Association of Lysozyme with Phospholipid Vesicles is Accompanied by Membrane Surface Dehydration

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Abstract. Lysozyme is a globular protein which is known to bind to negatively charged phospholipid vesicles. In order to study the relationship between binding of the protein and the subsequent destabilization of the phospholipid vesicles a set of experiments was performed using phospholipid monolayers and vesicles. Using microelectrophoresis the binding of lysozyme to phospholipid vesicles made of PS was determined. At low ionic strength and mild acidic pH of the solution lysozyme reduced the magnitude of the negative zeta potential of PS vesicles at lower concentrations compared to neutral pH and high ionic strength. In contrast, the bound fraction of lysozyme to PS vesicles was nearly constant at acidic and neutral pH. At low pH, the binding of lysozyme was accompanied by a strong aggregation of the vesicles. Lysozyme binding to PS vesicles is accompanied by its penetration into the PL monolayer. This was measured by surface tension and film balance measurements at low pH and low ionic strength. The interaction of lysozyme with negatively charged vesicles lead to a decrease of the vesicle surface hydration as measured by the shift of the emission peak of the fluorescent probe DPE. The binding of bis-ANS increased at low pH after addition of lysozyme to the vesicles. This indicates that more hydrophobic patches of the lysozyme-PS complex are exposed at low pH. At low pH the binding process of lysozyme to PS vesicles was followed by an extensive intermixing of phospholipids between the aggregated vesicles, accompanied by a massive leakage of the aqueous content of vesicles.

Key words: Lysozyme — Liposomes — Aggregation — Fusion — Fluorescence spectroscopy — Electrophoretic mobility

Abbreviations: PC: phosphatidylcholine; PS: phosphatidylserine; PL: phospholipid; DPE: *N*-(5-dimethylaminonaphtalene-1-sulphonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine; NBD-PE: *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine; Rh-PE: *N*-(lissami-

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ne rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine; ANTS: 8-aminonaphtalene-1,3,6-trisulfonic acid; DPX: *p*-xylene-*bis*-pyridinium bromide; MLV: multilamellar vesicles; SUV: small unilamellar vesicles; LUV: large unilamellar vesicles; mol. wt.: molecular weight; EPM: electrophoretic mobility; HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulfonic acid; bis-ANS: 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid; AMPSO: 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid; MES: 2-[N-morpholino]ethanesulfonic acid; 1,8-ANS: 1-anilinonaphtalene-8-sulfonic acid; PA: phosphatidic acid

Introduction

Protein-induced fusion is an important event in intra- and intercellular processes such as viral entry, and exo- and endocytic transport. Whereas artificial fusion events induced by cations or polymers have been subject of research for many years, the peptide- and protein induced fusion came into the focus during the last decade only.

Several water-soluble proteins have been used to investigate the mechanisms of their binding to PL vesicles under different circumstances, subsequently leading to PL vesicle aggregation and fusion. Lysozyme (Posse et al. 1990; Arnold et al. 1992; Dimitrova and Matsumura 1997), cytochrome *c* (Pinheiro and Watts 1994), annexins (Köhler et al.1997), albumin (Schenkman et al. 1981; Garcia et al. 1983), myelin basic protein (Cajal et al. 1997; ter Beest and Hoekstra 1993), clathrin (Maezawa et al. 1989), belong to this group of proteins. Lysozyme induces fusion of acidic PL vesicles under definite conditions. Arvinte et al. (1986, 1987) coupled lysozyme to neutral PL vesicles and measured fusion with erythrocytes at low pH. From these measurements the authors concluded that the maximum of lysozyme fusion activity is in the range of about pH 5.0. In a recent paper Arnold et al. (1992) reported that the lysozyme-induced fusion of PS vesicles is pH dependent and the maximum occurs at pH 5.0. Additionally, using energy transfer measurements and monolayer techniques they observed a penetration of lysozyme into the PL membrane. On the contrary, Posse et al. (1990) reported fusion efficiency for lysozyme over a broad pH range (pH 4.0–9.0). These authors did observe no internal content mixing of the vesicles, but a massive PL mixing accompanied by vesicle leakage.

In the present paper we will further outline the mechanisms of the interaction of lysozyme with phospholipid membranes especially under the view of surface dehydration in the aggregation and fusion process of negatively charged PL membranes. Lysozyme- bilayer interactions have been considered a paradigm for the electrostatic binding of peripheral proteins to biological membranes. As a consequence the mechanism of these associations has been intensively studied *in vitro*, using both lipid monolayers and lipid vesicles as model systems.

Lysozyme has been shown to bind strongly via electrostatic interactions to negatively charged membrane surfaces, and it has been established that such binding may have both structural and functional role, mediated by effects on the conformation of the protein as well as on the structure of the membrane surface.

