PHOSPHATIDYLINOSITOL MAY SERVE AS THE HYDROPHOBIC ANCHOR FOR IMMOBILIZATION OF PROTEINS ON LIPOSOME SURFACE

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Received 21 December 1981

1. Introduction

Liposomes have gained increasing importance as a means for the targeting of drugs [1–3]. Accumulation of liposomes with protein immobilized on their surface-specific macromolecules in desired regions has been described in both in vivo and in vitro systems [4,5]. Simple and quantitative methods of affinity proteins binding with the liposome surface without affecting the protein specific properties and liposome integrity should be developed. Several methods of protein immobilization on liposomes are known (review [6]) amongst which chemical methods seem to be the most attractive. According to these methods, proteins are directly chemically coupled with activated liposomes [7,8] or are incorporated into the liposome membrane after preliminary chemical modification with hydrophobic residues [9–11]. In the latter case, the nature of hydrophobic modifier becomes very important for it should be easily available and physiologically acceptable. Thus, for example, *N*-(N^{α}-iodoacetyl, N^{ϵ}-dansyllysyl)-phosphatidylethanolamine used in [9] was specifically synthesized, although its toxity is not known. We have shown that non-membrane hydrophilic protein–enzyme α-chymotrypsin can be successfully incorporated in liposome membrane during liposome formation by the detergent-dialysis method after preliminary modification of protein amino-groups with oxidized phosphatidylinositol. A high degree of protein binding is achieved and the enzyme thus immobilized preserves its ability to interact with high-M_r protein inhibitor.

2. Materials and methods

Egg yolk phosphatidylcholine was the product of Kharkov bacterial preparations plant. Saturated phosphatidylcholines, phosphatidylinositol (sodium salt), sodium cholate, α-chymotrypsin and its pancreatic protein inhibitor were Sigma products. Specific substrate, *N*-acetyl-L-tyrosine ethyl ester (ATEE), was supplied by Koch-Light and dialysis tubes were from Union Carbide.

2.1. *Oxidation of phosphatidylinositol*

Phosphatidylinositol (2 mg) was dissolved in 2 ml 0.01 M acetate buffer (pH 5.6) and the solution supplemented with 1.5-, 10- or 20-fold molar excess of $NaIO_4$. The mixture was stirred for 10–15 h at 20°C or 4°C in the dark. Then a 10-fold molar excess (to periodate) of ethyleneglycol was added and mixture was incubated in the dark for 3–4 h. Micelles of modified phosphatidylinositol were separated by gel-filtration on minicolumns with Sephadex G-50 in a centrifuge [12]. During this process phospholipid was transferred in carbonate buffer (pH 9.2).

2.2. *Modification of α-chymotrypsin*

The solution of oxidized phosphatidylinositol (1 ml) was added into 4 ml α-chymotrypsin solution (final conc. 5×10^{-5} M) in 0.05 M carbonate buffer (pH 9.2) and the mixture was incubated overnight at 4°C. Then, 5 mg sodium borohydride were added and mixture was incubated for additional 12–15 h. Low-M_r compounds were separated by gel-filtration on Sephadex G-50 in the centrifuge. Modified α-chymotrypsin bound with micelles of oxidized phosphatidylinositol was separated from native protein by gel-filtration on Sephadex G-100. Additional purification of modified protein from non-bound phosphatidylinositol was performed on a Whatman phosphocellulose column equilibrated with 10^{-2} M acetate buffer

Published by Elsevier Biomedical Press
0014593/82/0000–0000/$02.75

(pH 4), containing 0.5% of Triton X-100 (ICN). Protein on the column was washed with Triton-containing buffer, then with Triton-free buffer and was then eluted with 50 mM phosphate (pH 8). When necessary, modified protein was dialyzed against 5 mM Na_2HPO_4 buffer (pH 8) containing 0.145 M NaCl before binding experiments.

2.3. *Preparation of liposomes and protein immobilization*

Liposome preparation by the cholate dialysis method with simultaneous protein immobilization was performed as in [10]. The solution of modified α-chymotrypsin was mixed with the solution of phosphatidylcholine and trace amounts of [^{3}H]cholesterol in the 5 mM phosphate buffer (pH 8), containing 0.145 M NaCl and 2% of sodium cholate and the mixture was dialyzed against a 500-fold excess of the same buffer without cholate.

