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Lysozyme interactions with phospholipid vesicles: relationships with fusion and release of aqueous content

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Abstract

We have previously demonstrated that lysozyme induced fusion of negatively charged phospholipid vesicles and have stressed the importance of electrostatic interactions (Posse, E. et al. (1990) Biochim. Biophys. Acta 1024, 390–394). Using centrifugation and fluorescence polarization techniques, we show, in the present paper that lysozyme interacts with negatively charged liposomes (PC/PA, 9:1), but also with neutral liposomes (pure PC). Moreover, the ionic strength and pH of the media did not modify the protein–liposomes interactions. Such interactions induce the spontaneous release of encapsulated Tb-DPA complex in liposomes. Release and fusion of PC/PA liposomes were observed. As indicated by kinetic studies and substrate curves, fusion and release are two uncoupled processes. Taking these and previous results into account we suggest a hypothetical mechanism where a relationship between aggregation, leakage and fusion of liposomes induced by lysozyme interaction is established.

Key words: Lysozyme; Liposome; Protein–liposome interaction; Fusion; Tb-DPA release

1. Introduction

Lysozyme is a small basic protein capable of promoting bacterial aggregation and loss of viability independent of cell lysis [1]. It is well known that this protein can hydrolyze the peptidoglycan layer in the cell walls of sensitive bacteria; however, a subsequent action of the lysozyme on the cell membrane can not be ruled out. A study of the interaction between lysozyme and a major membrane component phospholipid is essential for a systematic approach to understanding the action mechanism of lysozyme. A previous paper showed that lysozyme was able to increase the permeability of sonically dispersed liposomes [2]. On the other hand, pH-dependent fusogenic activity of lysozyme covalently bound to liposomes when incubated with either erythrocyte ghost, or mouse liver nuclei has been described by Arvinte et al. [3,4]. Recently, it has been shown in our laboratory that lysozyme caused fusion of phospholipid vesicles, and we suggested that electrostatic interactions between the protein and lipid vesicles could be an important event in the process of lysozyme-induced membrane fusion [5].

In the present paper we analyze the interactions of lysozyme with small unilamellar vesicles. Such interaction induces the release of aqueous content of liposomes alone or leakage followed of vesicle fusion, depending upon the vesicle charge, ionic strength and pH of the media. Finally, a model showing a relationship between protein interactions, aggregation, leakage and fusion effects is proposed.

2. Materials and methods

2.1. Materials

Native lysozyme (grade I) from chicken egg white (Sigma, St. Louis, MO) was dissolved in appropriate

Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; DPA, dipicolinic acid; Tb, terbium; CA9C, cholesteryl anthracene 9-carboxylate; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; RET, resonance energy transfer; EDTA, ethylenediaminetetraacetic acid (sodium salt).

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buffer just before use. PC and PA were prepared as described previously [6]. $TbCl_3$ and DPA were purchased from Aldrich, (Milwaukee, WI). CA9C and NBD-PE were from Molecular Probes (Eugene, OR) and fluorescamine from Sigma.

2.2. Preparation of liposomes

Multilamellar vesicles were prepared by hydrating a dried lipid film with 20 mM Tris-HCl buffer (pH 7.6), 20 mM acetate buffer (pH 5.5) or 20 mM carbonate buffer (pH 10), according to the experiment to be performed, followed by vortexing for 5 min.

Small unilamellar phospholipid vesicles were prepared following the method of Batzri and Korn [7]. Appropriate lipid mixture (0.1 μmol) was dried under nitrogen and dissolved in 25 μl of ethanol. The ethanolic solution was injected into 2.0 ml of acetate, Tris-HCl or carbonate buffer (depending on the desired pH). To prepare vesicles containing Tb-DPA complex trapped within, the ethanolic solution of phospholipids was injected into the appropriate buffer containing 15 mM $TbCl_3$ and 150 mM DPA (sodium salt). Nonencapsulated material was eliminated by gel filtration on Sephadex G-75 as described earlier [8]. Vesicles for RET assay were obtained by mixing 1 μmol of phospholipid with either 0.01 μmol CA9C or 0.03 mmol NBD-PE previous to evaporation.

