A new hydrophobic anchor for the attachment of proteins to liposomal membranes

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The model enzyme α-chymotrypsin covalently modified by a phosphatidylethanolamine derivative has been attached to liposomal membranes in high yield. A maximal protein/lipid ratio of 5.4×10^{-3} mol enzyme/mol lipid was achieved.

Liposome *Phosphatidylethanolamine* *Immobilization* *α-Chymotrypsin* *Liposome targeting*

1. INTRODUCTION

Attachment of hydrophilic proteins to liposomal surfaces has become important during recent years for the use of liposomes as drug-targeting devices [1] and for the preparation of immunogenic liposomes [2]. Among the immobilization techniques covalent modification of proteins with hydrophobic compounds which may serve as an anchor to lipid membranes seems to be the most effective [3,4]. A number of new methods have been published recently [5–11].

Here, we describe a new hydrophobic anchor, *N*-glutarylphosphatidylethanolamine (N-glut-PE) and its capacity to attach the model protein α-chymotrypsin efficiently to liposomal membranes.

2. MATERIALS AND METHODS

Egg PC was obtained from the Kharkov plant of bacterial preparations. Egg PE was isolated according to conventional methods and purified on a silica gel 60 column (Merck). α-CT was a product of Boehringer. Calcein was purchased from Koch-Light and radiochemicals from Amersham International.

Abbreviations: PE-phosphatidylethanolamine; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; α-CT, α-chymotrypsin; DMSO, dimethyl sulfoxide; ATEE, *N*-acetyl-L-tyrosine ethyl ester

2.1. *Synthesis of N-glut-PE and N-glut-[^{14}C]PE*

605 mg egg PE were dissolved in 60 ml chloroform, then an approx. 10-fold molar excess (940 mg) of glutaric acid anhydride was added and the mixture incubated at 20°C for 4–5 h in the presence of 150 μl pyridine. After this time no free amino groups were detectable by ninhydrin or 2,4,6-trinitrobenzenesulfonic acid. The product was isolated on a silica gel 60 column with a yield of 90%. The radioactively labeled anchor was synthesized by adding 0.038 μmol dioleoyl-L-α-phosphatidyl-[2-^{14}C]ethan-1-ol-amine (49 mCi/mmol) to the reaction mixture before incubation with glutaric acid anhydride. The specific activity of the isolated N-glut-[^{14}C]PE was 2.096 nCi/mg.

2.2. *Modification of α-chymotrypsin*

The desired amount of dry N-glut-PE (2–20 mg) was suspended in 150 μl DMSO, followed by addition of 2 ml of 0.15 M NaCl solution. After brief sonication (15 s) the pH was adjusted to 3.5 and, depending on the amount of N-glut-PE, 3–30 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiim-

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ide were added. After about 5 min, 1 ml of a solution of α-CT (2–40 mg/ml 0.1 M borate buffer, pH 8.5) was added and the mixture incubated for 2 h at 4°C.

2.3. *Preparation of liposomes and protein immobilization*

Pure egg PC liposomes (final PC concentration 15 mg/ml) were prepared by reversed-phase evaporation [12] from an emulsion of 3 ml diethyl ether containing the lipid and 1 ml aqueous solution of the protein modified immediately before addition. Traces of [^{14}C]cholesterol or ^{3}H-labeled DPPC were added as radioactive markers. In addition, 20 μl of a solution of calcein (40 mM in 0.1 M NaCl, 10 mM Hepes) were included to check the integrity of the liposomes. Unbound α-CT was separated from the liposomes by flotation in a discontinuous Ficoll 70 gradient [13]. After separation the liposomes were pipetted off the gradient in a minimal volume, typically 0.4 ml. In all experiments controls were carried out with native α-CT.

2.4. *Determination of the degree of modification*

α-CT (final concentration 20 mg/ml) was incubated with different amounts of N-glut-[^{14}C]PE in the same way as described above. To separate the modified protein from the non-covalently bound anchor it was precipitated with trichloroacetic and carefully washed 3 times with chloroform until the radioactivity in the supernatant was negligible. The denatured protein was dissolved by brief sonication in NaOH solution. Aliquots were taken for protein determination according to Lowry et al. [14] on a micro scale [15] and for liquid scintillation counting of ^{14}C radioactivity. From the results the molar ratio of protein to lipid was calculated.