Liposomes were separated from non-bound α-chymotrypsin by gel-filtration on a Sepharose CL-4B column (1.5 × 30 cm) or in the centrifuge using 5 ml columns. Minicolumns were first centrifuged for 10 min at 50 × *g*, then for 10 min at 500 × *g*. Of the sample 0.5 ml was applied on each minicolumn and centrifuged for 10 min at 50 × *g*, then for 10 min at 500 × *g*. As a result, free protein was retained on the column and liposomes with immobilized enzyme were collected from the bottom of the tube. In some cases free protein was separated from liposome by flotation in dextran solution as in [13]. Protein concentration in each fraction was determined following its enzymatic activity and lipid concentration (following ^{3}H-radioactivity). Liquid Scintillation System Mark III 6880 (Searle) was used. Catalytic activity measurements, apparent Michaelis constant determination and inhibition studies were performed as in [10].

3. Results and discussion

Periodate treatment activates phosphatidylinositol through carbohydrate unit oxidation with the formation of reactive aldehyde groups which can form Schiff bases with protein amino-groups. Schiff bases, in turn, can be reduced to stable –C–N bonds by treatment with sodium borohydride.

Upon solubilization of native or oxidized phosphatidylinositol sodium salt in experimental conditions a transparent solution is formed, containing, probably, small monolamellar liposomes (phosphatidylinositol does not form micelles, but only membrane-like bilayer structures [14]). The phospholipid structures formed are eluted in the void volume on Sephadex G-100 columns and modified with oxidized phosphatidylinositol α-chymotrypsin. α-Chymotrypsin, which is associated with these structures, can thus be separated from native, non-modified enzyme (see fig.1). It was found that non-oxidized phosphatidylinositol practically does not bind protein (i.e., modification does not occur) whereas oxidized phosphatidylinositol easily binds protein. The more rigid the conditions used for oxidation the higher the amounts of protein that are associated with phospholipid structures (cf: curves 1–3 in fig.1).

In optimal conditions up to 50% of enzymic activity was eluted in the void volume. From the known composition of the incubation mixture (0.2 mg phosphatidylinositol/ml and 5 × 10^{-5} M protein) it can be calculated that molar phospholipid–protein ratio in the aggregates formed is ~10:1. Such a high protein concentration makes doubtful the existence of membrane structures in experimental conditions; we are probably dealing with non-ordered aggregates consisting of native phospholipid, oxidized phospholipid and modified protein.

Modified α-chymotrypsin readily incorporates into liposome membranes during the process of liposome

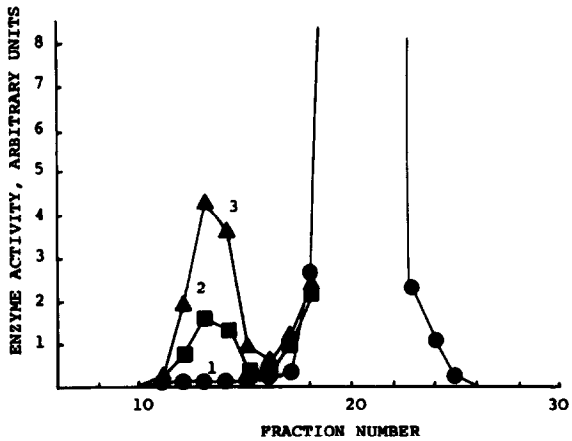

Fig.1. Gel-filtration on Sephadex G-100 of different preparations of α-chymotrypsin preincubated with phosphatidylinositol: (1) non-oxidized phosphatidylinositol; (2) phosphatidylinositol oxidized in mild conditions (4°C, 1.5-fold molar excess of periodate); (3) phosphatidylinositol oxidized in rigid conditions (20°C, 15-fold molar excess of periodate).