Spectroscopic studies have shown that the binding of lysozyme induces both protein structural changes involving the immediate environment as well as extensive alterations in the conformation of the protein backbone including loosening and destabilizing of the overall protein structure. Lysozyme binding has been demonstrated to alter the structure of the lipid phase of negatively charged membranes (Gulik-Krzywicki et al. 1969). Although the electrostatic interactions between negatively charged membranes and lysozyme are well documented and have been extensively discussed within the context of specific mechanisms of lysozyme binding, some results indicate the existence of hydrophobic interactions of the protein with the lipid bilayer. Strong experimental support for a hydrophobic component in the interaction has been provided by the finding that all bound lysozyme cannot completely dissociate from such membranes, either by increasing the ionic strength of the buffer solution, or by dilution of the bulk protein (Kimelberg and Papahadjopoulos 1971). Consistent with the electrostatic nature of the interaction, this effect occurs despite the fact that the initial binding itself is very sensitive to both the ionic strength of the solution and the amount of the charge in the lipid membrane.

In summary, there appears to be a general consensus on two points:

1. the initial binding step of lysozyme to the surface of the lipid membrane is governed by electrostatics,

2. subsequent to the initial stage of binding, changes in the structure of the protein and lipid components of the membrane occur, leading to a new and more complex situation involving penetration of lysozyme into the lipid phase.

A combination of methods was used in the present work to provide new insights in the action of lysozyme on phospholipid membranes.

Materials and Methods

Phosphatidylserine (PS), phosphatidylcholine (PC) (all from Sigma, St. Louis USA) were used without further purification. The PL used were pure as shown by thin layer chromatography. 4,4‘-dianilino-1,1‘-binaphthyl-5,5‘-disulfonic acid (bis-ANS), 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS), p-xylenebis(pyridiium bromide) (DPX) and fluorophore-labelled phospholipids *N*-(5-dimethylaminonaphtalene-1-sulphonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (DPE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (NBD-PE), *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (Rh-PE) were obtained from Molecular Probes. Egg white lysozyme was from Boehringer Mannheim, Germany.

The following buffer solutions were used: 10 mmol/l citrate, 0.1 mol/l NaCl, pH 4.0; 10 mmol/l MES, 0.1 mol/l NaCl, pH 6.0; 10 mmol/l HEPES, 0.1 mol/l NaCl, pH 7.4; 10 mmol/l HEPES, 0.1 mol/l NaCl, pH 8; 10 mmol/l AMPSO, 0.1 mol/l NaCl, pH 9.

Water was purified on a Barnsted nanopure equipment. Multilamellar vesicles (MLV) were obtained according to Bangham et al. (1974). Small unilamellar vesi-

cles (SUV) were prepared by hydrating the phospholipids in appropriate buffer, vortexing for 10 min and then sonicating for 10 min using a Branson W-250 tip sonifier under temperature control. Large unilamellar vesicles (LUV) were obtained after extrusion (5 times) through a Nucleopore polycarbonate membrane (0.1μm) using an extruder (Lipex Biomembranes) above the phase transition temperature of the phospholipid used. Phosphate determination was done according to Chen et al. (1956). Protein concentrations were determined using Bicinchoninic acid assay (Sigma).

Binding experiments

PS SUV were added to lysozyme at pH 4.0 and 7.4, incubated for one hour at 37℃, then centrifuged in a Beckman L8-60 ultracentrifuge using a SW 41 rotor with floating spacers at 300,000 $\times$ g for 1 hour. The concentration of lysozyme in the supernatant was determined using the bicinchoninic acid kit.

Monolayer experiments

Measurements of the surface tension were done on a Krüss K 12 D process tensiometer.After addition of an appropriate amount of PL in chloroform/hexane a monolayer was formed with a film pressure of about 25 mN/m. After stabilization ($\sim$ 15 min.) lysozyme was added to the subphase under constant stirring.

Alternatively, a thermostatted teflon through was used with a subphase volume of 30 or 80 ml and a surface area of about 17 or 34.7cm^2, respectively.

Two different modes of measurements were used:

1. The area of the spread phospholipid film was held constant. Film pressures were measured as a function of the lysozyme addition.

2. The internal film pressure was held constant (25 mN/m). Changes of the area of the spread phospholipid film were monitored.

Lysozyme was introduced to the stirred subphase behind the barrier.

Measurements of the surface dielectric constant

DPE was mixed with the phospholipids in chloroform at a phospholipid/DPE molar ratio of 200–300. The samples were evaporated, then suspended in the appropriate buffer solution. The fluorescence signal of DPE was detected by a spectrofluorimeter (Perkin-Elmer, LS-50). The excitation wavelength was 340 nm, the emission was measured in the range of 400–600 nm. From the shifts of the emission peak maxima the dielectric constants of the polar region of the lipid membrane were calculated using the Stokes equation which relates the wavelength at the maximum value of the emission spectrum and its dielectric properties (Kimura and Ikegami 1985).

