Biochimica et Biophysica Acta, 986 (1989) 310–314
Elsevier

BBAMEM 74610

Interaction of dipalmitoylphosphatidylcholine/cholesterol vesicles with ascorbate oxidase

G. Mossa [1], A. Di Giulio [2], L. Dini [3] and A. Finazzi-Agrò [4]

[1] *Istituto di Medicina Sperimentale del C.N.R.,* [2] *Dipartimento di Scienze Biomediche, Università di l'Aquila, Dipartimenti di* [3] *Biologia e* [4] *Medicina Sperimentale, Università di Roma Tor Vergata, Roma (Italy)*

(Received 26 May 1989)

Key words: Dipalmitoylphosphatidylcholine; Liposome; Ascorbate oxidase; Protein–lipid interaction; Electron microscopy

The possibility of entrapping the enzyme ascorbate oxidase into dipalmitoylphosphatidylcholine/cholesterol vesicles, in order to deliver it to cells, was studied. This work deals with the preparation and characterization by transmission electron microscopy of liposomes loaded with ascorbate oxidase. The vesicles were prepared by two different methods, namely by reverse-phase technique or by controlled detergent-dialysis technique. Liposomes obtained by controlled dialysis were found to be more homogeneous when observed by negative staining in electron microscopy. The size of the vesicles was smaller and the distribution profile was narrower for the protein-loaded than for the empty liposomes. Ascorbate oxidase seems to be distributed both inside and on the outside of vesicles as demonstrated by immunochemical and kinetic methods.

Introduction

Dipalmitoylphosphatidylcholine (DPPC)/cholesterol vesicles (liposomes) are useful models for biological membranes as well as targetable vectors for drugs and proteins. Recently they have been also used as enzymatic reactors for basic [1] and applied research [2].

The use of liposomes as protein vectors or as enzymic reactors requires a thorough characterization of the interaction between the bilayer and the entrapped protein, since they influence each other with changes of the lipid structure and of enzyme activity.

We have studied the possibility of entrapping the enzyme ascorbate oxidase into DPPC/cholesterol liposomes in order to deliver it to cells.

Ascorbate oxidase is a plant enzyme catalyzing very efficiently in vitro the reaction:

$$2\ \text{Ascorbate} + O_2 \rightarrow 2\ \text{Dehydroascorbate} + 2\ H_2O \qquad (1)$$

The incorporation of ascorbate oxidase into animal cells in culture can be easily detected, since this enzyme is not present in animals. The role of ascorbate in several biochemical reactions allows the prediction of a dramatic effect after the introduction of ascorbate oxidase into cells, which will drive Reaction 1 far to the right.

This paper deals with the preparation and characterization of ascorbate oxidase-loaded liposomes, using two different methods, i.e., the reverse-phase evaporation [3] and the detergent dialysis [4]. Evidence is given that the latter method yields more reproducible preparations of vesicles.

The effect of loading liposomes with ascorbate oxidase on their physicochemical properties and on the catalytic activity of the enzyme has been also investigated.

Materials and Methods

Chemicals

L-α-Dipalmitoylphosphatidylcholine (DPPC), cholesterol, bacitracin, Protein A and BSA were purchased from Sigma (St. Louis, MO, U.S.A.). EDTA, ascorbic acid, sodium cholate, diethyl ether and chloroform were purchased from Merck (Darmstadt, F.R.G.). Proteinase K was from Boehringer (Mannheim, F.R.G.). Ascorbate oxidase was prepared from green zucchini peelings with the method of Avigliano et al. [5].

Abbreviations: DPPC, dipalmatoylphosphatidylcholine; BSA, bovine serum albumin; REV, reverse-phase evaporation; DIAL, controlled detergent dialysis; PBS, phosphate-buffered saline; EM, electron microscopy.

Correspondence: A. Finazzi-Agrò, Dipartimento di Medicina Sperimentale, Università di Roma Tor Vergata, Via O. Raimondo, Roma, Italia.

Liposome preparation

The vesicles were prepared by two different methods, namely according to the Szoka and Papahadjopoulos technique (reverse-phase evaporation; REV liposomes) [3] and by controlled detergent-dialysis (DIAL liposomes) [4].

