INCORPORATION OF HYDROPHOBIC AMINOPEPTIDASE FROM HOG KIDNEY INTO EGG LECITHIN LIPOSOMES: NUMBER AND ORIENTATION OF AMINOPEPTIDASE MOLECULES IN THE LECITHIN VESICLES*

Hans WACKER, Fritz MÜLLER and Giorgio SEMENZA

Laboratorium für Biochemie, Eidgenössische Technische Hochschule, Universitätstrasse 16, CH 8006 Zürich, Switzerland

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1. Introduction

The brush border of renal tubuli contain an aminopeptidase, which is bound to, or is a building block of, the apical membrane [2,3]. Extraction with Triton X-100 yields an aggregated form of the enzyme which can be transformed into a low molecular weight form by the action of trypsin [4]. During this transformation a fragment of approximately 10 000 daltons is released; this probably represents the hydrophobic portion which anchors the protein to the lipid matrix of the original membrane [4]. This 'anchor' is likely to be of importance in reconstitution experiments, as has been shown for other amphipatic proteins [5]. Recently, a membrane-bound aminopeptidase from the small intestine has been shown to have a hydrophobic region [6].

Peptides cross the brush border membrane (of the small intestine) through route(s) which are different from those utilised by amino acids (for a review, see [7]). Utilising pure membrane vesicles from intestinal brush border membranes [8], it has been shown in this laboratory that peptides may cross the brush border at the same time as they are hydrolysed. The interest in studying the mode of incorporation of aminopeptidase into monolamellar vesicles of pure lipids is, therefore, obvious. To this goal we utilised aminopeptidase from (hog) kidney, rather than intestine, because the former can be obtained more easily in a fully undegraded form; this is due to the lack of very powerful proteases in kidney brush borders [4] although the two brush borders are very similar, both morphologically and biochemically.

We intend to present in the following, the successful reconstitution of the hydrophobic (non trypsin-treated) aminopeptidase from hog kidney into artificial liposomes.

2. Materials and methods

Detergent-solubilised aminopeptidase was prepared according to [4], its trypsin-solubilised form according to [9]. Egg lecithin, grade I, was obtained from Lipid-Products (South Nutfield, UK) and stored as a chloroform solution under nitrogen at −20°C. Cholic acid (Fluka, puriss.), four times recrystallised from acetone/water 4:1 (v/v), was neutralised with NaOH. [^{3}H] cholate was from NEN.

Aminopeptidase activity was assayed using L-leucine-4-nitroanalide as substrate [10]. Phosphate was determined according to [11] and cholate according to [12].

Protein-containing liposomes were obtained by drying a volume of the lecithin stock solution in chloroform, corresponding to 20 mg, in a test tube under a stream of nitrogen, and then for 2 h at 10^{-3} Torr. Cholate in buffer (10 mM Tris/HCl, pH 7.2 containing 0.1 M NaCl, 0.1 mM EDTA, and 0.02% NaN_3) was added. The clear solution was mixed with the enzyme, as reported for other proteins [12]. Final concentration was 2% cholate, 1% lecithin, and 0.1% protein. After overnight incubation at 4°C the mixture was passed through a Sephadex column to remove cholate [14].

*Part of the data were taken from the Diplomarbeit of F. Müller carried out in the winter term 1975/76 [1].

