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IMMOBILIZATION OF PROTEIN MOLECULES ON LIPOSOMES

ANCHORAGE BY ARTIFICIALLY BOUND UNSATURATED HYDROCARBON TAILS

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Abstract—A method for the immobilization of trypsin, a hydrophilic nonmembrane protein, on a liposomal surface has been developed. The technique consists of covalent coupling of linoleoyl residues to the protein globules and consequent binding of linoleoyl trypsin to liposomes by a detergent dilution method. The immobilized protein preserved its biological functions: specific esterolytic catalytic activity and ability to bind to a macromolecular trypsin protein inhibitor. Liposomes carrying immobilized trypsin were able to sequester glucose with the same efficiency as liposomes without trypsin.

According to the current point of view, the most promising method for directing liposomes towards particular cells is to modify the liposomes with molecules that have a specific affinity for these cells [1–3]. There are such proteins as immunoglobulins (or their fragments), enzymes with their specific sorptive sites, and lectins, which are potentially suitable for this purpose. Two approaches for the immobilization of nonmembrane hydrophilic proteins on liposomes were devised: noncovalent and covalent binding. Noncovalent binding (adsorption of proteins on, or insertion into, liposomal membrane [4–8]) is of limited utility (see Ref. 3 for the critical review). The covalent binding approach was developed by analogy to well known methods of enzyme immobilization on a variety of supports [9–12] and seems to be very promising.

Recently, a third approach to the immobilization of proteins on the liposomal surface was suggested, which is a combination of the first two approaches [13, 14]. It includes a covalent binding of long hydrophobic chains of palmitic acid to a protein and consequent adsorption of the protein onto the liposomal surface due to the protein tail–liposome hydrophobic interactions.

However, the use of palmitic acid derivatives as a hydrophobic anchor has led to a highly heterogeneous immobilization [13, 14]. More than half of the liposomes were protein-free after an attempted immobilization of palmitoyl chymotrypsin [14].

It is not clear why some of the liposomes did not bind protein molecules or bound much less than others. One of the possible suggestions is that the conformational properties of the hydrophobic tails may be important for the immobilization process. The average conformation of long saturated hydrocarbon chains should be rather coiled due to free rotations about each C–C bond. It is possible that such a shape may make it sterically difficult for the tail to penetrate the liposomal surface or to participate in lipid bilayer formation. Polyunsaturated hydrocarbon chains with their more expanded conformations might have advantages for these processes.

I report here the use of diunsaturated linoleoyl residues for the hydrophobization of trypsin. This method leads to a homogeneous and extensive immobilization of the protein on the liposomes.

MATERIALS AND METHODS

Materials. The following were obtained from the Sigma Chemical Co. (St. Louis, MO): bovine trypsin (Cat. No. T8003), twice recrystallized with concentration of active sites of 63% [15], *N*-α-benzoyl-L-arginine ethyl ester (a specific substrate of trypsin), picryl sulfonic acid (titrant for H_2N groups of proteins), trypsin inhibitor from beef pancreas, egg yolk phosphatidyl choline (chromatographically purified), cholesterol (>99%), and cholic acid. Cloranhydrides were obtained from Supelco Inc. (Bellefonte, PA). [^{14}C]Cholesterol, [^{3}H]glucose and sodium [^{14}C]cholate were obtained from the Radiochemical Centre (Amersham, U.K.). Sepharose 4B CL was a commercial preparation of Pharmacia Fine Chemicals (Uppsala, Sweden). The rest of the reagents used in this work were analytical grade preparations.

Preparation of liposomes. A detergent-removal method was employed to prepare liposomes from egg yolk phosphatidyl choline and cholesterol (molar ratio 8:2) using [^{14}C]cholesterol as a membrane marker and [^{3}H]glucose as an inner aqueous space

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marker. Solutions of 1% sodium cholate, which contained lipid constituents (total 5 mM), trace amounts of radioactive markers, 0.145 M NaCl, 10^{-2} M Tris–HCl (pH 7.0), and, in some cases, 25 μM trypsin (native or modified) were passed through a Sepharose 4B CL column (1.5 × 50 cm). In preliminary experiments it had been shown that liposomes never contained more than 0.5% (molar) cholate from total liposomal lipids.

Measurement of trypsin catalytic activity. Trypsin activity was determined potentiometrically in a Radiometer titration system by the initial rate of catalytic hydrolysis of a 3 mM solution of the specific substrate *N*-α-benzoyl-L-arginine ethyl ester in 0.145 M NaCl, i.e. at the substrate saturation [16].

