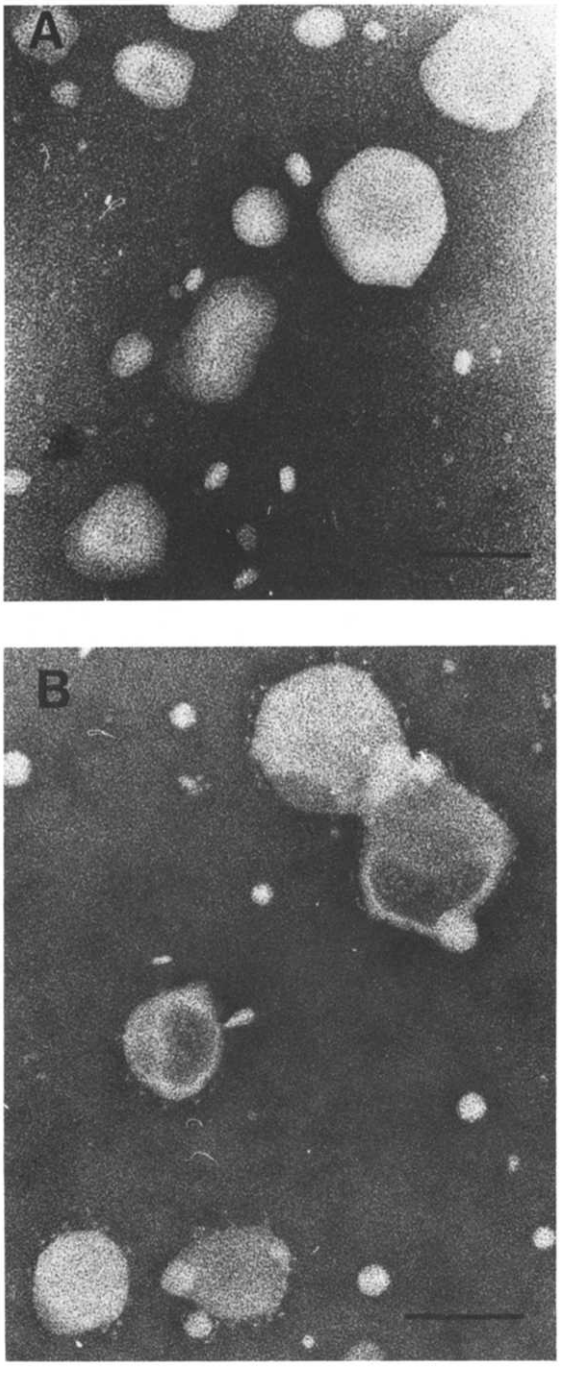

Fig. 4. Electron micrographs of negatively stained preparations of: liposomes, mixed with lysophosphatidylcholine, but without protein (A), reconstituted aminopeptidase (B). Bar 100 nm. The weight ratio of lipid to protein was 40.

observed when lysophosphadidylcholine is used as the detergent. This shift might represent a heterogeneity in the reconstituted vesicles. Thus, it is apparent from electron micrographs of negatively stained preparations that the very small vesicles (diameter 20–30 nm) do not contain protein (Fig. 4B).

When fusion of the micelles and the liposomes were performed at pH 7.0 about 30% of the protein added was incorporated into the lipid vesicles. Lowering pH resulted in an increase in the amount of protein incorporated into the lipid vesicles (Fig. 5). Fusion experiments at pH 5.2 resulted in incorporation of close to 100% of the protein added. pH-dependency of fusion is a well-known phenomenon, as also observed by White and Helenius [20] with fusion of Semliki Forest virus membrane with liposomes. No decrease in enzymatic activity of the aminopeptidase was observed by decreasing the pH during the time period of reconstitution.

Addition of Triton X-100 in a concentration of 0.05% to the protein-lipid recombinant did not remove any aminopeptidase activity from the