The controlled detergent dialysis was performed using a Liposomat apparatus (Dianorm Geräte, Munchen, F.R.G.). A chloroform solution (5 ml) containing 35 μmol DPPC, 15 μmol cholesterol and 100 μmol sodium cholate as detergent (lipid/detergent ratio = 1 : 2) was evaporated to dryness in a rotatory evaporator under vacuum at 35 °C. The suspension was made by adding 4 ml PBS alone or 4 ml PBS containing 1 mg ascorbate oxidase or BSA to prepare empty or loaded liposomes, respectively. Then the liposomes were formed by controlled dialysis of the detergent for 18 h. The liposomes were washed three times in PBS, followed by centrifugation at 12000 × *g* to remove the free enzyme. In some experiments, 1 mg ascorbate oxidase was added to empty liposomes in order to study the possible external binding of the enzyme.

REV liposomes were prepared by dissolving 35 μmol DPPC and 15 μmol cholesterol in 3 ml diethyl ether/chloroform (2 : 1, v/v); PBS (4 ml) with or without 1 mg ascorbate oxidase, was then added. The resulting two-phase suspension was sonicated for 3 min (30 s sonication and 30 s interval), while the temperature was kept at 5 °C. The organic phase was removed by evaporation under the experimental conditions described above. The liposome suspension was washed as in the case of DIAL liposomes.

Some preparations were also made by the method of Michelson [6], using a lipid cocktail containing also stearylamine (35 μmol DPPC, 5 μmol cholesterol and 10 μmol stearylamine).

Enzyme-activity measurements

Enzyme-activity measurements were performed in 0.1 M phosphate buffer (pH 6.0)/1 mM EDTA, using sodium ascorbate as substrate and following the oxygen consumption in a YSI model 5300 Biological Oxygen Monitor (YSI, Yellow Springs, OH, U.S.A.), while the temperature was kept at 25 °C.

In some experiments the liposomes were further extensively washed with PBS at various pH values (8.0; 7.4 and 5.5) before use in kinetic experiments.

Electron microscopy

Small aliquots of liposomes were negatively stained with a 2% phosphotungstic acid solution (pH 7.0). For a better spreading of liposomes, the Formvar carbon-coated grids were treated with a 1% bacitracin solution, according to the method of Gregory and Pirie [7].

Samples were observed under a Philips EM 400 transmission electron microscope. Grids were scanned at low magnification to select regions having a good distribution of vesicles and an even deposit of stain. The electron micrographs were taken randomly in these areas.

The diameters of individual vesicles were measured with a caliber on micrographs taken randomly and printed at 2.5-times the negative enlargements. The diameter of the disc multiplied by 0.75 was equal to the diameter of the equivalent sphere [8].

The immunolocalization of ascorbate oxidase on the surface of liposomes was done according to the following procedure. After a washing with phosphate buffer, liposomes were incubated in PBS containing 0.02 M glycine to quench free aldehyde groups. Anti-ascorbate oxidase antibodies (25 μg/ml in PBS/1% BSA) were incubated overnight with liposomes at room temperature. The antigen–antibody complex was localized by using Protein A-Au 5. The specificity of binding was tested by omitting the incubation with antibodies or by adding protein A without gold.

Results

Kinetic measurements

The oxidase activity toward ascorbate of liposomes loaded with ascorbate oxidase was studied by oxygen uptake. Ascorbate was efficiently oxidized by ascorbate oxidase-loaded REV and DIAL liposomes. However, the K_M value for the enzyme entrapped into liposomes was slightly but significantly higher than that of free enzyme ($(1.8 \pm 0.1) \cdot 10^{-4}$ M vs. $(1.4 \pm 0.5) \cdot 10^{-4}$ M). The addition of detergents to liposomes brought the K_M value back to that of free enzyme ($(1.3 \pm 0.1) \cdot 10^{-4}$ M).

A few experiments conducted with cationic liposomes gave essentially identical results.

When intact liposomes were treated with proteinase K, the oxidase activity ([ascorbate] = 2 mM) dropped to about 20% of the original after 24 h incubation. The addition of sodium cholate restored this activity to about 60% of the initial value. Control experiments showed that the activity of liposomes incubated without proteinase K declined only 20% in 24 h. The addition of detergent in this case caused a 140% increase of activity. Thus it appears that about 40–60% of the ascorbate oxidase is cryptic in intact liposomes.