Phospholipid mixing assay (NBD-PE/Rh-PE)

The PL mixing of the vesicles was monitored by the fluorescence energy transfer method, using NBD-PE and Rh-PE (Struck et al. 1981). The vesicles were composed of the appropriate phospholipid and 1% (mol/mol) of both NBD-PE and Rh-PE. One part (0.05 mmol lipid) of the fluorophore-incorporated vesicles and

two parts (0.1 mmol lipid) of the unlabelled vesicles were suspended in 2 ml of the appropriate buffer solution (without/with lysozyme). The fluorescence measurements were carried out by exciting at 470 nm and recording the fluorescence from 500 to 620 nm. The recording started after the system had almost reached equilibrium ($\sim$ 1 min.). The extent of phospholipid mixing M was calculated from the emission intensities of NBD-PE at 525 nm and Rh-PE at 578 nm:

$$M = (I_{525}/I_{578}) - (I^0_{525}/I^0_{578})$$

where I was the fluorescence amplitude from the sample containing lysozyme, and I^0 that without lysozyme.

The experiments were done at room temperature. The 100 % value of PL mixing was defined as the fluorescence amplitude obtained after solubilization of the vesicles in 0.1 % Triton X-100.

Vesicle leakage assay (ANTS/DPX)

For the preparation of LUV containing ANTS and DPX, the phospholipids were evaporated and subsequently suspended in the appropriate buffer solution containing 12.5 mmol/l ANTS, 45 mmol/l DPX, 20 mmol/l NaCl. MLV were produced by 5 freeze-thaw cycles. LUV were prepared by extrusion through polycarbonate membranes on a Lipex extruder. Free dyes were removed by passage through a Sephadex G-75 column. The excitation wavelength was set to 360 nm, the total fluorescence emission was measured at wavelength $>$ 540 nm using an emission filter at 530 nm. 100% leakage was measured after addition of 0.1 %(w/v) Triton X-100 (Ellens et al. 1985).

Laser electrophoresis and light scattering

The electrophoretic mobilities of PS LUV were measured using a Malvern Zetasizer 4 device. The modulation frequency of 250 Hz was used. The electrode current was 7.5 mA for 0.1 mol/l NaCl buffer. The 90° light scattering was measured using the same sample. Multimodal analysis was used for the deconvolution of the data.

Measurement of hydrophobic sites

Bis-ANS was added as aqueous solution to the sample. The excitation wavelength was 390 nm, and the emission intensity was measured at 500 nm.

Results

Binding

The binding of lysozyme to PS SUV was measured by an ultracentrifugation based assay. In Fig. 1 the results are given for pH 4.0 and pH 7.4 and two different ionic strengths. At the low ionic strength (10 mmol/l NaCl) the binding of lysozyme to PS SUV decreased with the increasing pH. At pH 4.0 about 0.028 mol lysozyme

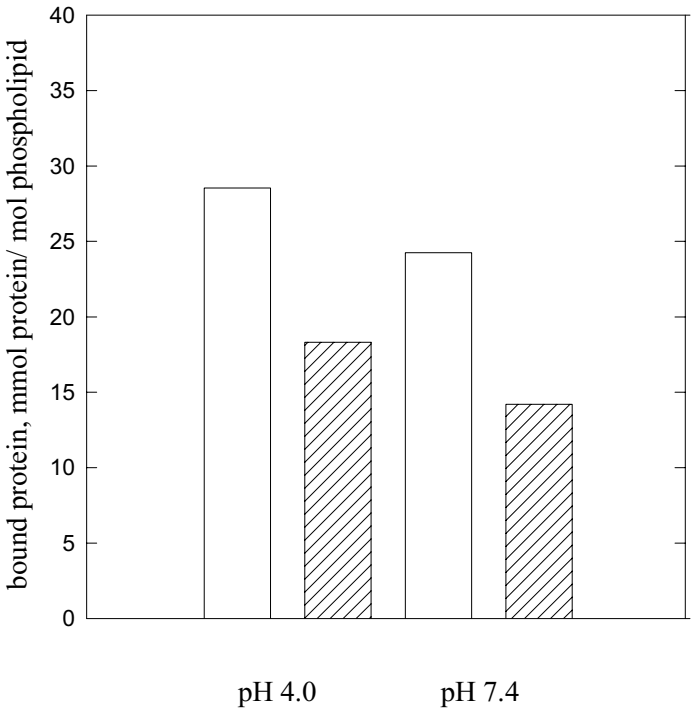

Figure 1. Lysozyme binding to PS SUV at pH 4.0 and pH 7.4 at different ionic strengths at a lysozyme/PS molar ratio of 0.35. (10 mmol/l NaCl open bars, 100 mmol/l NaCl dashed bars). 25 °C.

was bound to 1 mol PS. This is equal to 1 mol lysozyme to 40 mol of total phospholipid. Taking into account that 2/3 of the phospholipids are located on the outer monolayer of the vesicles, only 26 mol phospholipids are associated with one mol lysozyme. Assuming the area per phospholipid to be 0.7 nm^2, this equals 18.2 nm^2. The lysozyme molecule has an ellipsoidal shape with dimensions of $4.5 \times 3 \times 3$ nm. The maximum area is then about 13.5 nm^2.

