Volume 214, number 1, 13–16 FEB 04562 April 1987

Thiolation of preformed liposomes with iminothiolane

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Received 3 February 1987

Preformed phosphatidylethanolamine-containing liposomes were thiolated with 2-iminothiolane (Traut's reagent) and subsequently activated by mixed disulfide formation with 5,5'-dithiobis(2-nitrobenzoic acid). Up to 65% of amino groups of the outer liposomal lamella, corresponding to 230 SH-groups per vesicle, were modified. Covalent attachment of thiolated α-chymotrypsin to these thiol-liposomes via S-S bridges yielded a protein/lipid ratio of 3.6×10^{-4} mol enzyme/mol lipid.

Thiol-liposome; 2-Iminothiolane; Membrane thiolation; Immobilization; α-Chymotrypsin

1. INTRODUCTION

During recent years, semisynthetic methods have been developed to graft liposomes with SH-groups [1]. Interest in thiolated lipid vesicles is at least 3-fold: (i) formation of cleavable crosslinks with proteins via S-S bridges for the purpose of drug targeting [2]; (ii) study of intracellular movements of thio analogues of membrane lipids by spectrophotometric titration with DTNBS [3,4]; and (iii) preparation of functionalized vesicles with the aim to make highly organized ensembles of bioorganic molecules to be used as reagents in biomimetic chemistry [5].

In hitherto existing methods phosphatidylethanolamine [1,2] or stearylamine [6] are condensed with Carlsson's heterobifunctional reagent SPDP to produce *N*-3-(2-pyridyldithio)propionyl derivatives which, after incorporation in liposomal membranes, can be either reduced to the 3-mercaptopropionyl compound or reacted with thiolated proteins to attach them covalently via thiol-disulfide exchange to liposomal surface [7,8].

In the present paper we describe a new efficient method of thiolation of preformed PE-containing liposomes with 2-iminothiolane, a cyclic thioimido ester readily soluble in water (systematic name: 2-iminotetrahydrothiophene) which has been used till now only in protein chemistry [9].

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Abbreviations: DTNBS, 5,5'-dithiobis(2-nitrobenzoic acid); SPDP, *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate; PDP-, *N*-3-(2-pyridyldithio)propionyl-; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; ATEE, acetyltyrosine ethyl ester; α-CT, α-chymotrypsin; DTE, dithiothreitol; TNBS, 2,4,6-trinitrobenzene sulfonic acid; SUV, small unilamellar vesicle

2. MATERIALS AND METHODS

N-Hydroxysuccinimidyl-3-(2-pyridyldithio)propionate and all separation gels were obtained from Pharmacia, Uppsala. Iminothiolane was purchased from Pierce, Rockford, IL. Crystalline α-chymotrypsin from bovine pancreas and *N*-acetyl-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

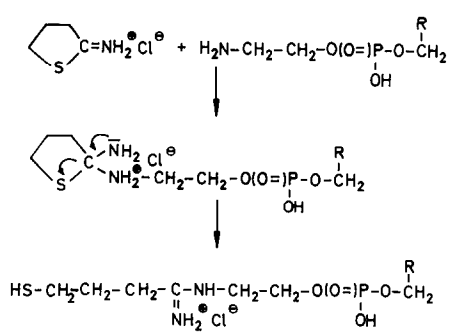
L-tyrosine ethyl ester came from Koch & Light Laboratories, and calcein from Merck, Darmstadt. PC was obtained from the Kharkov plant of bacterial preparations and PE from Calbiochem. [^{14}C]Cholesterol was synthesized at the All-Union Research Institute of Cardiology, Moscow.

Buffers: borate buffer – 0.112 M borate/NaOH, pH 9.5, 5 mM EDTA, 0.15 M NaCl; Tris buffer – 0.065 M Tris-HCl, pH 8.5, 1 mM EDTA, 0.15 M NaCl.

2.1. *Preparation of thiolated liposomes*

31.5 mg egg-PC in chloroform/methanol (9:1), 3.5 mg DPPE in benzene and 0.2 μCi [^{14}C]cholesterol in chloroform were mixed, the organic solvents flush-evaporated with argon and solvent traces removed under vacuum. After hydration with 3 ml borate buffer and brief vortex-mixing the lipid dispersion was sonicated for 35 min at 25°C with a probe sonicator and thereafter titanium traces removed by centrifugation at 11000 × g for 5 min. The percentage of amino groups exposed at the outer liposomal surface was determined (see section 2.4) and aliquots of the dispersion reacted with 2-iminothiolane hydrochloride according to scheme 1. Typically, 500 μl of SUVs (egg-PC/DPPE, 9:1) were shaken for 40 min at 23°C with 15 or 25 μl of a freshly prepared 0.5 M iminothiolane solution in borate buffer in a closed vessel under argon. Thereafter, modified liposomes were separated from unreacted iminothiolane on a Sephadex G-75 minicolumn which was equilibrated with deoxygenated Tris buffer.

The eluted fractions were immediately reacted with 1/5 of their volume with 20 mM DTNBS in



Scheme 1. Amidination reaction of PE with 2-iminothiolane.

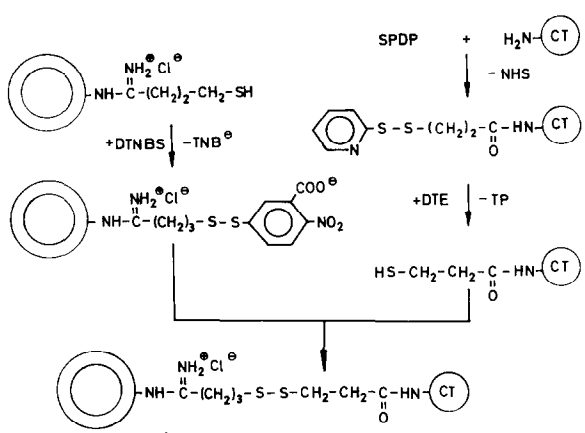
Tris buffer. The SH-group content was then evaluated from properly corrected absorbance readings and, at the same time, thiol groups were blocked and activated for thiol/disulfide exchange reactions.