2.3. Labeling procedure

Fluorescamine-labeled lysozyme was prepared by adding 30 μl of fluorescamine in acetone (40 mg/ml) to 3.0 ml of buffer (20 mM phosphate (pH 7.4)) containing 12 mg of lysozyme. The free dye reagent was separated from the conjugated material by filtration through a Sephadex G-25 column equilibrated and eluted with 20 mM phosphate buffer (pH 7.4).

2.4. Binding experiments

A procedure involving centrifugation and an assay based on fluorescence polarization were used to determine potential association of lysozyme to lipid vesicles. The former procedure was done with multilamellar vesicles. Phospholipids (0.3 μmol) were incubated with 0.02 μmole of lysozyme in acetate, Tris-HCl or carbonate buffer (depending on the desired pH) for 10 min at 37°C. Subsequently, the vesicles were centrifuged for 15 min in an Eppendorf centrifuge. Residual protein was determined in the supernatant fraction, as a measure of the extent of binding. Blanks, obtained by centrifugation of lysozyme without liposomes, were always subtracted to correct the values of precipitated protein.

Alternatively, binding of lysozyme to the unilamellar phospholipid vesicles was determined by an assay based on polarization of fluorescence. The fluorescence polarization (P) of a sample of fluorescamine-labeled lysozyme ($5 \cdot 10^{-6}$ M) alone and in the presence of unilamellar lipid vesicles (50 μM) was measured at different pH. Fluorescence polarization measurements were carried out on a 4048c SLM spectrofluorometer. Excitation wavelength was set at 390 nm and the emission was passed through a 3–73 cutoff Corning filter. The polarization values were calculated by $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}), I_{\parallel}$ and $I_{\perp}$ being the fluorescence intensities recorded with the analyzing polarizer oriented parallel and perpendicular to the direction of the excitation beam, respectively [9].

2.5. Leakage experiments

Release of phospholipid vesicles content was measured by following decrease of Tb-DPA complex fluorescence upon its release into the external media containing 1.0 mM EDTA. The excitation wavelength was 280 nm and the emission followed through a 3–71 Corning filter at 550 nm in an Aminco Bowman fluorometer. The leakage was initiated by addition of different amounts of lysozyme. The fluorescence scale was calibrated such that the 0% release corresponds to the Tb-DPA fluorescence without protein, and 100% release to the fluorescence obtained in the presence of 0.1% Triton X-100 to achieve the complete release of the complex.

2.6. Fusion assay

Vesicle fusion was estimated by a method described by Uster and Deamer [10] based on resonance energy transfer. The RET assays monitor the energy transfer between the donor (CA9C) and acceptor (NBD-PE) which were incorporated in separate vesicle populations. When both fluorescent lipids were in separate vesicles no energy transfer occurs. Fusion of vesicles results in intermixing of the lipids and increase of resonance energy transfer. The percentage of RET that is proportional to the extent of fusion was calculated according to the following equation:

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

Where F_o and F are the donor fluorescence in the absence and in the presence of lysozyme respectively, and F_s the fluorescence of donor when both donor and acceptor are in the same vesicle population.

2.7. Additional assays

Protein concentration was determined by tryptophan fluorescence emission intensities when the sam-

ples were excited at 280 nm and/or by the method of Lowry et al. [11]. Lipid phosphate was determined by the colorimetric method of Ames [12]. Aggregation was followed measuring light scattering at 90°, with the emission and excitation wavelength set up at 370 nm.

3. Results and discussion

3.1. Association of lysozyme to liposomes

The association of lysozyme with phospholipid vesicles was studied by two different methods: (i) a procedure involving centrifugation separation of bound and unbound protein and (ii) an assay based on fluorescence polarization determinations.

