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Lysozyme induced fusion of negatively charged phospholipid vesicles

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Lysozyme promotes fusion of negatively charged phospholipid vesicles prepared by ethanolic injection. Vesicle fusion was a leaky process as revealed by the release of encapsulated carboxyfluorescein or Tb-DPA complex. Extensive proteolysis of lysozyme inhibited the fusion process. The fusion process was critically dependent on the medium ionic strength; 100 mM of any salt was sufficient to inhibit totally the fusion activity of the protein. The high efficiency of lysozyme (80% RET) was almost constant in the pH range from 4.0 to 9.0, but it was sharply diminished when the pH of the medium was at the isoelectric point of the protein (p*I* 11.0). Fusion induced by chemically modified lysozyme, showed that the pH profile changed according to the isoelectric point of the protein derivative. These observations stress the importance of electrostatic interactions in the process of fusion induced by lysozyme.

Membrane fusion is an ubiquitous and essential event in cell biology, and it has been extensively studied in recent years [1–3]. Although fusion requirements for simple membrane model systems are often far from those known to be required for biological membrane, most of fusion studies have been carried out with liposomes.

Kimelberg and Papahadjopoulos [4] have examined the ability of lysozyme to interact with lipid model membranes, showing that the protein markedly increased the Na^+ permeability of sonically dispersed vesicles. Iacono et al. [5] reported that lysozyme is capable of promoting bacterial aggregation and loss of viability independent of cell lysis. On the other hand, Arvinte et al. [6,7] reported the pH-dependent fusogenic activity of lysozyme covalently bound to neutral liposomes when incubated with either erythrocyte ghost, or mouse liver nuclei. Finally studies with RET and light scattering from our laboratory suggested the liposomal fusion ability of lysozyme [8].

The present study reveals that lysozyme indeed caused fusion of phospholipid vesicles at pH 10 or below. Using gel chromatography, light scattering, electron microscopy and changes of resonance energy transfer between fluorescent lipid probes, we show that larger structures do, infact, represent fused bilayer. It is also, suggested that electrostatic interactions between the protein and lipid bilayer could be the most important early event in the process of the lysozyme- induced membrane fusion. Confirmative experiment was carried out with chemically modified lysozyme.

Native lysozyme (grade I) from chicken egg white (Sigma Chemical Co., St. Louis, MO) was dissolved in appropriate buffer just before use. The digestion of lysozyme with pronase VIII was carried out in 20 mM Tris-HCl buffer (pH 7.4) in a ratio lysozyme/pronase 2:1 (w/w) for 60 min at 37°C. Succinylation of lysozyme was performed as described elsewhere [9]. Amidation of carboxyl groups of succinyl-lysozyme was carried out as described by Hoare and Koshland [10]. The isoelectric point of native and chemically modified lysozyme were estimated by a method developed by Yang and Langer [11].

PC, PA, and the fluorescent lipids *N*-NBD-PE and

Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; CA9C, cholesteryl anthracene 9-carboxylate; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; DPA, dipicolinic acid; RET, resonance energy transfer; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide; DPC, dicetyl phosphate; Chol, cholesterol; EDTA, ethylenediaminetetraacetic acid (sodium salt).

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CA9C were prepared as described previously [8]. Total lipid concentration was determined by phosphate analysis according to Ames [12]. The preparation of unilamellar phospholipid vesicles was done following the method of Batzri and Korn [13]. Briefly, 1 μmol of appropriated lipid mixture was dried under nitrogen and dissolved in 0.25 ml of ethanol. The ethanolic solution (25 μl) was injected into 2.0 ml of 20 mM Tris-HCl (pH 7.4). 'Mock-fused' (100% probe intermixing) vesicles were obtained by including both probes in the same lipid mixture. To prepare vesicles containing carboxyfluorescein, Tb, DPA, or the Tb-DPA complex trapped within, phospholipids (PC/PA, 9:1 molar ratio) were injected into 20 mM Tris-HCl buffer (pH 7.4) containing, respectively, the following: (a) 100 mM carboxyfluorescein; (b) 15 mM $TbCl_3$, and 150 mM sodium citrate; (c) 150 mM DPA (sodium salt); or (d) 15 mM $TbCl_3$ and 150 mM DPA (sodium salt). Non-encapsulated material was eliminated by gel filtration on Sephadex G-75 as described earlier [14].