2.2. *Preparation of PDP-α-CT*

To 5 mg α-CT, dissolved in 1 ml of 50 mM phosphate buffer (pH 7.6) and 0.1 M NaCl, were added dropwise 15 μl of SPDP in DMSO at 0°C (final concentration 0.31 mg/ml, corresponding to a 5 M excess over α-CT). Unreacted SPDP was removed by gel centrifugation through Sephadex G-25 minicolumns equilibrated with 0.1 M sodium acetate buffer, pH 4.6, 0.1 M NaCl. PDP-α-CT running through the column at 1000 × g, 4 min, was lyophilized and stored at −15°C.

2.3. *Coupling of thiolated protein to liposomes*

The coupling strategy is summarized in scheme 2. Immediately before coupling PDP-α-CT was reduced at pH 4.6 with DTE (final concentration 50 mM) for 20 min at room temperature. Excess DTE and liberated 2-thiopyridinone were removed by gel centrifugation as described in section 2.2. SH-α-CT was taken up in Tris buffer. 100 μl of SH-α-CT (0.55 mg/ml) and 100 μl of modified liposomes were shaken overnight at 4°C. The molar protein/lipid ratio in the reaction mixture was 4.3×10^{-3}. Isolation of α-CT-liposomes was



Scheme 2. Coupling of chymotrypsin to PE-containing liposomes via disulfide bridges. TP, 2-thiopyridinone; TNB^-, thionitrobenzoate; NHS, *N*-hydroxysuccinimide; CT, chymotrypsin.

done by gel centrifugation through Sepharose CL-6B minicolumns presaturated with lipid.

Unspecific protein binding to liposomes was evaluated by blocking the SH-groups of modified α-CT with iodoacetic acid amide.

2.4. *Other methods*

Lipid concentrations were calculated on the basis of added ^{14}C radioactivity.

Liposomal membrane integrity was checked after each preparative step by determination of the leakage of entrapped calcein. The calcein technique [10] and an Aminco SPF-500 fluorimeter were used to measure the captured aqueous volume of liposomes.

Protein concentrations were determined spectrophotometrically after addition of Coomassie brilliant blue G-250 according to Bradford [11].

Reaction of intact liposomes with TNBS yielded the outer amino group concentration, trinitrophenylation of PE after disrupture of liposomes with Triton X-100 the total amino group concentration [12]. The amount of modified and liposome-bound α-CT was calculated from the catalytic activity towards ATEE. Esterolytic activity was determined by automatic titration at constant pH.

3. RESULTS AND DISCUSSION

The mean captured aqueous volume of liposomes was 0.33 μl/μmol lipid, i.e. in the expected range of SUV preparations. On average, 72% of DPPE was found to be located in the outer lamella of the vesicles.

The results of vesicle thiolation with 2-iminothiolane are summarized in table 1. No SH-groups were detected with DTNBS on control liposomes from which DPPE was omitted. The half-life of liposomal thiol groups under working conditions (pH 8.5, 23°C) was 5 h. Both calcein latency tests and captured volume estimations proved that the modifications at the outer surface of DPPE liposomes did not impair their permeability.

The mean modification of α-CT was 2.2 PDP-groups/mol protein. Reduction with DTE at acid pH (pH 4.6) before coupling was expected to remove preferentially 2-thiopyridinone, whereas intramolecular disulfide bridges should be less sensitive. Nevertheless, there was a certain loss of activity (cf. table 2).

Coupling of SH-α-CT to *N*-phosphatidylethanolamine mercaptobutyramidine vesicles afforded 3.6×10^{-4} mol active enzyme/mol total lipid, which might be compared with 3.4×10^{-5} mol α-CT/mol lipid for unspecific binding in the control experiment. This is 10-fold less than found with highly efficient methods of protein conjugation to liposomes [13] but within the range of most commonly used techniques [14,15] which is around 10^{-4} mol protein/mol lipid. The average diameter of liposomes, determined by negative electron microscopy (not shown), was 25 nm. From this value it can be calculated that about 20 chymotrypsin molecules are attached to one liposome.

Summarizing the results, we conclude, that with 2-iminothiolane thiol groups can be introduced efficiently into amino lipid containing phospholipid

Table 1

Degree of conversion of DPPE in preformed SUVs into *N*-phosphatidylethanolamine mercaptobutyramidine by 2-iminothiolane

Lipid conc. (mM)	Molar excess of imino-thiolane over outer NH_2-groups	Modified lipid (% of total)	Modified NH_2-groups (% of outer NH_2-groups)
17.0	10.0	2.9	40
17.0	10.0	2.5	35
16.7	16.6	4.6	65
16.7	16.6	4.3	56

The SD values less than 15% of the values determined

Table 2

Loss of activity of chymotrypsin during thiolation with SPDP

Sample	% activity towards ATEE
Native α-CT	100.0
PDP-α-CT (2.2 PDP-residues per molecule)	90.7
SH-α-CT	59.3

The SD values less than 5% of the values determined

bilayers. Because of its excellent water solubility iminothiolane, in contrast to SDPD, is a reagent suitable for thiolation of plasma membranes of living cells. Such modified cells may then be used to make cleavable coimmobilized cell/enzyme systems, to follow transmembrane lipid movements and to study the action of phospholipases C and D on intact cells by DTNBS titration of their water soluble products.

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