The centrifugation methods involve the direct determination of the net amount of bound lysozyme to multilamellar vesicles. The association of lysozyme to performed multilamellar liposomes composed of PC, PC/PA (9:1) and PC/PA (9:1) in the presence of 0.1 M NaCl was measured at pH 5.5, 7.6 and 10.0. The protein to phospholipid ratio was fixed at 0.06 μg of protein/μmol of phospholipid. The results depicted in Fig. 1 show that lysozyme adsorbed substantially to PC and PC/PA liposomes at all three pH values tested. The effect of increasing ionic strength did not change the results, although a considerable reduction of the association was observed at pH 10.0. Upon addition of increasing amount of phospholipid vesicles, the binding of lysozyme increased almost linearly (result not shown). To determine interaction of lysozyme with unilamellar vesicles, an indirect procedure was employed: fluorescence polarization. Since the fluorescence polarization value of a molecule is related to its rotation relaxation times, we can suppose that the association of labeled lysozyme to liposomes induces a concomitant increase of the polarization values of fluorescence labeled lysozyme at different pH values. As can be seen in Table 1, the labeled protein presented identical polarization values at acidic, neutral and basic pH. However, these polarization values were significantly increased by the presence of neutral (PC) or negatively (PC/PA) charged liposomes. These results were not influenced by the presence of 100 mM NaCl. These studies do not let us make any quantification; however, both methods clearly show the interaction.

Previous reports have introduced several operation criteria to differentiate the electrostatic from hydrophobic protein–lipid interactions [13]. Electrostatic interactions should be disrupted when the ionic strength is increased. Because the lysozyme isoelectric point is around 11, we can suppose that over the wide pH range studied, the protein is positively charged and electrostatic interactions with the negative head group of phosphatidic acid take place. However, the association of lysozyme to PC vesicles and PC/PA vesicles in the presence of 0.10 M NaCl clearly shows the importance of the hydrophobic interactions between the protein and the phospholipid vesicles.

Table 1
Association of fluorescamine labeled lysozyme to unilamellar phospholipid vesicles

Sample	Fluorescence polarization		
	pH 5.5	pH 7.6	pH 10.0
Lysozyme-fluorescamine			
Alone	0.255 ± 0.008	0.241 ± 0.001	0.243 ± 0.009
+ PC-vesicles	0.326 ± 0.004 [a]	0.292 ± 0.016 [c]	0.268 ± 0.005 [c]
+ PC/PA-vesicles	0.348 ± 0.004 [b]	0.310 ± 0.012 [a]	0.295 ± 0.014 [d]
+ NaCl (0.1 M)	0.244 ± 0.005	0.245 ± 0.003	0.241 ± 0.008
+ NaCl (0.1 M) and PC/PA-vesicles	0.315 ± 0.003	0.322 ± 0.008 [a]	0.298 ± 0.017 [a]

Association was estimated by fluorescence polarization assay. Final protein concentration was $5 \cdot 10^{-6}$ M. Phospholipid vesicles (50 μM) were prepared in 20 mM acetate buffer (pH 5.5), 20 mM phosphate buffer (pH 7.6) or 20 mM carbonate buffer (pH 10.0). Values are means of five experiments.
[a] $p < 0.005$, [b] $p < 0.001$, [c] $p < 0.025$, [d] $p < 0.010$. These values of p were obtained by comparison with the corresponding control to respective pH.

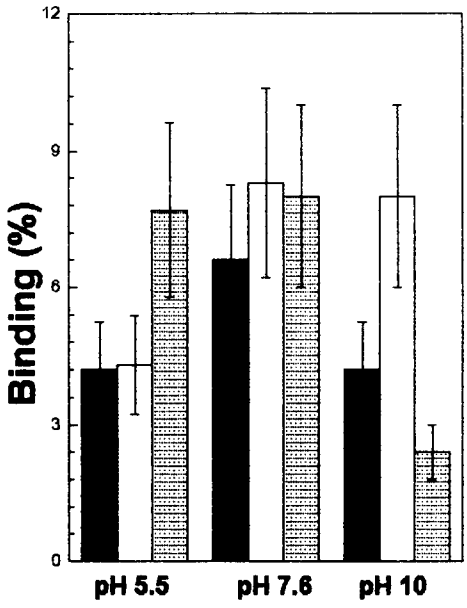

Fig. 1. pH and lipid-dependent binding of lysozyme to multilamellar vesicles. 200 nmol PC (black bars), PC/PA (9:1) (white bars) or PC/PA (9:1) in the presence of NaCl 0.1 M (gray bars) were incubated with lysozyme (280 mg) at the indicated pH. The extent of binding was determined by centrifugation method, as described in Materials and methods. These results are means ± S.E. of quintuplicate experiments.