Vesicles fusion was estimated by a method based on RET described by Uster and Deamer [15]. The %RET, which is proportional to the extent of fusion was calculated according to the following equation:

$$\%\text{RET} = [(F_0 - F)/(F_0 - F_s)] \times 100$$

where F_0 is the fluorescence of CA9C (λ_{ex} 370, λ_{em} 470 nm) in the absence and F in the presence of lysozyme, and F_s the CA9C fluorescence of the 'mock-fused' vesicles.

Intermixing of vesicle content during fusion was determined by the procedure of Wilschut and Papahadjopoulos [16] using the Tb/DPA assay. Leakage of vesicle content was monitored following the release of carboxyfluorescein [17] or the Tb-DPA complex [18]. The fluorescence intensity of the Tb-DPA complex was followed at 545 nm with the excitation wavelength at 275 nm, and the fluorescence of carboxyfluorescein was monitored with excitation and emission monochromators set at 470 nm and 525 nm, respectively.

Steady-state emission spectra were obtained by using a 4048 C SLM spectrofluorometer. Light scattering changes were recorded with an Aminco Bowman spectrofluorometer with excitation and emission monochromators set at 370 nm.

The previous suggestion of our laboratory using light scattering and RET criterion was not conclusive about the capability of the lysozyme to induce liposomal fusion. These results can also be explained by vesicle aggregation and mixing of lipids between the outer monolayers of different vesicles without concomitant fusion of the vesices. To determine the reliability of the previous hypothesis we eluted through a Sepharose 4B column, CA9C-labeled phospholipid vesicles treated with and without lysozyme. Chromatograms of the untreated liposomes showed mainly a broad included peak, meanwhile, large amounts (85%) of labeled vesicles appeared in the void volume fractions after treatment with lysozyme (not shown). This result clearly indicates that lysozyme treatment caused a large increase of the phospholipid vesicles due to the formation of aggregated and/or fused particles. The morphology of the vesicles was examined by negative stain electron microscopy, and the average diameter of the vesicles were measured. Before the addition of lysozyme, (PC/PA, 9:1) vesicles were unilamellar and rather homogeneous in size with an average diameter ranging from 150 to 300 Å (Fig. 1A). Addition of lysozyme resulted in appearance of vesicles shown in Fig. 1B. The size distribution of treated vesicles were in the range of 800–1000 Å. The mean diameter was 4-times the original one. According to these results, it appears likely that the larger vesicles in the preparations are formed by fusion of smaller ones.

Since vesicle fusion involves the mixing of bilayer lipids and the mixing of aqueous content, we attempted to measure both processes. The mixing of membrane bilayer was studied by RET assay. Both, the donor CA9C [15] and acceptor *N*-NBD-PE [19] show no detectable exchange in unfused vesicles, over the course of hours. The %RET increased abruptly after 5 min at 37°C incubation of vesicles in the presence of lysozyme, indicating membrane fusion. The presence of lysozyme did affect neither the CA9C-labeled vesicle fluorescence nor the 'mock-fused' vesicle fluorescent. Proteolytic treatment of lysozyme previous to the fusion assay inhibited the lysozyme effect. Pronase VIII by itself did not produce diminution of CA9C fluorescence emission (results not shown).

Studies of intermixing of the aqueous contents evaluated through the Tb^{3+}-DPA complex fail to detect any coalescence of vesicle content (not shown). Probably the vesicle content leaks before the complex mix into vesicles obtained by fusion. To investigate this possibility we studied the leakage of aqueous content following (I) the release of fluorescent Tb-DPA complex to the external medium where EDTA was present, and (II) the release of carboxyfluorescein self-quenched into vesicles. Fig. 2 shows the time course of aqueous release from (PC/PA, 9:1) vesicles caused by lysozyme. As can be seen, a large leakage of vesicle content was observed by both methods. Moreover, the leakage of vesicle content was very fast and it was completed in 30 s.