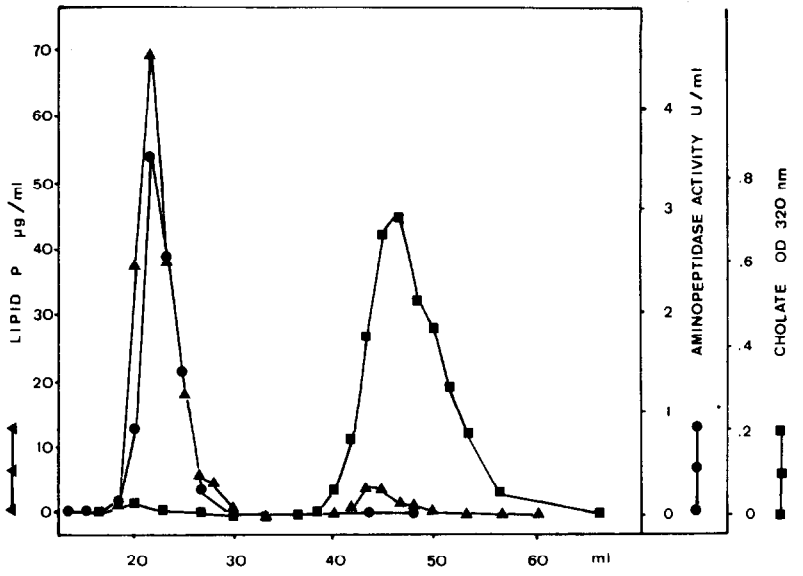

Fig.1. Removal of cholate by gel filtration. The column (Sephadex G 50, medium, 1.7 × 18 cm) was equilibrated and eluted at room temperature with 10 mM Tris HCl, pH 7.2, containing 100 mM NaCl, 0.1 mM EDTA and 0.02% NaN_3. Aminopeptidase, 1 mg, egg lecithin, 10 mg, cholate, 20 mg, in 1 ml of above buffer were applied. Flow rate: 20 ml/h; 1.6 ml per fraction. Enzyme recovery was 80–90%.

3. Results and discussion

3.1. *Reconstitution*

Fig.1 shows the elution pattern of the cholate–lecithin–protein mixture from a Sephadex G-50 column. 99.9% of the cholate could be removed by this procedure as determined with [^{3}H]cholate on the pooled enzyme peak. Electron microscopy showed vesicles with sizes varying between 200 and 1000 Å and a few multilamellar aggregates (fig.2A).