Table 1
The binding of native and modified α-chymotrypsin with liposomes

Sample	Protein (M)	Lipid (mg/ml)	Bound protein (mol/mol lipid)
Pure, non-modified α-chymotrypsin	2.5×10^{-5}	2.5	1.3×10^{-5}
Non-modified α-chymotrypsin in the presence of non-oxidized phosphatidylinositol	1.7×10^{-5}	2.5	5.3×10^{-5}
α-Chymotrypsin, modified with oxidized phosphatidylinositol	(a) 5.3×10^{-6}	2.5	1.8×10^{-4}
	(b) 1.06×10^{-5}	2.5	1.1×10^{-3}
	(c) 1.06×10^{-5}	1.25	2.4×10^{-3}

formation by the detergent-dialysis method. The degree of binding depends on protein and phosphatidylinositol concentrations (see table 1) and in optimal conditions it can be as high as 2.4×10^{-3} mol enzyme/mol lipid. It means that in the case, for example, of monolamellar liposomes with diam. ~1000 Å, containing 10^5 lipid molecules [15], 240 molecules of active enzyme are bound/single liposome, and simple calculations show that 20% of liposome surface is coated with the protein (assuming that the liposome membrane is impermeable for substrate and products of its decomposition, and we are dealing only with the protein located on the outer surface of liposome [10]).

The method developed is specially designed for construction of drug targeting systems, and therefore the specific properties of the bound protein should be maximally preserved in the process of immobilization, or at least, the changes in these properties, which are the result of modification or immobilization, should be known. From this point of view an enzyme can serve as a very convenient model, because its ability to bind specific substrates and inhibitors or to catalyze chemical reactions can be easily characterized quantitatively.

Modification and immobilization only slightly change catalytic activity of the native enzyme and its apparent Michaelis constant (K_m) in the reaction of ATEE hydrolysis: after modification and immobilization, the enzyme preserves of ~70% of the initial activity and K_m-values for native and immobilized enzyme are 0.7 and 0.4 mM, respectively. The changes in catalytic constant (K_{cat}) (which in the case of modified enzyme was determined by titration of enzyme active centers with *p*-nitrophenylacetate [16]) are also within the limits of experimental error.

The study of the ability of modified and immobilized α-chymotrypsin to interact with high-M_r pancreatic inhibitor (M_r ~ 6000) has shown that modification does not change this ability and even in the immobilized state >90% of active enzyme can still be inhibited (see fig.2). The observed high degree of preservation of enzyme-specific properties can be explained by low modification degree (unfortunately, it cannot be shown directly by, for example, titrating protein-free amino-groups, due to experimental diffi-

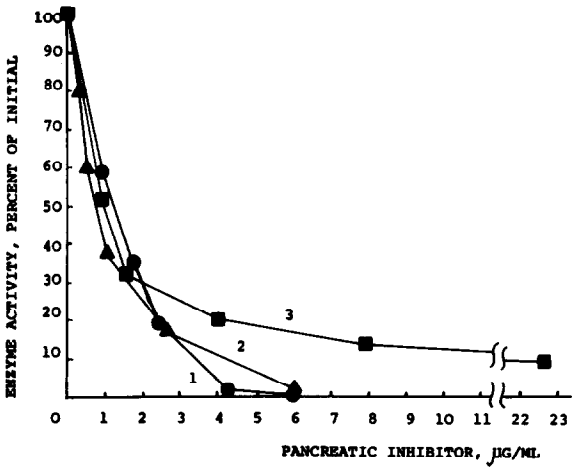

Fig.2. Inhibition of α-chymotrypsin with protein pancreatic inhibitor: (1) native enzyme; (2) enzyme modified with oxidized phosphatidylinositol; (3) enzyme immobilized on liposome surface after modification.

culties connected with the existence of the two-phase system) or by the fact that the phosphatidylinositol residue serves as a 'spacer' group separating the molecule of the immobilized enzyme from the liposome surface and thus eliminating steric hindrances [8].

4. Conclusions

(1) Phosphatidylinositol can serve as an hydrophobic anchor for immobilization of hydrophilic proteins on liposome surface. Binding degree reaches 2.4×10^{-3} mol protein/mol lipid.

(2) About 90% of immobilized protein preserves specific affinity towards a high-M_r ligand.

(3) Since phosphatidylinositol is a natural non-toxic phospholipid the method developed may be useful in drug targeting experiments.

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