3.2. Charge dependence of liposome aggregation

The presence of $5 \cdot 10^{-6}$ M lysozyme strongly increased the 90° light scattering of a sample of PC/PA liposomes, as compared with the scattering level of PC liposomes. When 150 mM NaCl was added to the mixture of PC/PA liposomes the scattering was nearly the same as that the PC liposomes (Fig. 2). These results suggested that aggregation of liposomes induced by lysozyme is strongly dependent on the negatives charges of liposomes; moreover, electrostatic forces could play an important role since high ionic strength inhibited the aggregation process.

3.3. Leakage and fusion experiment

Since the association of proteins to lipid bilayers frequently alters the membrane integrity and changes the permeability, we examined the release of encapsulated fluorescent complex Tb-DPA from liposomes induced by lysozyme association. Fig. 3A shows a typical time-course of aqueous content leakage from PC/PA vesicles induced by lysozyme at pH 7.6. In the concentration range of lysozyme tested, the Tb-DPA complex release occurred within the first 15 s. With our experimental system it was impossible to follow shorter kinetic events. After the initial burst the fluorescence level remained constant. Identical results have been obtained at pH 5.5 and 10.0 (not shown). We also measured the time-course of PC/PA vesicles fusion induced by $5 \cdot 10^{-6}$ M lysozyme (inset, Fig. 3A). Comparisons of kinetic release of aqueous content and fusion clearly indicate that release occurs before vesicle fusion is completed. When maximum liposome leakage was expressed as a function of protein concentration, the effect of lysozyme was seen to be almost linear and did not reach saturation even at a protein concentration of $3 \cdot 10^{-5}$ M (Fig. 3B). On the other hand, dependence of extent of fusion on protein concentration shows a plateau at $5 \cdot 10^{-6}$ M lysozyme (inset, Fig. 3B). These studies show that leakage and fusion are two uncoupled processes with different kinetics and substrate saturation curves. Further studies were made to examine the influence of the ionic strength and liposome composition on the leakage process induced by lysozyme. Table 2 shows the maximal release of aqueous content from neutral (PC), negatively charged (PC/PA, 9:1) and negatively charged liposomes in the presence of 0.1 M NaCl, at different pH values. Measurements with these liposomes gave identical percentage release of Tb-DPA complex in all cases. A comparison between leakage and fusion of PC/PA vesicles induced by lysozyme at pH 7.4, is also included in Table 2. Although the enzyme induced leakage from vesicles with different lipid composition, fusion occurs as we described previously [5], but only with negatively charged vesicles (PC/PA, 9:1) without NaCl. We attributed this result to the fact that fusion was a leaky process. In this paper we show that lysozyme

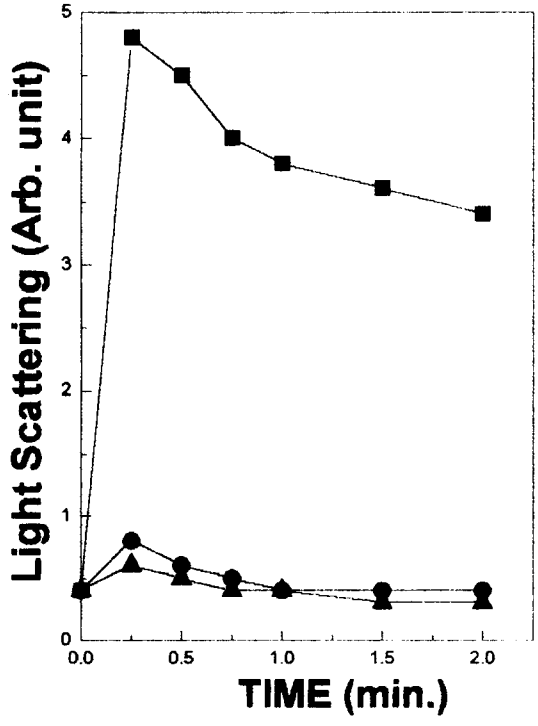

Fig. 2. Kinetic of lysozyme-induced aggregation of PC (▲), PC/PA (■) and PC/PA liposomes in the presence of 150 mM NaCl (●).