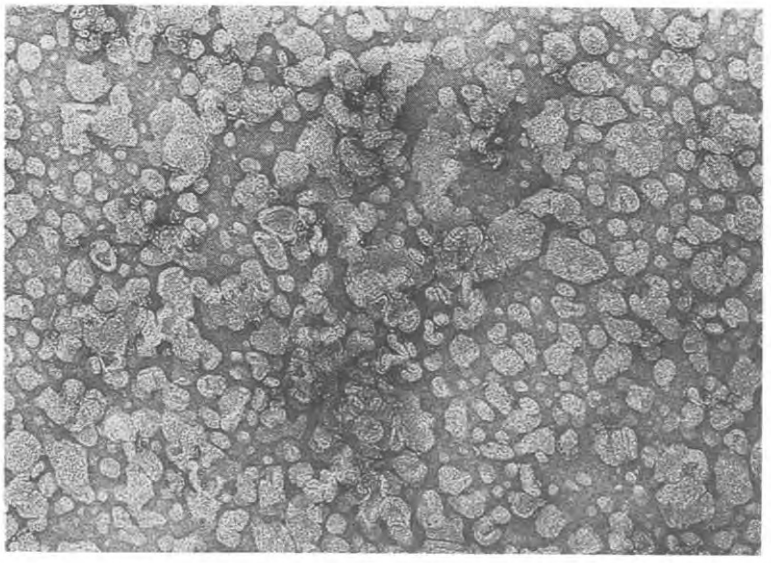

Fig.2a

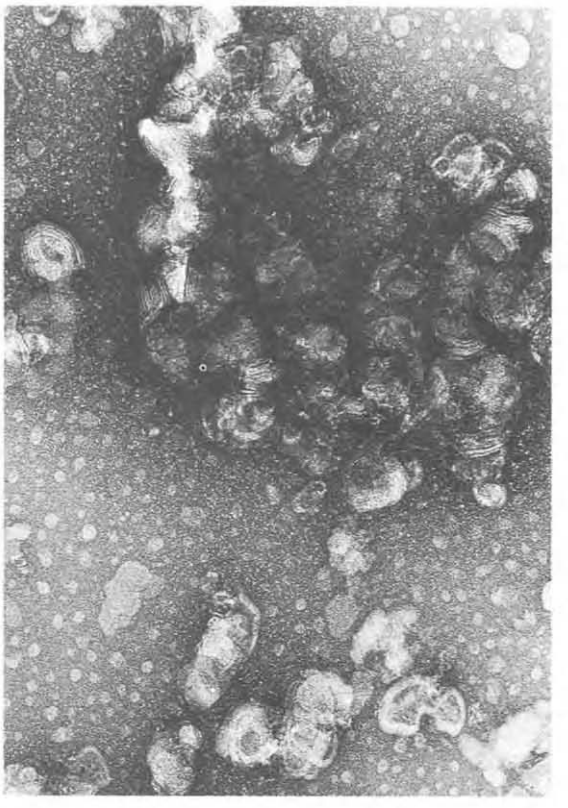

Fig. 2b

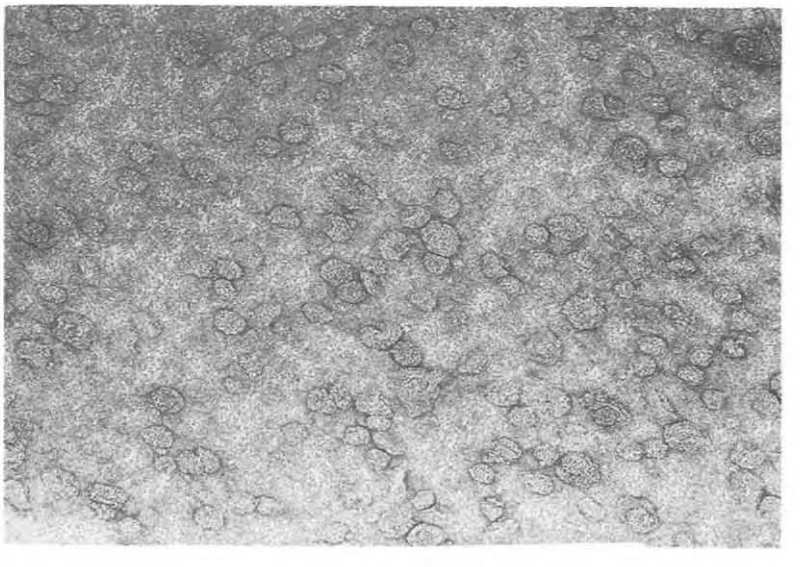

Fig. 2c

Fig. 2. Electron micrographs of reconstituted vesicles. Negative staining was performed with 2% uranyl acetate. Grids (400 mesh) coated with Formvar were floated on the surface of a droplet of sample for 1 min and then transferred to a drop of uranyl acetate solution. After 5 min excess fluid was blotted off with filter paper. The samples were observed in a Philips 301 electron microscope. Magnification: × 120 000. (A) Sample after removal of cholate on a Sephadex column (fig.1). (B) Front peak of the Sepharose chromatography (fig.3A). (C) Retarded peak of the Sepharose column (fig.3A).

3.2. *Fractionation of protein-containing liposomes*

The front peak from Sephadex G-50 of fig.1, which contained all the aminopeptidase activity, was concentrated by vacuum dialysis and fractionated on Sepharose 4B (fig.3). The elution pattern corroborated the heterogeneity which had been seen in the electron micrographs of the same fraction (fig.2A): Some material (approx. 10% of the total lecithin and approx. 20% of the total aminopeptidase activity) emerged in the void volume V_0, while the rest was retarded; the phosphate/enzymatic activity changed throughout the peak. It should be noted that even co-elution of lipid and protein could not by itself be taken as evidence for enzyme–lipid interaction, because liposomes prepared by the same method in the absence of protein, or aminopeptidase aggregates, when processed individually emerged with almost identical elution volumes (compare fig.3 A,B,C,D.). Liposomes made of lecithin alone do not contain any material that is excluded from the column [14].