2.5. *Other methods*

A possible loss of membrane integrity caused by the incorporation of the modified protein was evaluated by measuring the leakage of the entrapped fluorescent marker calcein [16]. Lipid concentrations were calculated on the basis of added ^{14}C or ^{3}H radioactivity. The amount of active enzyme, bound to the surface of liposomes, was determined by its catalytic activity with ATEE as specific substrate [17].

3. RESULTS AND DISCUSSION

N-glut-PE reacts in the presence of water-soluble carbodiimides with free amino groups of proteins to form amide bonds (fig.1). α-CT has 14 lysines and 3 free α-amino groups, so that, theoretically, a degree of modification of 17 could be achieved. The experimental results are summarized in table 1. It can be seen, that in spite of a large excess of N-glut-PE, only about 6 NH_2 groups react with anchor molecules. This corresponds closely to the result obtained by reacting the enzyme with palmitoyl chloride [17].

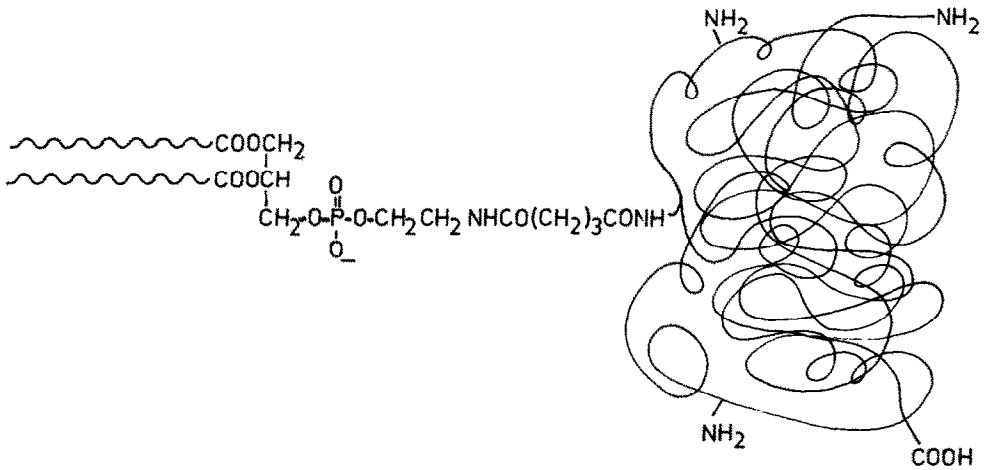

Fig.1. Schematic representation of protein modified with *N*-glutarylphosphatidylethanolamine.

Table 1

Degree of modification of α-CT

mol anchor/mol enzyme initial ratio	mol covalently bound anchor/mol enzyme
5	5.5; 5.5
9	5.5; 6.7
18	5.1; 6.3

The results of protein coupling to liposomes depending on the initial protein and anchor concentrations are shown in table 2. Control experiments were done with the unmodified enzyme. To exclude the possibility that large protein aggregates copurify with the liposomes in the flotation step, an additional control was done: hydrophobically modified enzyme was subjected to the liposome formation and isolation procedure but no lecithin was added. After centrifugation in the Ficoll gradient nearly all protein sedimented at the bottom of the tube. The protein concentration in the fraction where liposomes concentrate was only 0.35×10^{-6} mol/l. This is in the same range as determined for nonspecific adsorption of the enzyme to liposomes, thus excluding any substantial aggregate formation by cross-linking.

The highest incorporation of protein in liposomes, amounting to 5.47×10^{-3} mol protein/mol lipid, was obtained at an initial molar ratio of anchor to protein of 7.5 (cf. table 2). This corresponds to 125 μg protein/μmol lipid and is about 100-times more than that bound in the control experiment with unmodified enzyme.

The yield of binding under the most favorable conditions was 50% of the total protein added. After addition of Triton X-100 the activity increased approx. 2-fold, whereas the activity of control preparations with native enzyme increased to even higher values (table 3). This suggests that the modified enzyme is symmetrically distributed between the inner and outer monolayer of the membrane, whereas the native hydrophilic enzyme is mainly entrapped in the aqueous space of the lipid vesicles.

The average diameter of liposomes, determined by negative stain electron microscopy, was 250 nm (not shown). Using this value, we estimated that under optimal conditions 500–700 molecules of active enzyme are bound on average to the surface of a single liposome. Despite a number of attempts, we are unable to increase the amount of bound active enzyme above 5.47×10^{-3} mol protein/mol lipid.