The percentage of RET and light scattering increased proportionately to the lysozyme concentration, reaching a 'plateau' at concentration of $4 \cdot 10^{-6}$ M (Fig. 3). We have examined the dependence of the negative density charge of the vesicles. Lipid mixing was not observed with neutral vesicles (pure PC). PC vesicles did not mix even at lysozyme concentrations of 10^{-4} M. Neutral

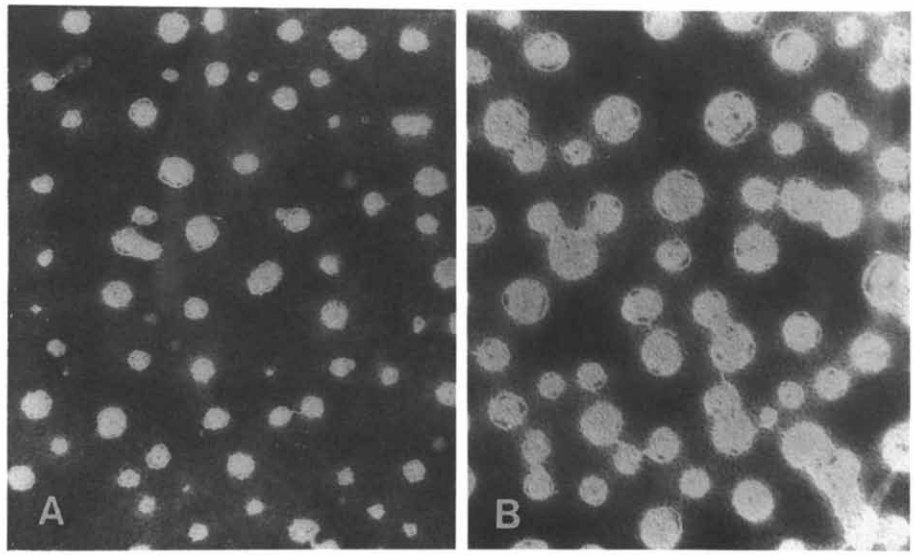

Fig. 1. Lysozyme-induced fusion studied by electron microscopy. Phospholipid vesicles (PC/PA, 9:1) were prepared, as described in the text. An aliquot of vesicles without treatment (A), or treated with $5 \cdot 10^{-6}$ M lysozyme (B) was loaded onto formvar-coated grids. After approximately 1 min, the grids were blotted. A drop of 2% phosphotungstic acid (pH 6.5) was applied. After 1 min, the grids were blotted again, allowed to dry, and examined in a Zeiss, EM 109, 50 kV. Magnification: 80000×.

lipids together with acidic phospholipids (PA) form vesicles capable to mix in the presence of lysozyme. The fusogenic activity of lysozyme increased almost proportionally to the negative charge of vesicles. Similar results were obtained by using DCP instead of PA as negatively charged lipid (not shown). We have also examined the dependence of lipid mixing on the ionic strength. Fig. 4A shows the effect of increasing different salt (NaCl, KCl and LiCl) concentration on the mixing of (PC/PA, 9:1)-vesicles at pH 7.4. It is clear that lipid mixing was drastically diminished as the salt concentration increased. Since vesicle aggregation is reverted by high salt concentration and on the contrary fusion is not, we could confirm that a true liposomal fusion occurs in the presence of lysozyme. As can be seen in Fig. 4B, addition of 0.1 M NaCl after the fusion process was completed, slightly affected lipid mixing (determined by RET assay and light scattering). Moreover, the elution pattern of fused vesicles chromatographed on Sepharose 4B was not reverted by the presence of salt. Identical results were obtained by treatment with pronase VIII after liposomal fusion was obtained by the presence of lysozyme (not shown).