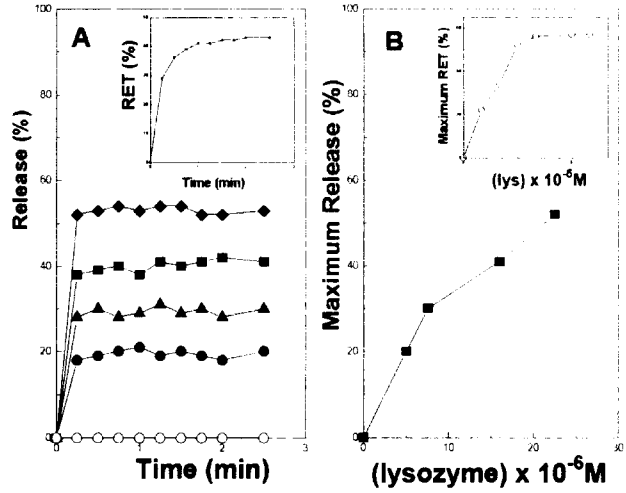

Fig. 3. (A) Time dependence of phospholipid vesicles aqueous content leakage induced by lysozyme. 50 μM PC/PA (9:1) vesicles containing Tb-DPA complex entrapped within were prepared as described in Materials and methods. Release was induced by addition of 0 M (○), $4 \cdot 10^{-6}$ M (●), $7.5 \cdot 10^{-6}$ M (▲), $15 \cdot 10^{-6}$ M (■) and $22.5 \cdot 10^{-6}$ M (♦) lysozyme and incubation at 37°C. (Inset) Time dependence of lipid intermixing induced by $5 \cdot 10^{-6}$ M lysozyme. (B) Dependence of maximal release of liposomal content as a function of lysozyme concentration. (Inset) Dependence of maximal lipid intermixing (% RET) as a function of lysozyme concentration.

Table 2
Effect of vesicles charge and ionic strength on the release of aqueous content and fusion of liposomes induced by lysozyme at different pH

	Release (%)			RET (%)
	pH 5.5	pH 7.4	pH 10.0	pH 7.4
PC vesicles	32±4	35±6	30±4	0
PC/PA vesicles	33±5	35±3	37±7	67±10
PC/PA vesicles +0.1 M NaCl	31±5	30±2	34±5	0

Small unilamellar vesicles (50 μM phospholipid) were prepared according to Materials and methods in 20 mM acetate (pH 5.5), phosphate (pH 7.6) or carbonate (pH 10.0) buffers. Vesicle mixture was incubated 5 min at 37°C with $7.5 \cdot 10^{-6}$ M lysozyme.
Values ± S.E. are means of five experiments.

was able to interact with uncharged vesicles, inducing leakage without fusion.

3.4. Relationship between association, leakage and fusion

The experiments described here as well as previous results published recently, together with theoretical considerations enable us to propose a model where association of lysozyme with small unilamellar vesicles could be related to leakage and fusion of these vesicles.

Phospholipid small unilamellar vesicles bearing negative charges (PC/PA, 9:1) are stable structures that do not aggregate and fuse spontaneously, because of electrostatic repulsion (Fig. 4). Although vesicles consisting of neutral phospholipids (PC vesicles) have a tendency to aggregate following Van der Waals attraction, the hydration forces between phospholipid bilayers seprated from each other slightly are so strong that they normally keep the membranes separated [14]. Lysozyme is a basic protein and under pH 10 (isoelectric point = 10.7) is positively charged. Associations of the protein occur with both negatively charged and neutral small unilamellar vesicles (Table 2 and Fig. 1). Experiments of fluorescence polarization suggest that hydrophobic interaction could take place (Table 1). However, polar interactions can not be ruled out. This observation agrees with the mechanism proposed by Kimelberg [2].