Covalent modification of trypsin by acyl chlorides. A 20-μmole sample of a chloroanhydride was added to 10 ml of a 50 μM trypsin solution containing 30 mM sodium cholate, 0.145 M NaCl, and 20 mM benzamidine, a competitive trypsin inhibitor, at pH 8.0 and 0°. Benzamidine was added to prevent autolysis of the enzyme. The mixture was irradiated by ultrasound for 1 min (for mixing) and then incubated for 5 min at 0°, pH 8.0. In this time interval all excessive chloroanhydride underwent hydrolysis (potentiometric data). Titration of H_2N-groups of the protein with picryl sulfonic acid [17] was done to determine the quantity of amino groups acylated by linoleoyl chloride.

Electron microscopy. The electron microscopy studies were performed on a JEM-100C electron microscope. The suspension of liposomes was supplied on a grid covered with formwar film. Samples were negatively contrasted with 5% uranyl acetate.

Statistical treatment of results. Results are represented as means ± standard error of the mean; N is the number of independent experiments.

RESULTS AND DISCUSSION

Covalent modification of trypsin by linoleoyl chloride and palmitoyl chloride. After the modification and centrifugation of the enzyme solution, about 63–68% of the enzyme activity remained in the supernatant fraction. Titration of the enzyme active sites [15] has shown that the inactivation was not due to a change of catalytic properties but was a result of a disappearance of some of the active sites. Active enzyme had the same catalytic constant for *N*-α-benzoyl-L-arginine ethyl ester enzymatic hydrolysis. The number of linoleoyl chains coupled per one trypsin molecule was estimated to be 2.2 ± 0.3, N = 6 (titration by picryl sulfonic acid). The number of palmitoyl chains per trypsin molecule was 2.4 ± 0.4 (N = 4). Native bovine trypsin contains 16 H_2N-groups per molecule [16].

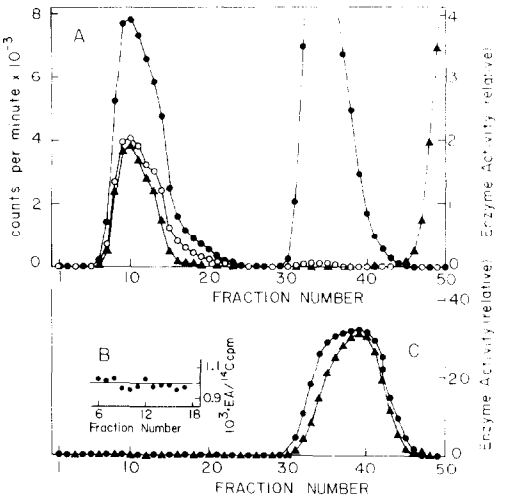

Fig. 1. Immobilization of linoleoyl trypsin on liposomes by a detergent-removal method. (A) Gel chromatography of 25 μM linoleoyl trypsin (●) in an aqueous solution containing 0.145 M NaCl, 0.01 M Tris–HCl (pH 7.0), 4 mM egg yolk phosphatidyl choline, 1 mM cholesterol, 23 mM (1%) sodium cholate and trace amounts of [^{3}H]glucose, 8.5 Ci/mmole (▲), and [^{14}C]cholesterol, 43 Ci/mmole (○). (B) Calculated from the Fig. 1A ratio of trypsin enzymatic activity (EA) to the quantity of radioactive cholesterol. (C) Gel chromatography of 25 μM linoleoyl trypsin (●) or 25 μM native trypsin (▲) dissolved in aqueous solution containing 0.145 M NaCl and 0.01 M Tris–HCl (pH 7.0).

Table 1. Immobilization of trypsin molecules on liposomes*

Preparation	Amount of bound enzyme activity (% initially added to the detergent solution)	Estimated number of trypsin molecules/10^5 lipid molecules	Amount of entrapped glucose (% initially added to the detergent solution)	Enzyme activity remaining after inhibition by an excess† of pancreatic trypsin inhibitor (%)
Liposomes with native trypsin‡	0.6 ± 0.1	3.2	0.51 ± 0.07	49 ± 8
Liposomes with palmitoyl trypsin‡	0.9 ± 0.2	4.8	0.52 ± 0.09	37 ± 4
Liposomes with linoleoyl trypsin‡	12 ± 2	62	0.50 ± 0.09	35 ± 3
Native trypsin in a solution				0
Palmitoyl trypsin in a solution				1.0 ± 0.3
Linoleoyl trypsin in a solution				0.9 ± 0.3

* Results of three independent experiments.
† Twenty-fold molar excess; the same results were obtained for 50- and 150-fold molar excesses of the inhibitor.
‡ All liposome-containing chromatographic fractions were mixed together.