Experiments to study the pH dependence of the lipid mixing process revealed that phospholipid vesicle mixing occurs over a wide range (pH 4.0 to pH 9.0) without

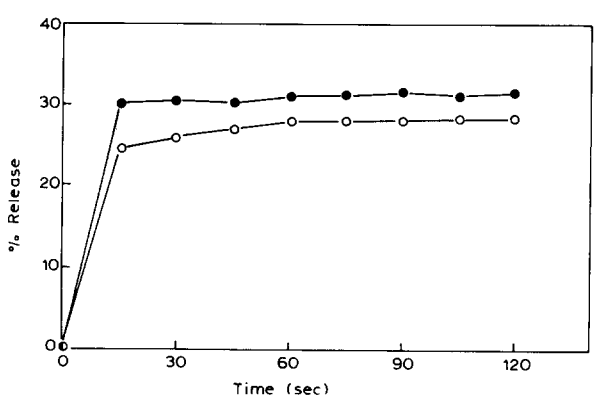

Fig. 2. Kinetic release of contents of phospholipid vesicles induced by lysozyme. PA/PC (9:1)-vesicles containing carboxyfluorescein or Tb-DPA complex entrapped within were prepared as described in the text. Carboxyfluorescein (●———●) or Tb-DPA complex (○———○) release were induced by addition of $5 \cdot 10^{-6}$ M lysozyme, and incubation at 37°C. 100% release was obtained by the presence of 0.1% Triton X-100.

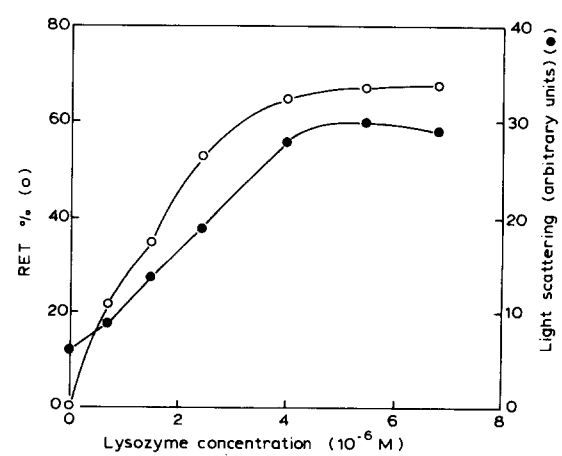

Fig. 3. Dependence of the extent of fusion on lysozyme concentration. Phospholipid vesicles were prepared as described in the text. Fusion was initiated by the addition of a desired concentration of lysozyme. Fluorescence energy transfer was calculated as described.

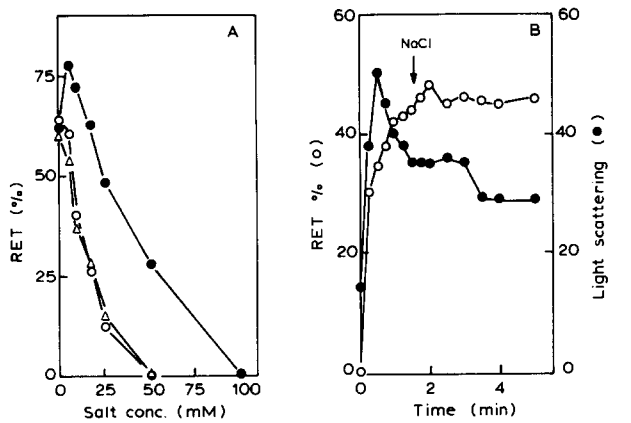

Fig. 4. (A) Dependence of liposome fusion on ionic strength. Liposomes (PC/PA, 9:1) prepared at pH 7.4 were incubated at 37°C for 5 min in the presence of $5 \cdot 10^{-6}$ M of lysozyme and different salt concentration. NaCl (○——○), KCl (●——●), LiCl (△——△). (B) Time dependence of lipid intermixing as induced by lysozyme and effect of NaCl. Fusion was initiated by addition of $5 \cdot 10^{-6}$ M lysozyme (final concentration). The arrows indicate the addition of 100 mM NaCl.