Electron microscopy of ascorbate oxidase-loaded liposomes

The liposomes prepared by controlled detergent dialysis (Fig. 1b) were found to be more homogeneous than those obtained by the reverse-phase method (Fig. 1a).

The membrane thickness was in the range expected for unilamellar liposomes. The REV liposomes were more prone to fuse into larger vesicles (not shown). Therefore we studied in greater detail only the DIAL liposomes, which were stable for at least 10 days when

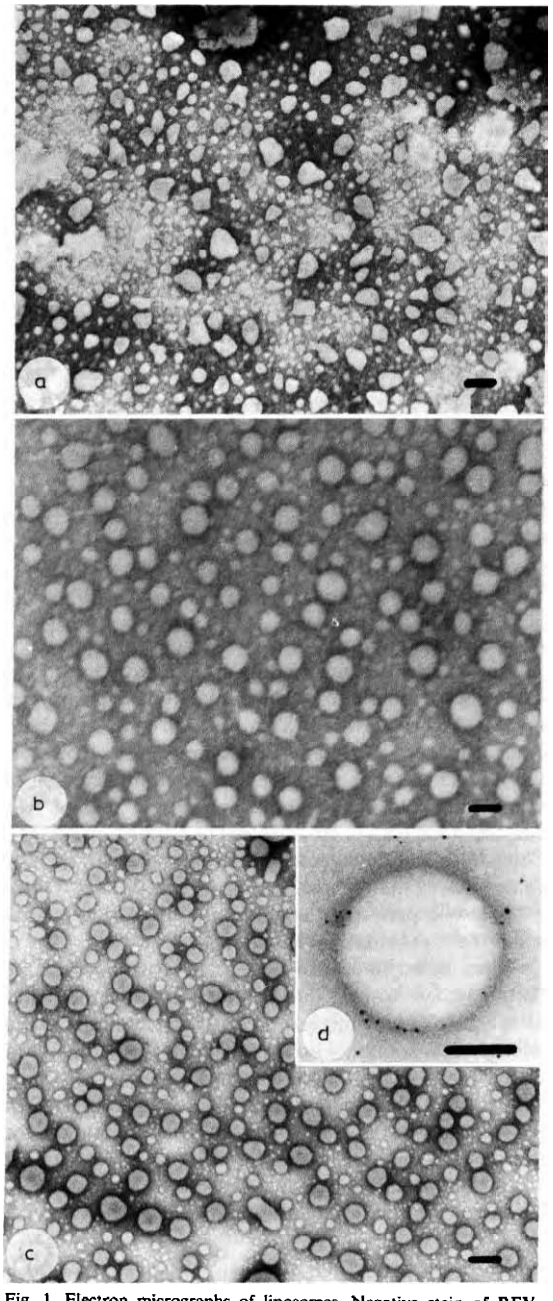

Fig. 1. Electron micrographs of liposomes. Negative stain of REV vesicles (a); negative stain of DIAL vesicles (b); negative stain of DIAL vesicles loaded with ascorbate oxidase enzyme (c); immunolocalization of ascorbate oxidase on the DIAL vesicles (d). The bar in the lower right corner is 0.1 μm.

stored at 4°C. As shown in Fig. 1c, the DIAL liposomes loaded with ascorbate oxidase had a lower mean diameter with respect to the empty ones. They also showed a tendency to assemble in a characteristic ordered pattern (not shown). Fig. 2 shows the size-distribution profiles for empty liposomes and for those loaded with protein. The presence of ascorbate oxidase dramatically reduces the mean vesicle diameter from 110 to 52 nm. Both values are in the typical range of unilamellar vesicles [9]. The size distribution profile is more homogeneous for the loaded than for the empty liposomes. In both cases, the distribution is narrower after gel-filtration on Sephadex G-25, but without significant change of the mean value.

A shrinking effect similar to that of ascorbate oxidase on vesicles was observed upon loading liposomes with bovine serum albumin (not shown).

The presence of external ascorbate oxidase adhering to the vesicle surface, suggested by kinetic experiments and by the effect of proteinase K, could not be directly demonstrated by EM. Thus, we treated the ascorbate oxidase-loaded vesicles with rabbit anti-ascorbate oxidase antibodies counterstained by the protein-A-gold method. Fig. 1d shows that there is some ascorbate oxidase sticking to the external surface of the liposomes as revealed by the dots of the electron-dense colloidal gold. Gold precipitation inside the vesicles could not be observed because the membrane is impermeable to the antibodies.