At pH 7.4 and 9.0 (data not shown) the amount of bound lysozyme to PS was somewhat smaller than at pH 4.0, but lysozyme still bound to PS.

The amount of bound lysozyme per PS in solutions of high ionic strength was smaller at all pH values measured. This result suggests the importance of electrostatic attraction forces between lysozyme and the PS membrane.

Microelectrophoresis

In buffer solutions containing 100 mmol/l NaCl a zeta potential of about −45 mV was measured for PS SUV at pH 7.4. This value is smaller in magnitude than reported for multilamellar PS vesicles (Eisenberg et al. 1979). This may be due

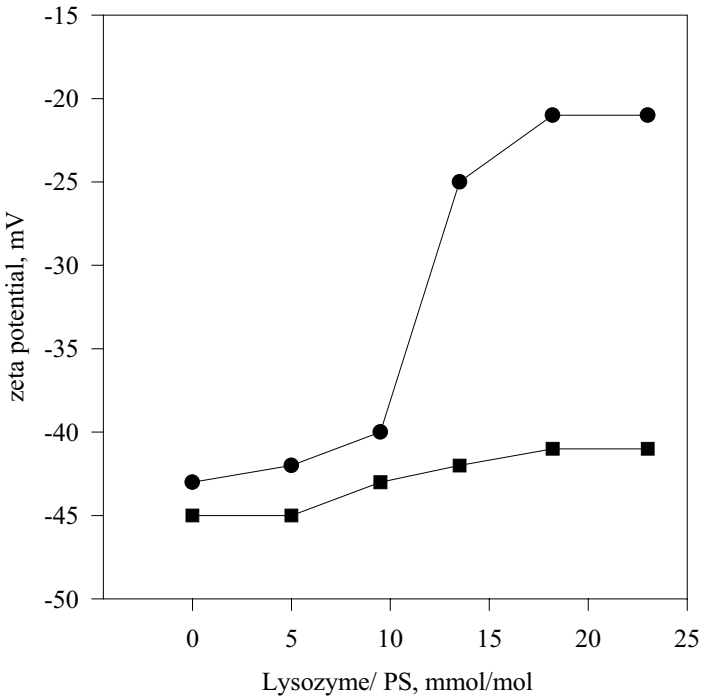

Figure 2. Zeta potentials of PS SUV in dependence on the lysozyme concentration at pH 4.0 (●) and pH 7.4 (■). 100 mmol/l NaCl, 25 °C

to the use of SUV (diameter about 50–60 nm) instead of MLV (diameter roughly several μm). A possible explanation for these differences in zeta potential between MLV and SUV was recently given by Egorova et al. (1992). The decrease of the solution pH does not result in a drastic change of the measured zeta potential (zeta potential −43 mV for pH 4.0). For PS the dissociation steps were reported to be at pH 3.0–3.5 for the carboxyl group, and at pH 3.5–3.7 for the phosphate group (Papahadjopoulos 1968).

At low pH, the addition of lysozyme to PS SUV resulted in a more positive zeta potential of the vesicles (Fig. 2). At pH 4.0 the calculated zeta potentials changed for PS from −43 mV to −20 mV in the presence of 18–23 μg/ml lysozyme. A drastic reduction of the zeta potential magnitude occured at lysozyme to PS molar ratios larger than 0.01. Measurements at different ionic strength revealed qualitatively the same behaviour. Measurements at pH 7.4 resulted in a moderate decrease of the EPM value after addition of lysozyme to the PS vesicles, the zeta potential decreases from −45 mV to −42 mV at lysozyme to PS ratios larger than 0.02.

On the other hand, addition of lysozyme to PC vesicles at pH 7.4 and 4.0

did not change the zeta potential of the vesicles indicating a complete reduction of binding.

The addition of denaturated lysozyme (heated to 60 ℃ for one hour) did not induce any change of the EPM of PS SUV (also at different pH). Addition of natural inhibitors of lysozyme, N-acetyl-glucosamine or N-acetyl-galactosamine, inhibited the binding of lysozyme to PS vesicles at pH 4.0 in a concentration dependent manner. This behaviour was also observed for the negatively charged polymer dextran sulfate.

This inhibition occurs because of the binding of these negatively charged molecules to the positively charged surface regions of lysozyme preventing an attachment to the negatively charged PS surface. It has been demonstrated in other reports that there is no binding of DS to the negatively charged phospholipid surfaces (Krumbiegel and Arnold 1990).

Light scattering

At low pH, the addition of lysozyme to PS SUV was accompanied by a strong increase in particle size indicating aggregation of vesicles (data not shown). This process is connected with electrostatic interactions of lysozyme with the vesicle surfaces, because the addition of the negatively charged dextran sulfate inhibited the aggregation. Addition of denaturated lysozyme did not lead to vesicle aggregation either.

Particle size changes at pH 4.0 revealed increases of the particle diameter from 50 nm diameter to about 400 nm in the presence of 1–2 μmol/l lysozyme. At pH 7.4 the increase of the particle size occured only at much higher lysozyme concentrations ($>$ 100 μg/ml). The aggregation process is connected with several changes in the physico-chemical characteristics which shall be described below.