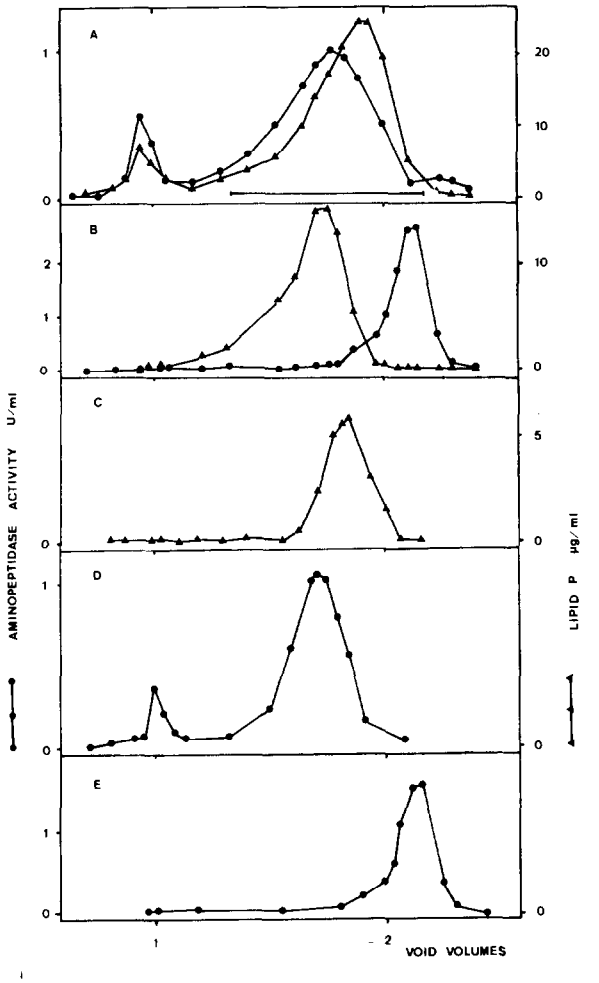

Fig.3. Gel filtration on Sepharose 4B of the lipid–protein mixture after removal of cholate. The column (2.0 × 33 cm) was equilibrated and eluted with buffer, at a flow rate of 4 ml/h. Samples of approx. 1 mg protein and 10 mg lecithin in 1 ml of buffer were applied. Enzyme recovery was 85–90%. (A) Detergent form of aminopeptidase plus lecithin after removal of cholate by gel filtration from Sephadex G50, (fig.1). (B) Trypsin-solubilised aminopeptidase submitted to an identical treatment. (C) Lecithin liposomes, without protein, prepared by the same procedure [14]. (D) Detergent form of aminopeptidase. (E) Trypsin-solubilised aminopeptidase.

The two peaks from the Sepharose chromatography of hydrophobic aminopeptidase plus lecithin (fig.3A) were examined, after negative staining, with the electron microscope. The front peak consisted of multilammellar aggregates (fig.2B), whereas the retarded, heterogeneous peak consisted of vesicles of diameter ranging from 250 to 300 Å (fig.2C).

Since gel filtration alone could not prove interaction of protein and lipids (see above), and the elution profiles of aminopeptidase activity and phosphate indicated inhomogeneity of the retarded peak, gradient centrifugation was used for further fractionation and characterisation.

Fig.4 shows a gradient centrifugation of the retarded peak from the Sepharose column. Several peaks are now apparent; that at the top of the gradient contained phosphate but no aminopeptidase activity (it thus consisted of protein-free lecithin liposomes), fig.4B. The peak at the bottom of the gradient showed enzyme activity only (unreacted peptidase), fig.4C. Three more peaks contained both phosphate and aminopeptidase activity and banded at a density value higher than pure phospholipid but lower than the protein. This would be expected for a lipid–protein complex.

After an additional density gradient centrifugation, each of the three peaks, which contained both phosphate and aminopeptidase activity, yielded protein/phosphate molar ratios which were constant throughout the fractions of each peak and corresponded to 5000–6000, 2500–2600, and 1400–1900 molecules of lecithin per molecule of protein, respectively. Protein-free liposomes prepared similarly from lecithin have a Stoke's radius of 150 Å [14], i.e. they are composed of approx. 5800 lecithin molecules per