changing significantly (Fig. 5). However, it decreased sharply when the medium pH was increased above 9.0, being null at the isoelectric point of lysozyme (pH 11.0). On the other hand succinyl-lysozyme (all detectable amino groups succinylated) with a p*I* of 4.6 showed mixing ability only at pH values lower than 5.0, and the methyl ester glycyl-succinyl derivative (carboxyl groups blocked) with p*I* greater than 11.0 presented a pH dependence curve basically similar to those for native lysozyme.

In the present study we have shown that lysozyme at low concentration induces the leakage of vesicular content and increase the size of the negatively charged phospholipid vesicles. Electron microscopy study

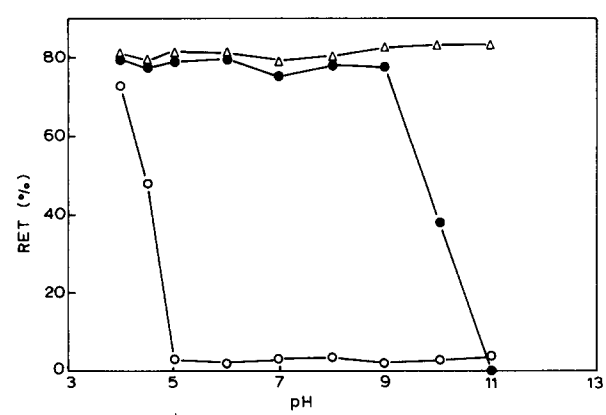

Fig. 5. Dependence of liposome fusion on medium pH. Liposomes (PC/PA, 9:1) prepared at different pH values were incubated at 37°C for 5 min in the presence of $5 \cdot 10^{-6}$ M of either lysozyme (●——●), succinyl-lysozyme (○——○), or methyl ester glycyl-succinyl-lysozyme (△——△). pH values between 4.0 and 6.0 were obtained with 20 mM acetate-acetic acid buffer; pH = 7.0–8.0 with 20 mM Tris-HCl buffer; and pH = 9.0–11.0 with 20 mM glycine-NaOH buffer.

showed clearly the appearance of a new larger population of vesicles, as a consequence of fusion. The facts that light scattering, RET changes and elution pattern on Sepharose 4-B of lysozyme-treated vesicles were irreversible upon the addition of 0.1 M NaCl and treatment with pronase, strongly support the liposomal fusion possibility. We fail to detect any coalescence of content and attributed this to the fact that fusion was leaky, however, the extent of leakiness (30%) is incompatible with the extend of membrane mixing (80%). This incompatibility was also observed by other investigators, and can be attributed to the fact that extensive aggregation may be accompanied by probe exchange, which will induce an overestimation of fusion [20].

The facts that lysozyme had no effect on uncharged pure PC-vesicles and that the enzyme-induced fusion of negatively charged vesicles was inhibited by increasing the medium ionic strength strongly indicated that an electrostatic interaction takes place initially. In agreement with this hypothesis lysozyme effect disappeared by increasing the pH near to the isoelectric point of the protein. The same results were obtained with lysozyme derivatives; that is succinyl-lysozyme with a p*I* of 4.6 promote fusion only at pH below 4.5 and the methyl ester glycyl-succinyl-lysozyme (p*I* above 11.0) promoted fusion even at pH 11.0. These results are quite similar to those found with insulin [22], and are totally opposed to those found with glyceraldehyde-3-phosphate dehydrogenase [23], since this enzyme is able to fuse uncharged vesicles. It is bilieved that the antimicrobial activity of this protein results from its ability to hydrolys the murein layer in prokaryotic cell walls.

The results presented here suggest that physiological lysozyme effect could not be limited to the enzymatic activity of the enzyme. We can suppose that the lysozyme–membrane interaction may play also an important role.

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