Trypsin immobolization. According to the electron microscopy data, multilamellar liposomes 40–100 nm in diameter were obtained. The results of immobilization are represented in Fig. 1 and Table 1. Native trypsin binds to liposomes in small amounts. Only 0.6% of the originally added enzyme activity could be detected on intact liposomes. Destruction of liposomes by Triton X-100 (final concentration 0.1%, v/v) led to a 12-fold increase of the enzyme activity. The results fit a model in which the vast majority of trypsin molecules are sequestered (encapsulated) inside liposomes and are inaccessible (or not easily accessible) for a hydrophilic substrate (*N*-α-benzoyl-L-arginine ethyl ester), while trypsin molecules which are located on the outer liposome surface (associated with the lipid bilayer) catalyze the hydrolysis. The liposomes were able to sequester a water-soluble hydrophilic marker, glucose, which is consistent with the model. We cannot completely rule out the possibility of substrate (*N*-α-benzoyl-L-arginine ethyl ester) influx into liposomes and participation of the entrapped enzyme in the substrate hydrolysis. Therefore, the enzymatic activity measured in the native trypsin–liposome complex before lysis may not be completely associated with the trypsin molecules located on the outer liposome surface.

Binding of palmitoyl trypsin was also poor (Table 1), probably due to reasons discussed in the beginning of the paper.

Quite different results were obtained for liposome immobilization of linoleoyl trypsin. About 12% of the initially added enzyme activity was detected on intact liposomes (twenty times more than in the case of native trypsin). After destruction of the liposomes, the enzyme activity increased only 1.4 times. Linoleoyl-trypsin-containing liposomes were also able to sequester glucose. These results are in good agreement with the fact that about 65% of the immobilized trypsin was inhibited by a high molecular weight inhibitor and, therefore, was located on the outer liposome surface (see below).

Inhibition of trypsin by the protein inhibitor from bovine pancreas. Trypsin inhibitor from bovine pancreas is well known to associate with trypsin in a 1:1 complex with a very high association constant of about $10^{10}\,M^{-1}$ [18]. The protein specifically binds to the trypsin active site and is a competitive inhibitor. In our study we used this model ligand for two purposes. First, we wished to examine the ability of liposomes with immobolized trypsin to bind to a macromolecular compound. Second, it was a way to estimate the lower limit for the number of trypsin molecules on a liposome surface. Trypsin molecules sequestered by the lipid bilayer would not bind the ligand as well as the trypsin molecules that were located on the liposome surface and extended into the bilayer. The data are represented in Table 1. About 65% of the linoleoyl-immobolized trypsin interacted with the inhibitor; therefore, their active sites were located on the liposomal outer surface. Another 35% of the active sites were apparently hidden inside the liposomes or within the lipid bilayer.

Our observation of preferential immobilization of linoleoyl trypsin on the outer liposomal surface is not an unusual fact. Analogous results were obtained for other amphiphilic liposome constituents with cumbersome hydrophilic heads [19–21]. If we use theoretically estimated geometrical liposome parameters [22] and assume equal catalytic activities for immobilized and free enzymes, we can estimate there are about 3–5 linoleoyl trypsin molecules per unilamellar liposome (60 nm diameter). Figure 1 demonstrates that approximately the same activity of linoleoyl trypsin per 10^5 lipid molecules was coupled to liposomes in all Sepharose chromatography fractions.

Concluding remarks. The method of protein immobilization on phospholipid bilayer is of interest from several points of view. First, liposomes carrying on their surfaces proteins with specific affinities look promising for directing drugs to certain target cells *in vivo* and *in vitro*. Second, liposomes covered by non-antigenic proteins appear to be useful for avoiding the problem of liposome removal from the blood by the liver and spleen. Third, the possibility of incorporating nonmembrane proteins into lipid bilayers can be of interest for introducing protein antigens into cell plasma membranes by fusing cells with proteoliposomes as in the case of lipid antigens [23] and membrane proteins [24].

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