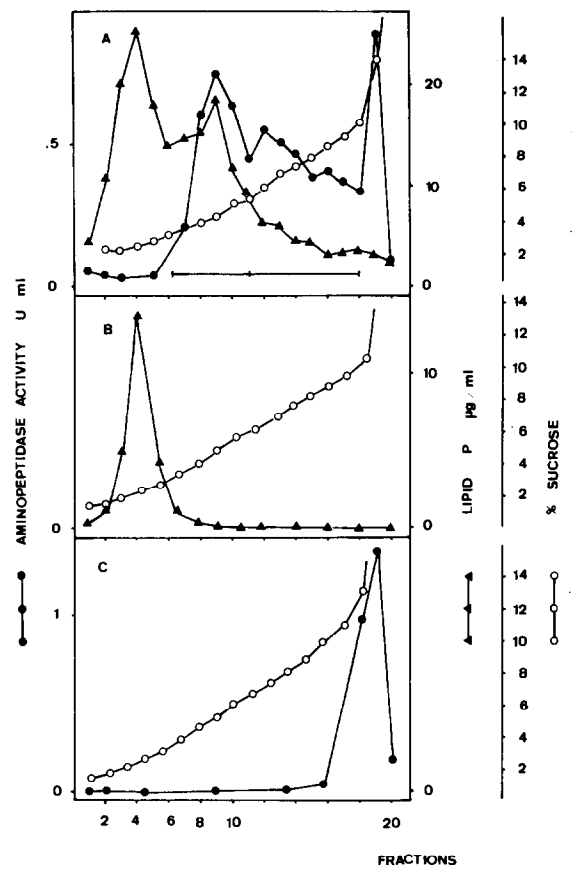

Fig.4. Gradient centrifugation under quasi-equilibrium conditions. A linear sucrose gradient (0–12% sucrose) was made in a 5 ml cellulose nitrate centrifuge tube. The sample, in 0.15–0.25 ml buffer, was layered on top of the gradient. After centrifugation for 20 h at 25 000 rev/min (58 000 *g*) in a SW 50.1 rotor of a Beckman Spinco 50 B centrifuge the gradient was displaced upwards with a 40% sucrose solution which was pumped through a steel needle in the bottom of the tube. Fractions of 0.25 ml were collected. Sucrose concentration was read with an Abbe hand refractometer (Atago optical works Co., Ltd.). (A) Aliquot from the retarded peak of fig.3A containing approx. 1 mg lipid and 0.1 mg protein. (B) Pure egg lecithin liposomes, approx. 1 mg, prepared by the cholate-Sephadex method [14]. (C) Detergent form of aminopeptidase without lipids added, approx. 0.1 mg.

liposome (assuming a bilayer thickness of 50 Å and an area of 70 Å^2 per lecithin molecule). Assuming that the aminopeptidase-containing liposomes are composed of a similar number of lecithin molecules (which is made likely by the small difference, if any, in their V_e values on Sepharose 4B, see fig.3A, and in the electron micrograph, see fig.2C), the phospholipid/aminopeptidase ratios given above correspond to liposome populations where each liposome contains either 1,2 or 3 aminopeptidase dimers of 280 000 daltons [4], respectively (see also [15]).

When trypsin-solubilised, rather than detergent-solubilised, aminopeptidase was subjected to the same procedure, the aminopeptidase activity was clearly separated from the liposomes (fig.3B); the enzyme and the lipids emerged from the column with the same elution volume as when each of them was chromatographed alone (fig.3C, E), and no material appeared at the void volume. Thus, trypsin-solubilised aminopeptidase did not interact with lecithin. This observation is at partial variance with what is known about the papain-solubilised intestinal sucrase-isomaltase, which still aggregates at moderately high ionic strength [16] (although less than the detergent-solubilised form does [17]) and can still bind to lipids [18,19] - even if the latter carry a negative charge [19] and in the presence of high ionic strength [19,20]. Presumably, papain treatment of sucrase-isomaltase does not totally remove its hydrophobic part, whereas trypsin treatment of aminopeptidase totally, or almost totally, does so.

3.3. *On the orientation of hydrophobic aminopeptidase in monolamellar liposomes*

This problem was tackled in a two-fold approach: (i) by the susceptibility of liposome-bound aminopeptidase to trypsin; this detached the enzyme from the liposome; (ii) by measuring the aminopeptidase activity of enzyme-containing liposomes in the presence and absence of lytic concentrations of detergents.