The ascorbate oxidase activity of intact liposomes was never reduced to zero, even after five washings at three different pH values. EM could not be performed

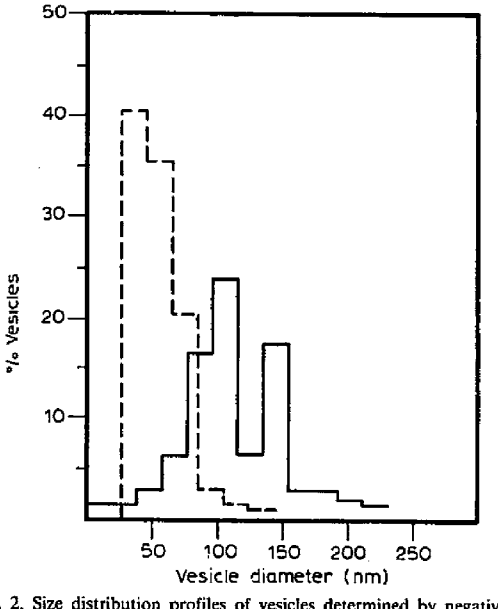

Fig. 2. Size distribution profiles of vesicles determined by negative stain electron microscopy. Solid line: empty vesicles; dashed line: ascorbate oxidase-loaded vesicles. 450 samples of both types of DIAL vesicles were measured.

on these liposomes due to the damage induced by repeated washings.

Discussion

The use of liposomes as vehicles for drugs offers many interesting theorical and practical aspects. The pharmacodynamics and the targeting of liposomes have received much attention. This interest is related to the possibility of enriching cells with macromolecules (proteins and DNA) in order to change their enzymatic or genetic profile. The enrichment can be made both in vitro and in vivo, though in the latter condition the usual capture of liposomes by the reticulo-endothelial system must be taken into account [10]. In either case, a key step of the enrichment process is the fusion of liposomes with the target cells followed by the unloading of their content inside the cell. This step critically depends on the composition of the lipid shell. It also depends on the interaction between the vesicles and the macromolecules carried. In fact, DNA and proteins may interact in several ways with lipids. In particular, proteins may be entrapped inside the vesicle, may interact with the inner or outer lipid leaflet or may sit across the lipid bilayer. Each of these possibilities results in different kinetics of fusion and in different targeting into the cell.

The present paper deals with the characterization of ascorbate oxidase-loaded liposomes as a preliminary step toward the goal of enriching cells with this enzyme. The enrichment of cells with ascorbate oxidase is of interest since it may affect the cellular level of ascorbate and therefore all the processes involving it.

First we tried different recipes for preparation of homogeneous liposomes. Electron microscopy was used to control the quality of vesicles obtained by different methods. Negative staining [11] and freeze-fracture [12] electron microscopy have been used to study the vesicle structure. Olson et al. [8] reported that both techniques gave essentially the same results regarding the structure of phosphatidylcholine and phosphatidylserine vesicles. We found that the DIAL liposomes had a narrower distribution of diameter with respect to REV liposomes. Furthermore, they have a lesser tendency to coalesce into larger vesicles. After 47 h at room temperature, the mean diameter and distribution of the DIAL vesicles was almost unchanged. The presence of ascorbate oxidase reduced the mean diameter of the vesicles by about 50%. The effect was not specific of ascorbate oxidase, since bovine serum albumine similarly reduced the diameter of the vesicles. Instead, the external addition of either protein did not affect the dimension of the vesicles once they were formed. This may indicate that only a protein located inside the vesicle is able to shrink the volume, perhaps through a change in surface tension. The presence of ascorbate oxidase inside the vesicles is confirmed by kinetic data showing that: (i) about 50% oxidase activity of ascorbate oxidase-loaded liposomes is cryptic, but it can be released by addition of detergents; (ii) the K_M of ascorbate oxidase is increased slightly but significantly when ascorbate oxidase is embedded into liposomes. The K_M value is brought back to that of the soluble enzyme after disruption of liposomes by detergents.