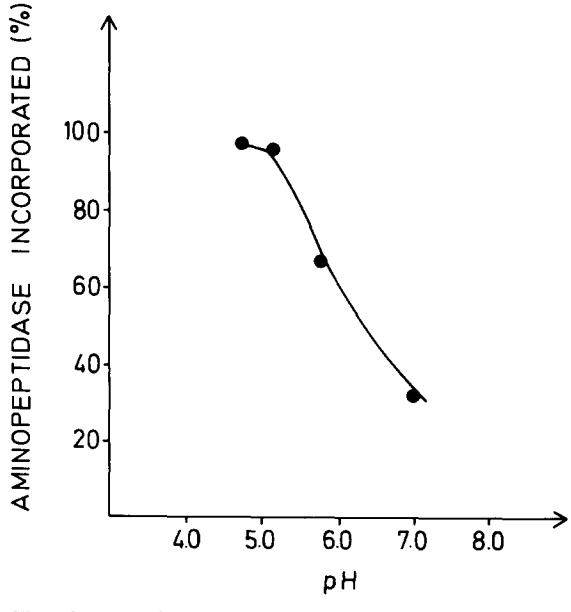

Fig. 5. pH dependency of fusion of aminopeptidase-lysophospholipid micelles with liposomes. Freshly prepared liposomes and aminopeptidase-lysophosphatidylcholine micelles were mixed. Aliquots were adjusted to pH values between 5 and 7 by addition of 1% acetic acid. After sucrose gradient centrifugation of each sample the amount of protein incorporated into the lipid was determined.

TABLE II

ASSOCIATION OF AMINOPEPTIDASE ACTIVITY WITH LIPID VESICLES AFTER TREATMENT WITH TRITON X-100 OR TRYPSIN

The protein-lipid recombinant formed by fusion at pH 5.5 as described in Materials and Methods was centrifuged at 100000 × *g* in a Beckman Airfuge. The vesicles were suspended without washing in buffer or buffer containing either Triton X-100 (0.05% or 1.00%) or trypsin (0.15 mg) and recentrifuged at 100000 × *g* for 1 h. The aminopeptidase activity and the radioactivity (lipid) was measured in the supernatant and the pellet. The results are the percentage of the initial activity and radioactivity associated with the vesicles.

	In pellet		In supernatant	
	Aminopeptidase activity	Lipid	Aminopeptidase activity	Lipid
Buffer	86	90	14	10
+0.05% Triton X-100	86	89	14	11
+1.00% Triton X-100	–	–	100	98
+Trypsin	14	96	86	4

vesicles, indicating that no aminopeptidase activity was loosely bound to the vesicles, whereas addition of 1% Triton X-100 to the recombinant solubilized the aminopeptidase, but did not increase the enzyme activity (Table II). The activity was inhibited completely by addition of antibody against aminopeptidase, to the recombinant and trypsin digestion released more than 80% of the aminopeptidase activity from the vesicles.

Electron microscopy

Reconstitution of the aminopeptidase with liposomes was visualized by negatively staining followed by electron microscopy. In the protein-lipid recombinant in contrast to liposomes without added protein, but containing lysophospholipids, a number of particles were seen protuding from the membrane (Fig.4). The morphology of the extraneous part of the molecule is similar to the morphology observed in negatively stained preparations of aminopeptidase incorporated into liposomes by the use of the sodium deoxycholate-dialysis method [15]. The interior of the vesicles, both with and without protein, is generally free of staining reagent, indicating that the vesicles are impermeable to these reagents, which are hydrophilic and have molecular weights of about 3500.

General conclusions

The present report describes a method for reconstitution of a protein into lipid vesicles by use of natural detergents. The method is based on fusion of protein-lysophospholipid micelles with liposomes prepared by sonication. The method is used for reconstitution of the intestinal microvillus enzyme, aminopeptidase N into lipid vesicles prepared from microvillus membrane lipids, which is a mixture of neutral lipid, phospholipids, neutral glycolipids and ganglioside in the weight ratio of 18 : 50 : 29 : 2 [13]. A protein-lipid recombinant is obtained according to analysis by gel permeation chromatography and sucrose density gradient centrifugation. Trypsin digestion of the protein-lipid recombinant released all the activity from the vesicles. These results together with inhibition studies by anti-aminopeptidase and lack of activation by addition of Triton X-100, to the recombinant indicate that the aminopeptidase is inserted undirectionally into the membrane. The incorporation is visualized by negative staining followed by electron microscopy. In the micrographs, the aminopeptidase appear as a structure similar to the results obtained when aminopeptidase is inserted into lipid vesicles by the sodium deoxycholate dialysis method [15], and similar to the model of the aminopeptidase in the intact microvillus membrane [16].

Lysophospholipids are intermediates in lipid metabolism and are natural, but minor, constitutents of all biological membranes, and thus have advantages in reconstitution experiments over organic solvent and artificial detergent. This method of reconstitution could thus be used especially in cases where the protein is sensitive to

synthetic detergents [21]. We have developed the present method for incorporation of proteins into a natural membrane like the endoplasmic reticulum, a membrane which contains the enzymes of the phospholipid synthesis, and thus the incorporated lysophospholipid will be converted to a non-detergent form, namely a phospholipid. Used in this way the method may be valuable tool in studies of how certain newly synthesized proteins are inserted into membranes.

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