Monolayer experiments

In Fig. 3, the decrease of the surface tension of a PS monolayer caused by lysozyme is shown for different pH and ionic strengths. The experiments were carried out at an initial surface pressure of 25 mN/m. Addition of a bulk lysozyme concentration of 3 μg/ml resulted in a decrease of the surface tension of about 10 mN/m at pH 4.0 (10 mmol/l NaCl). Low pH conditions, but higher ionic strength (50 and 100 mmol/l NaCl) resulted in smaller changes of the surface tension ($\sim$ 7.5 and 6 mN/m, respectively).

Especially at higher pH values (pH 7.4 and 9.0) the regulation of the lysozyme induced change of surface tension by the ionic strength was obvious. At these pH values, the surface tension of the PS monolayer was reduced by maximally 8 mN/m after addition of 3 μg/ml lysozyme in 10 mmol/l NaCl containing solutions, whereas at high ionic strength (100 mmol/l NaCl) the effect was vanishing (maximum 1 mN/m).

In the experiments illustrated in Fig. 3 the surface area was held constant, the following experiments were carried out using a constant pressure in the monolayer.

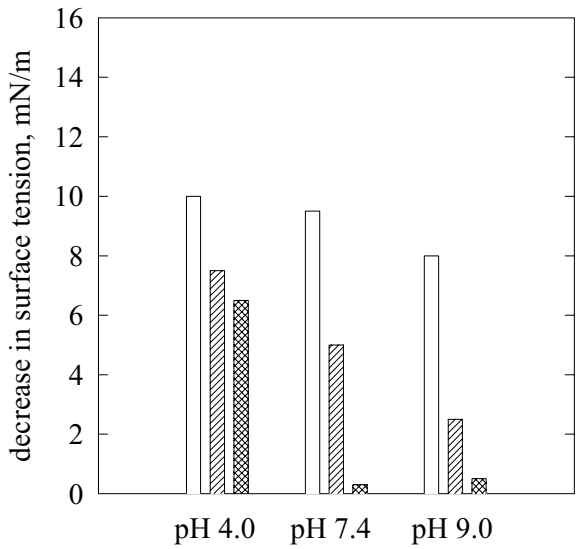

Figure 3. Decrease of surface tension of a PS monolayer after addition of 3 μg/ml lysozyme into the subphase. The ionic strength of the buffer was 10 mmol/l NaCl (open bars), 50 mmol/l NaCl (dashed bars) and 100 mmol/l NaCl (crossed bars). Measurements were done at 25 ℃, the initial film pressure was 25 mN/m.

The change of the surface area was measured after addition of lysozyme at different pH as shown in Fig. 4.

At low ionic strength the area increase of the PS monolayer was larger than 50% independent of the pH of the subphase after addition of 10 μg/ml lysozyme. This is a result of at least partial penetration of lysozyme into the monolayer. At high ionic strength the change in surface area was moderate (pH 4.5 about 0.5%, pH 7.4 about 1.5%).

For the low ionic strength conditions the amount of penetrated lysozyme into the PS monolayer was only small. In our experiments, the change of the PS surface area was about 20.4 cm^2 (in 10 mmol/l NaCl, pH 4.5), equal to 2.04×10^{15} nm^2. In the subphase about 1.65×10^{16} lysozyme molecules were present. The lysozyme molecule has an ellipsoidal shape with dimensions $4.5 \times 3 \times 3$ nm. So, in theory the maximal area of all lysozyme molecules in the surface could be 2.22×10^{17} nm^2. The comparison with the measured values shows that only a part of the lysozyme molecules penetrated into the monolayer.

PL-mixing assay

Using the NBD-PE/Rh-PE fluorescence assay the lysozyme induced PL mixing of PS LUV was measured. The results are given in Fig. 5. The maximal changes of the phospholipid mixing M are given for pH 4.0 and 7.4. At pH 4.0 lysozyme at

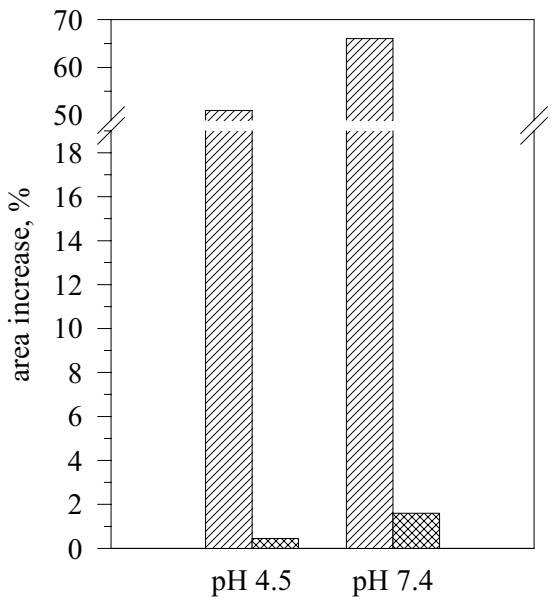

Figure 4. Surface area increase of a PS monolayer after addition of 10 μg/ml lysozyme at different pH and ionic strengths (10 mmol/l NaCl dashed bars, 100 mmol/l NaCl crossed bars). The temperature of the subphase was 25 ℃. The film pressure was held constant at 25 mN/m.

a concentration lower than 10 μg/ml induced a strong PL mixing of the vesicles (30–60 %). At pH 7.4 the same concentrations of lysozyme did not induce any detectable phospholipid mixing of PS LUV. Using higher lysozyme concentrations ($>$ 200 μg/ml) PL mixing could be also observed under these conditions.