At an initial molar ratio of anchor to protein of 7.5 the activity loss of the enzyme caused by the reaction with the anchor was 54%. A 5-fold variation of the initial molar ratio of anchor to protein changed the activity loss by a factor of only 1.7.

To achieve a high amount of liposome-bound enzyme a sufficiently high initial protein concentration is also necessary (table 2). As stated above, it was not possible to increase the amount of bound enzymatically active protein above 5.47×10^{-3} mol protein/mol lipid. On the contrary, this value dropped when the initial molar ratio of anchor to protein was increased to 15 (table 2). Obviously, it was not possible to separate the non-

Table 2

Protein coupling to liposomes

Concentration in reaction mixture			mol protein coupled/mol lipid ($\times 10^5$)	mol unmodified protein coupled/mol lipid ($\times 10^5$)
α-Chymotrypsin (mg/ml)	Anchor (mg/ml)	mol anchor/mol protein		
1	2	60	9	0.8
10	2	6	135	5.7
20	1	1.5	78	5.4
20	2	3	88	
20	5	7.5	547	
20	10	15	279	

Table 3

Increase of enzyme activity after addition of Triton X-100

Concentration in reaction mixture during modification		mol protein coupled/ mol lipid[a] ($\times 10^3$)	mol protein coupled/ mol lipid[a] (after addition of Triton X-100) ($\times 10^3$)
α-Chymotrypsin (mg/ml)	Anchor (mg/ml)		
1	2	0.09	1.91
10	2	1.35	3.02
20	1	0.78	1.44
20	2	0.88	2.11
20	5	5.47	10.39
20	10	2.79	4.15
1	–	0.008	0.03
10	–	0.057	0.36
20	–	0.054	0.27

[a] Calculated from activity measurements

covalently bound excess of anchor by flotation entailing enzyme inhibition. Indeed, we could show that a considerable amount of hydrophobic modification reagent is irreversibly adsorbed to the protein in aqueous solution and could be removed only by treatment with denaturing organic solvent (fig.2). A general conclusion is to avoid an excess of hydrophobic modification reagents when modifying biologically active proteins before incorporation in lipid vesicles. The results of calcein leakage measurements are assembled in table 4. It is evident that there is no correlation between increasing amounts of bound protein and leakage rates which are throughout comparable with those of control liposomes.

In summary, we showed that N-glut-PE can serve as an easy to handle hydrophobic anchor for the attachment of proteins to liposomal membranes in high yield while preserving liposomal integrity. We also demonstrated that knowledge of

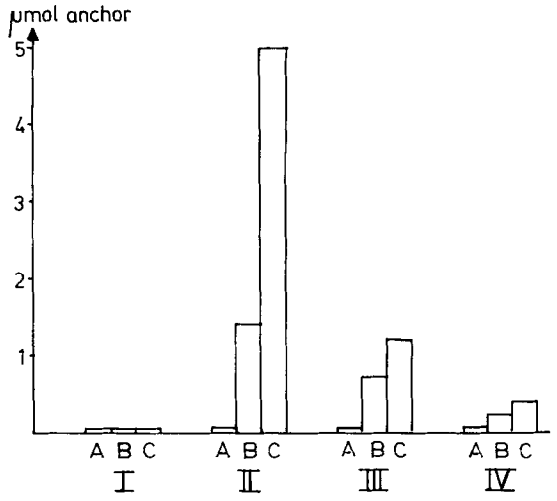

Fig.2. Removal of non-covalent bound anchor by washing with chloroform. μmol anchor calculated according to measurement of *N*-glutaryl[^{14}C]phosphatidylethanolamine. A–C, three different experiments. I, amount of anchor in the trichloroacetic acid supernatant; II, amount of anchor in the first chloroform washing; III, amount of anchor in the second chloroform washing; IV, amount of anchor in the third chloroform washing.

Table 4

Calcein leakage measurements

mol protein/mol lipid ($\times 10^5$)	Leakage of entrapped calcein in % of initially encapsulated concentration after 48 h
88	11.3
547	7.7
279	16.7
No protein	16.2

the maximally attainable degree of modification of the protein is a prerequisite for manipulating the amount of protein which can be attached to lipid vesicles.

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