The higher K_M value of ascorbate oxidase in liposomes may be due to a slower diffusion of ascorbate across the lipid bilayer than in the bulk solution. Ascorbate oxidase activity might therefore be used to study the diffusion of ascorbate through the membranes as a function of their composition for a better understanding of ascorbate transport and metabolism in cells. A more quantitative study of ascorbate transport through the vesicle membrane has been prevented by the presence of some ascorbate oxidase partly or fully exposed to the external medium. In fact, incubation of ascorbate oxidase-loaded intact liposomes with a proteolytic enzyme significantly reduced the liposome-bound ascorbate oxidase activity. Similarly it has been reported that the treatment of rat-brain adenylate cyclase, incorporated into phospholipid vesicles [13], with soluble proteases or immobilized trypsin, destroys 80–90% of the enzyme activity. A further proof of externally bound ascorbate oxidase was obtained by gold immunostaining in electron microscopy (Fig. 1d). Similar kinetic and immuno-localization results were obtained by adding ascorbate oxidase to preformed vesicles indicating that some enzyme sticks on the external surface of the vesicle. The binding of external ascorbate oxidase to liposomes is relatively tight, since extensive washing or gel-filtration of liposomes was unable to reduce the activity of ascorbate oxidase to zero. It is still possibie that some ascorbate oxidase is located across the vesicle membrane with the active site exposed to the external solvent.

The insertion of ascorbate oxidase into vesicles may be affected by its distribution coefficient between the buffer and the lipid phase. This possibility must be further tested by studying the efficiency of insertion as a function of the lipid cocktail used to prepare the liposomes, though Ostro and Giacomoni [14] already disclaimed this hypothesis. Another possibility [15] is that the efficiency of entrapment depends on the molecular weight of the protein. Previous work [16] has shown that catalase (M_r 240000) and superoxide dismutase (M_r 33000), which are both very soluble in water, are entrapped into liposomes with very different yields. This finding is in keeping with the observations of Adrian and Huang [17]. Both hypotheses are currently under investigation in order to obtain vesicles more suitable as a bioreactor for the study of kinetics of entrapped ascorbate oxidase and for the enrichment of cells with ascorbate oxidase.

References

1 Sada, E., Katoh, S., Terashima, M. and Tsukiyama, K. (1988) Biotech. Bioeng. 32, 826–830.
2 Torchilin, V.P. (1987) Adv. Drug Del. Rev. 1, 41–86.
3 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
4 Weder, H.G. and Zumbuehl, O. (1984) in Liposome Technology (Gregoriadis, G., ed.), Vol. 1, pp. 79–107, CRC Press Inc., Boca Raton, FL.
5 Avigliano, L., Gerosa, P., Rotilio, G., Finazzi-Agrò, A., Calabrese, L. and Mondovì, B. (1972) It. J. Biochem. 21, 248–255.
6 Michelson, A.M., Puget, K., Durosay, P. and Rousselet, A. (1980) in Biological and Clinical Aspects of Superoxide Dismutase (Bannister, W.H. and Bannister, J.V., eds.), pp. 348–366, Elsevier, New York.
7 Gregory, D.W. and Pirie, B.J.S. (1973) J. Microsc. (Oxford) 99, 251–255.
8 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
9 Fendler, J.H. (1982) Membrane Mimetic Chemistry, pp. 113–183, J. Wiley and Sons, New York.
10 Allen, T.M. and Chom, A. (1987) FEBS Lett. 223, 42–46.
11 Szoka, E. and Papahadjopoulos, D. (1981) in Liposomes: from Physical Structure to Therapeutic Applications (Knight, C.G., ed.), pp. 51–82, Elsevier/North-Holland, Amsterdam.
12 Hope, M.J., Bally, M.B., Mayer, L.D., Janoff, A.S. and Cullis, P.R. (1986) Chem. Phys. Lipids 40, 89–107.
13 Hebdon, G.M., Le Vine, H., Minard, R.B., Sahyoun, N.E., Schmitges, C.J. and Cuatrecasas, P. (1979) J. Biol. Chem. 254, 10459–10465.
14 Ostro, M.J. and Giacomoni, D. (1983) in Liposomes (Ostro, M.J., ed.), p. 169, Marcel Dekker, New York.
15 Mam, G.V. and Newton, P. (1975) Ann. N.Y. Acad. Sci. 258, 243–263.
16 Finazzi-Agrò, A., Di Giulio, A., Amicosante, G. and Crifò, C. (1986) Photochem. Photobiol. 43, 409–412.
17 Adrian, G. and Huang, L. (1979) Biochemistry 18, 5610–5614.