Dextran sulfate 500 inhibited the PL mixing induced by lysozyme. Denaturated lysozyme (heating for 30 min. in 60 ℃ water bath) did not induce PL mixing of PS LUV at pH 4.0.

ANTS/DPX leakage assay

Coencapsulated ANTS and DPX show a low fluorescence intensity because of collisional quenching in the used concentration range. As a result of leakage the concentration of both components will be diluted in the total sample. Fig. 5 shows the results of these experiments. PS vesicles leaked very rapidly after the addition of low amounts of lysozyme at pH 4.0. The extent of leakage reached 60% at the lysozyme concentrations of 20 μg/ml. At pH 7.4 much higher concentrations of lysozyme (for instance 250 μg/ml lysozyme to reach 40% leakage) were necessary to achieve the same leakage extents compared to pH 4.0.

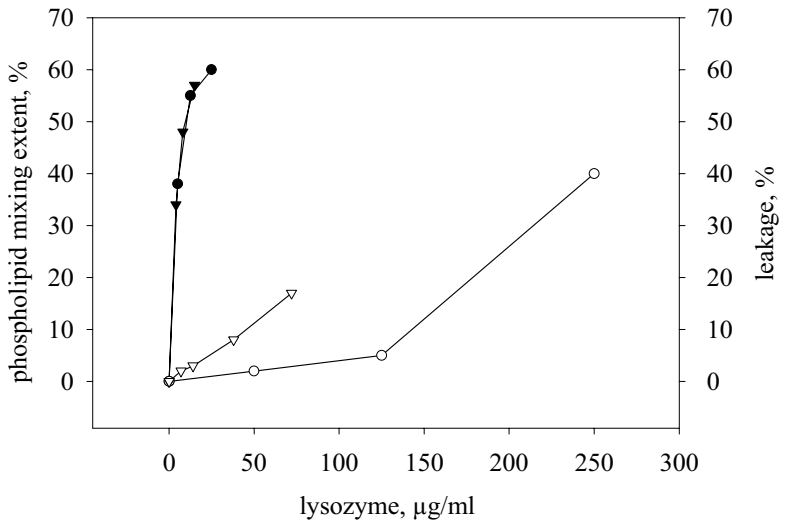

Figure 5. Phospholipid mixing extent (▼) as measured by the NBD-PE/Rh-PE assay (left axis) and the leakage extent (●) measured by the ANTS/DPX assay (right axis) of PS SUV in dependence on lysozyme concentration. Filled symbols: pH 4. 0, open symbols pH 7. 4. 0. 1 mol/l NaCl, 25 °C

Surface dielectric constant

The aggregation process of PS SUV induced by lysozyme at low pH was accompanied by a blue shift of the emission maximum of the fluorescent probe DPE incorporated in the PS membrane (see Fig. 6). The surface dielectric constant was calculated according to Kimura and Ikegami (1985). The largest changes of the surface dielectric constant were obtained in buffers of low pH.

At pH 5.0 the surface dielectric constant of PS SUV without addition of proteins was about 26. This corresponds to values reported by Ohki and Arnold (1990). Increasing concentrations of lysozyme resulted in a decrease of the surface dielectric constant. At 20 mmol/l and 100 mmol/l NaCl the decrease of the surface dielectric constant was more enhanced in comparison to samples containing 500 mmol/l NaCl indicating the role of electrostatic interaction forces between lysozyme and the PL surface.

Bis-ANS fluorescence

The three-dimensional structure of lysozyme can change when free protein binds to phospholipid membranes (Gulik- Krzywicki et al. 1969). Conformational changes may govern and reflect the expression of specific protein properties, such as the ability to induce membrane perturbation and subsequently to cause vesicle aggregation and fusion. To examine whether the pH dependent functional distinctions of