The interaction of the hydrophobic protein domain with the phospholipid bilayer could provoke local fluctuations in lipid packing with a subsequent leakage of the aqueous content. Furthermore, the protein domain emerging from the lipid bilayer, will give an additional positive character to the surface vesicles. As a result, when lysozyme interacts with negatively charged vesicles, both negative charges of the phospholipid head group and positive charges of protein domain would facilitate vesicle cross-linking and subsequently aggregation (see Fig. 4).

Since fusion of phospholipid vesicles consists of two distinct coupled steps, formation of vesicle aggregates and the fusion reaction itself, the association of lysozyme with neutral vesicles will not induce fusion because aggregations of vesicles do not occur (see Fig. 2). Association with negatively charged vesicles facilitates vesicle aggregation and appearance of defects, due to the presence of the protein domain immersed into the lipid bilayer. The defects are brought 'opposed' to each other because of vesicle aggregation. This configuration would render a structure which is energetically unfavorable as a result of local dehydration, phase transition or lateral phase separation, which will finally conduce to fusion.

Several agents that induce fusion, among these basic proteins, have received much attention. The major questions asked are, how do these agents act and is there a common mechanism of fusion? The membrane fusion activity of polycationic proteins was investigated in several systems that use negatively charged membranes. Cytochrome *c* [15], myelin basic protein [16], as well as synthetic polycations such as polylysine [17] and polyhistidine [18], among others, have fusion activity accompanied by an increase of membrane permeability. The interaction of myelin basic protein with large unilamellar vesicles and subsequent aggregation, release and fusion, was extensively examined by Terbeest and Hoekstra [19]. Our results obtained with lysozyme show great similarity to those found with myelin basic

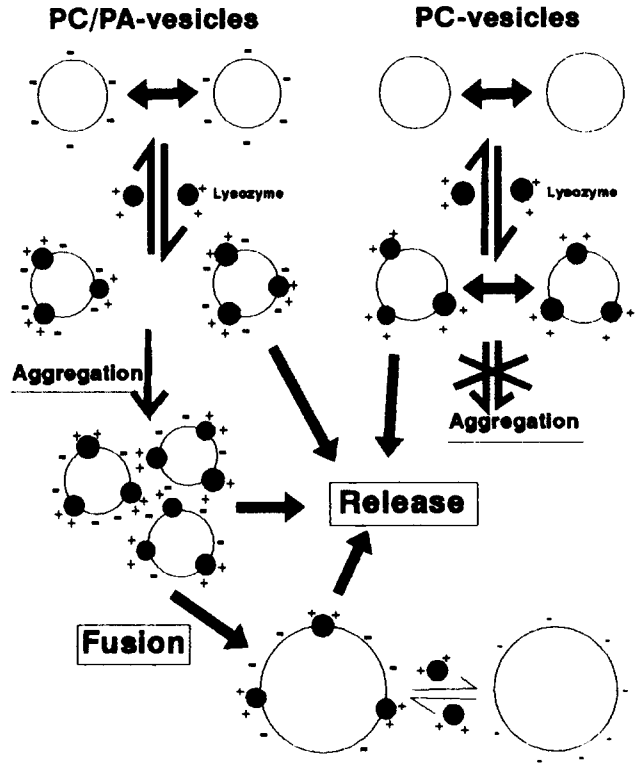

Fig. 4. Schematic drawing showing the molecular interaction of lysozyme with uncharged (pure PC) and negatively charged (PC/PA, 9:1) phospholipid vesicles. Relationships with fusion and leakage of aqueous content.

protein, since both proteins interact with liposomes inducing leakage and aggregation in a separate process. However, some differences concerning lipid mixing have been found, fundamentally due to the size of unilamellar vesicles and different lipid compositions employed.

The results presented here do not allow us to establish the molecular mechanism of fusion induced by lysozyme. Further studies, in progress in our laboratory, will enable us to understand better the role of the lipid phase, radii of curvature of vesicles as well as the identification of the protein domain responsible for protein–lipid interaction and phospholipid vesicle fusion.

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