(i) Two fractions were taken as indicated in fig.4A, the first from the peak having the highest protein/lipid ratio, the other from both other peaks with a lower ratio. Half of each fraction was incubated with trypsin, the other half served as reference in gradient centrifugation under equilibrium conditions. The results are shown in fig.5. The first lipid-protein complex was essentially homogeneous and banded at a density of approximately 1.048 g/ml which is in the density range of low density serum lipoproteins. After tryptic digestion the phosphate peak was shifted to the (lower) density of protein-free lecithin vesicles (fig.5C),

while the enzyme activity banded at a density greater than 1.1. (compare fig.5D). The fractions containing 2 or more molecules of protein per liposome banded at 1.06 and 1.08 g/ml. After tryptic digestion most of the aminopeptidase activity banded again at a density greater than 1.1, although some still banded at a low density. This indicated that most protein, but not all, had been released from the liposomes, and that perhaps some liposomes containing one aminopeptidase molecule only were formed (figs.6A and B).

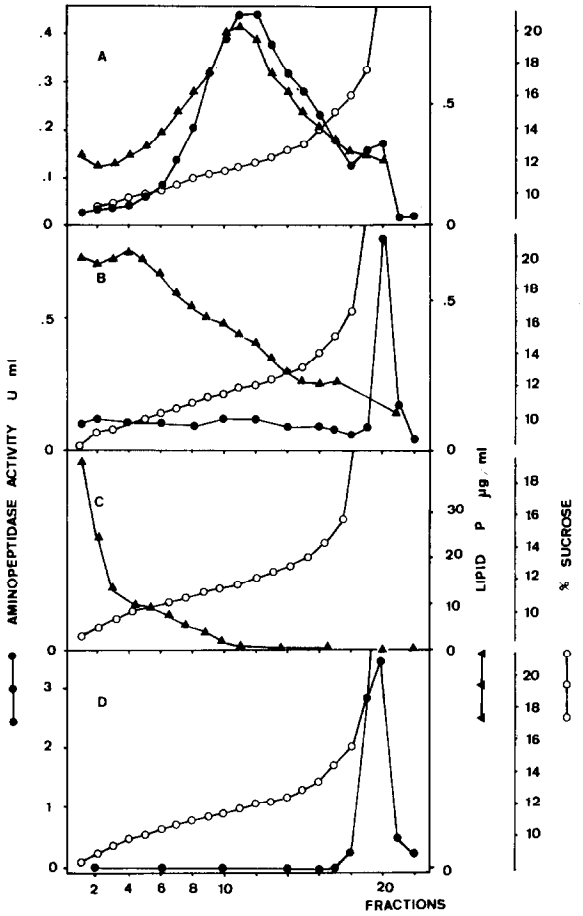

Fig.5. Gradient recentrifugation under equilibrium conditions of the peak from fig.4A with a lipid/protein molar ratio of 6000:1 (fractions 7–11) before and after tryptic digestion. Fractions 7–11 from fig.4A recentrifuged as described below under 5A; the peak obtained was divided into two aliquots and processed as follows: (A) One aliquot approx. 0.05 mg protein, 0.8 mg lecithin in 0.2 ml 20% sucrose) which served as control, was placed at the bottom of a 5 ml centrifuge tube. Over it, a linear sucrose gradient (9–14% sucrose) was formed. Centrifugation for 16 h at 40 000 rev/min. (B) The same sample as in fig.5A was incubated for 60 min at room temperature with 0.5 mg trypsin. After addition of sucrose to 20%, the sample was centrifuged as in 5A. (C) Egg lecithin liposomes [14], approx. 1 mg, in 0.2 ml 20% sucrose. (D) Trypsin-solubilised aminopeptidase (0.14 mg) in 0.2 ml sucrose.