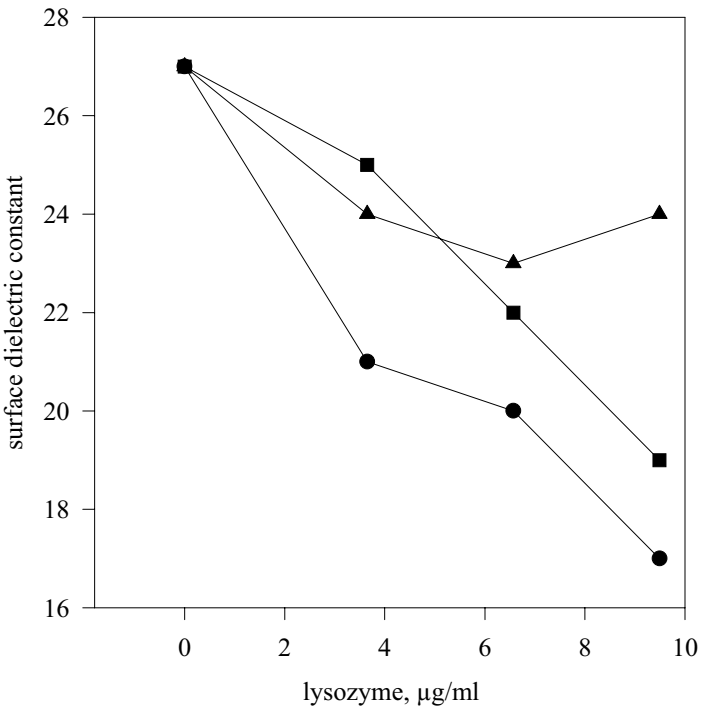

Figure 6. Calculated surface dielectric constants (measured by the emission shift of DPE) of PS SUV in dependence on lysozyme concentration at pH 4. 0 (filled symbols) at different ionic strengths (● 20 mmol/l NaCl, ■ 100 mmol/l NaCl, ▲ 500 mmol/l NaCl).

lysozyme were related to structural parameters, we studied such structural changes using bis-ANS.

The fluorescence properties of bis-ANS depend on the quantity of hydrophobic binding sites in the sample. The probe is virtually nonfluorescent in aqueous solutions, but becomes strongly fluorescent when bound to hydrophobic sites in proteins (Rosen and Weber 1969; Lambers et al. 1984). We measured the binding of bis-ANS to PS liposomes, lysozyme and PS liposomes/lysozyme at different pH. The results are given in Fig. 7.

The addition of PS to bis-ANS containing buffer induced a small increase in fluorescence emission intensity over the whole pH range studied. When only lysozyme was injected into an aqueous solution containing bis-ANS, the fluorescence intensity increased considerably in dependence on pH. At pH 6.5 the fluorescence intensity increased about 3 times, whereas at lower pH the increase was about 12-fold (pH 4.0).

The addition of both PS SUV and lysozyme resulted in a higher fluorescence emission intensity at all pH values used. The binding of bis-ANS to the

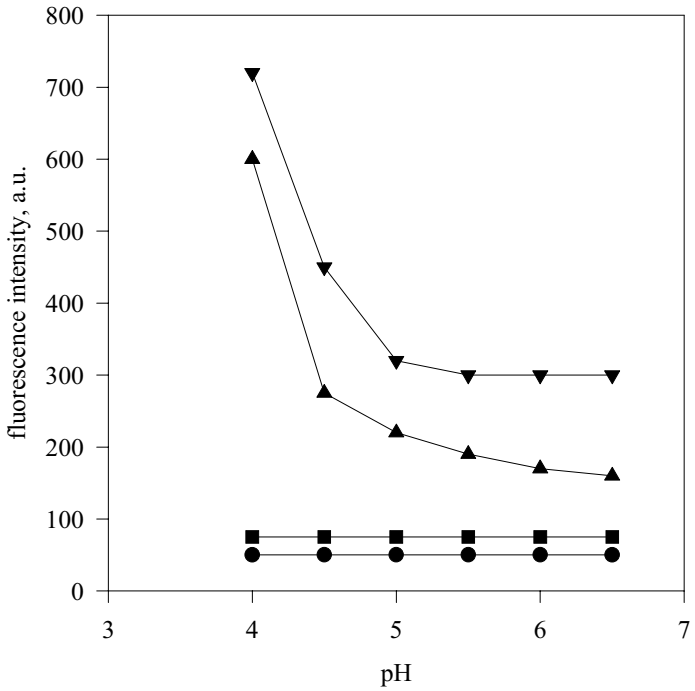

Figure 7. Fluorescence intensities of 12 μmol/l Bis-ANS (●) in the presence of 10 μmol/l PS SUV (■), or 2 μmol/l lysozyme (▲), 10 μmol/l SUV and 2 μmol/l lysozyme (▼) at different pH. Buffer: 100 mmol/l NaCl

PS/lysozyme system is regulated by electrostatic and hydrophobic attraction forces. Because bis-ANS is negatively charged the binding to lysozyme is regulated by pH. Lysozyme contains about 4 positive charges at pH 7.4, at pH 4 there are about 12–14 positive charges. The binding of bis-ANS to lysozyme at low pH should be much higher. As a control, 1,8-ANS was used, which reports preferentially the influence of changes of the surface potential. The fluorescence intensity of this probe did not change dramatically in the presence of lysozyme at different pH. Because the electrostatic component of the interaction of the analog probe 1,8-ANS with lysozyme is minimal at all pH, the drastic increase in bis ANS fluorescence resulted from changed hydrophobic properties of lysozyme. As outlined above change in fluorescence emission intensity is also caused by hydrophobic sites at the binding places. The further increase in bis-ANS fluorescence intensity in the presence of lysozyme and PS SUV at all pH seems to be a result of the exposure of additional hydrophobic sites.

Discussion

One basic conclusion to be drawn from our measurements is that lysozyme can bind to negatively charged phospholipid membranes. This binding is modulated mainly by electrostatic attraction forces modulated by pH and ionic strength and hydrophobic interaction forces (Marsh 1990). At pH 4.0 and pH 7.4 the amount of bound lysozyme per PL is approximately equal. So, at different pH the same amounts of bound lysozyme at different pH do not result in the same disturbation of the phospholipid vesicle integrity.

To point out this problem, the binding of lysozyme to PS SUV at 100 mmol/l NaCl at pH 4.0 and 7.4 is 0.018 and 0.014 mol/mol, respectively. The amount of bound lysozyme at low pH is slightly higher than at neutral pH. Looking for the PL mixing and leakage data of this system a clear difference can be observed. The PS vesicles fuse and leak dramatically at low pH, whereas at neutral pH the vesicles are relatively stable at comparable lysozyme concentrations.

From the electrophoretic data it can be concluded that the amount of charges on the lysozyme surface (at pH 4.0 about 12–14 positive charges) leads to a stronger neutralization of the PS surface charge. Assuming an equal amount of bound lysozyme at pH 7.4 (lysozyme surface charge about 3–4 positive charges) the reduction of the total surface charge of the lysozyme-PS vesicle complex is smaller. This is in accordance with measured data where the maximal binding capacity of phospholipid vesicles is regulated by the charge neutralization of the aggregates (Bergers et al. 1993).

As already shown by us (Arnold et al. 1992), lysozyme penetrates the PS monolayer at low pH. This penetration can be modulated by pH, lysozyme concentration and ionic strength. The penetration of lysozyme into the PL monolayer (shown above) and in the PS bilayer (Posse et al. 1990; Arnold et al. 1992) is accompanied by drastic changes of the protein and phospholipids in the vesicle bilayer. Using the emission properties of a headgroup-labeled phospholipid a drastic decrease of the PL surface dielectric constant was observed at low pH in the presence of lysozyme. It should be noted that the possibility cannot be excluded that lysozyme binds to the dansyl group directly. In this case a shift of the emission spectrum towards lower wavelength would be monitored. Additionally to known perturbations of the PL bilayer such as permeabilization (Kimelberg and Papahadjopoulos 1971; Posse et al. 1990) a strong reduction of water content near the headgroups at least in the contact regions was measured. These dehydrated regions are one prerequisite for the molecular attachment of opposing membrane surfaces. On the other hand, also lysozyme itself changes the exposure of hydrophobic sites at low pH. This was shown by changes of the tryptophan fluorescence in the process of binding to PL vesicles (Pap et al. 1996). In the present work these changes were measured using bis-ANS. These measurements also revealed that different membrane interaction modes may exist for lysozyme. One mode involves stable adsorption, which is electrostatically driven. The other one involves a dynamic mode of interaction, which is presumably hydrophobic in nature. This dynamic interaction and its hydropho-

bic character are reflected by changes in membrane permeability and in spectral changes, typical for hydrophobic interactions.

It is suspected that globular proteins at least change their conformation in part (Rankin et al. 1998) if they are in contact with PL membranes. It is not sure if this process is reversible. From the other class of peripheral proteins, the annexins, it is known that at low pH these proteins change the exposure of several groups of the protein to the external phase (Sopkova et al. 1994; Köhler et al. 1997). The binding characteristics of annexin V at low pH used in these studies are the same as those of the lysozyme/PS system used in the present study.

Several authors have reported that lysozyme interacts with negatively charged phospholipid membranes over a broad pH range (Kim and Kim 1989; Posse et al. 1990; Arnold et al. 1992; Bergers et al. 1993)

For instance, Posse et al. (1990) carried out resonance energy transfer measurements using NBD-PE and cholesteryl anthracene 9-carboxylate embedded in PA/PC (1:9) vesicles. They found phospholipid mixing induced by lysozyme at pH < 9. Compared to our results the discrepancies can be explained by the low ionic strength used in these experiments.

The interesting point is that aggregation, phospholipid mixing and leakage of PS vesicles do not occur despite of the binding of lysozyme at pH 7.4. This property is also observed in the presence of other negatively charged phospholipid vesicles (phosphatidic acid, phosphatidylglycerol).

It can be concluded from our data that lysozyme binds to negatively charged phospholipid vesicles over a broad pH range. The amount of binding is regulated by electrostatic forces. In dependence on pH and subsequently on total protein charge the bound protein induces a strong destabilization of the negatively charged phospholipid bilayers. The destabilization is connected with a reduced water content in the phospholipid headgroup region due to aggregation, exposure of hydrophobic patches and a total reorganization of the vesicles.

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