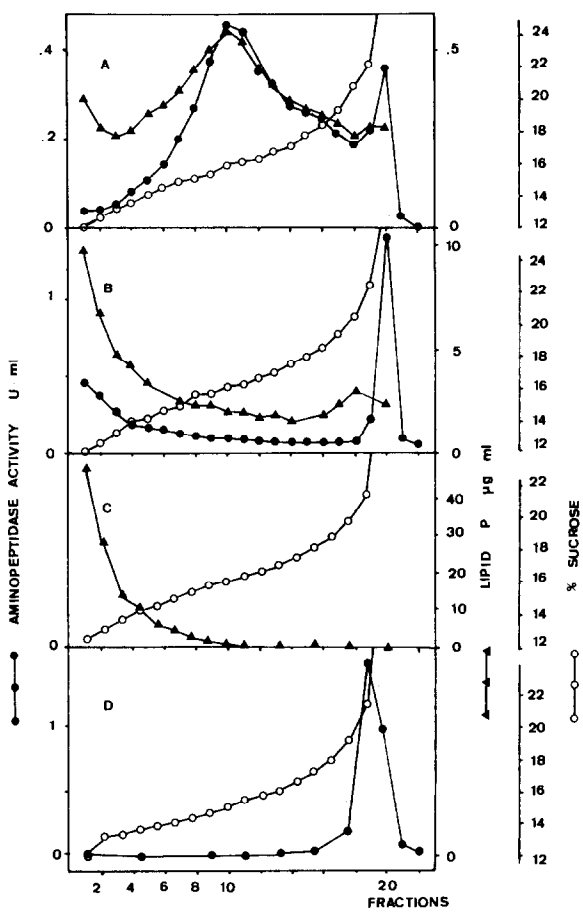

Fig.6. Gradient centrifugation under equilibrium condition of the fractions (12–18) from fig.4A with low lipid/protein ratio before and after tryptic digestion. Fractions 12–18 from 4A were recentrifuged as described below under 6A; the sample obtained was divided into two aliquots and processed as follows: (A) One aliquot (0.06 mg protein, 0.4 mg lecithin 0.2 ml 25% sucrose) served as control, and was placed at the bottom of a centrifuge tube. Over it, a linear sucrose gradient (13–19% sucrose) was made. Centrifugation as in fig.5. (B) The same sample as in fig.6A was incubated with 0.6 mg trypsin, as in fig.5B. After addition of sucrose to 25%, the sample was centrifuged as in 6A. (C) Egg lecithin liposomes [14], approx. 1 mg. (D) Trypsin-solubilised aminopeptidase, approx. 0.1 mg.

Table 1
Aminopeptidase activity of enzyme-containing liposomes in the presence and absence of lytic concentrations of Triton X-100 and cholate

Sample	Percent change in relative activity after detergent treatment.
Total liposomes (front peak on Sephadex, see fig.1)	0 (Triton)
id.	−10 (Cholate)
Liposomes containing one aminopeptidase molecule (from gradient centrifugation, fig.4A, fraction 9)	+ 2 (Triton)

These results showed, therefore, that detergent-solubilised aminopeptidase interacted with monolamellar lecithin liposomes and formed protein–lipid complexes of discrete protein/lipid ratios, while the trypsin-solubilised enzyme did not. In the original kidney brush-border membrane also, aminopeptidase is susceptible to solubilisation by proteolytic treatment [2].

(ii) Addition of lytic amounts of Triton or cholate to the aminopeptidase containing liposomes failed to increase the enzymatic activity (table 1), showing that the substrate had free access to the active site of aminopeptidase. This suggested an asymmetric distribution of the protein in the reconstituted system.

Acknowledgements

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Note added in press:

Pattus et al. (Biochem. Biophys. Res. Comm., 69 (1976) 718–723) have just reported the incorporation of the the detergent form of hog intestinal aminopeptidase into liposomes. They also failed to detect any interaction between trypsin-solubilised form of the enzyme and lipids. Among the major differences between their results and ours are: (i) the detergent form of the intestinal aminopeptidase is always contaminated with sizeable amounts of the trypsin form, which reduces the incorporation of the activity into lipids; (ii) the liposomes obtained by us were monolamellar, which permitted estabilishing the stoichiometry of interaction between enzyme and lecithin vesicles.

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