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Interaction of wheat α-thionin with large unilamellar vesicles

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Abstract

The interaction of the wheat antibacterial peptide α-thionin with large unilamellar vesicles has been investigated by means of fluorescence spectroscopy. Binding of the peptide to the vesicles is followed by the release of vesicle contents, vesicle aggregation, and lipid mixing. Vesicle fusion, i.e., mixing of the aqueous contents, was not observed. Peptide binding is governed by electrostatic interactions and shows no cooperativity. The amphipatic nature of wheat α-thionin seems to destabilize the membrane bilayer and trigger the aggregation of the vesicles and lipid mixing. The presence of distearoylphosphatidylethanolamine-poly(ethylene glycol 2000) (PEG-PE) within the membrane provides a steric barrier that inhibits vesicle aggregation and lipid mixing but does not prevent leakage. Vesicle leakage through discrete membrane channels is unlikely, because the release of encapsulated large fluorescent dextrans is very similar to that of 8-aminonaphthalene-1,3,6,trisulfonic acid (ANTS). A minimum number of 700 peptide molecules must bind to each vesicle to produce complete leakage, which suggests a mechanism in which the overall destabilization of the membrane is due to the formation of transient pores rather than discrete channels.

Keywords: fluorescence spectroscopy; lipid-protein interactions; liposomes; wheat α-thionin

All living beings have developed defence mechanisms that allow them to survive in hostile environments, which are often abundant in potential pathogens. One of the simplest and more widespread defense strategies consists of the production of small peptides with antimicrobial activity. Organisms as diverse as microorganisms, plants, insects and vertebrates, including humans, can produce oligopeptides with a wide range of antimicrobial activities (Haegele et al., 1995). These peptides are the product of single genes, and because of their small size they are produced by the host very rapidly and with a minimal input of energy and biomass (Broekaert et al., 1995). In higher plants, thionins constitute a well-defined group of low molecular weight peptides (García-Olmedo et al., 1989; Bohlmann & Apel, 1991) whose toxic effects on bacteria (Stuart & Harris, 1942; Fernández de Caleya et al., 1972; Molina et al., 1993; Caaveiro et al., 1997), fungi (Stuart & Harris, 1942; Bohlmann et al., 1988; Molina et al., 1993; Thevissen et al., 1996), yeast (Hernández-Lucas et al., 1974), and animal cells (Nakanishi et al., 1979; Carrasco et al., 1981) have been described. Apart from this toxicity, which could reflect a direct role in plant defence, many other biological activities have been observed (Florack & Stiekema, 1994) and, therefore, their biological role is still a matter of controversy. It is thought that most, if not all, of these effects are the consequence of their interaction with the target-cell membrane (Carrasco et al., 1981). This idea is strengthened by the fact that several thionins interact with model phospholipid bilayers (Gasanov et al., 1993; Thevissen et al., 1996; Caaveiro et al., 1997; Huang et al., 1997).

In the endosperm of hexaploid wheat (*Triticum aestivum* L.), three different thionins exist, namely, α_1, α_2, and β-thionin (Fernández de Caleya et al., 1976). These polypeptides have a molecular mass of about 5,000 and contain 45 amino acid residues. They have a high content of basic amino acid residues, which render the peptides positively charged at neutral pH and contain 8 cysteine residues that are involved in four disulfide bridges (Bohlmann & Apel, 1991). The three-dimensional structure has been solved in

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Abbreviations: LUV, large unilamellar vesicles; PC, egg phosphatidylcholine; PG, egg phosphatidylglycerol; RhB-PE, L-α-phosphatidylethanolanime-N-(lissamino rhodamine B sulfonyl); NBD-PE, 1,2-dimyristoyl-sn-glycero-3-phosphatidylethanolamine-N-[7-nitro-2-1,3-benzoxadiazol-4-yl]; PEG-PE, distearoylphosphatidylethanolamine-poly(ethylene glycol 2000); ANTS, 8-aminonaphthalene-1,3,6,trisulfonic acid; DPX, p-xylene-bis-pyridiniumbromide; FITC, fluorescein isothiocyanate; RP-HPLC, reverse-phase high-performance liquid chromatography; QELS, quasi-elastic light scattering; PS, phosphatidylserine; DPPG, dipalmitoylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol.

solution (Clore et al., 1987) and in crystalline form (Teeter et al., 1990). The molecule has the shape of the greek letter Γ, where the vertical arm consists of two antiparallel α-helices and the horizontal arm contains two antiparallel β strands and a β turn.

It has previously been shown that wheat thionin interacts with cell membranes (Gasanov et al., 1993) and model membranes (Caaveiro et al., 1997). In this work, we try to study in detail the interaction of wheat α-thionin with large unilamellar vesicles (LUV) of different lipid compositions in an attempt to understand its mechanism of action. We have used different fluorescent probes to measure various aspects of the protein-lipid interaction, e.g., release of encapsulated fluorophores, lipid mixing, vesicle fusion, and aggregation.

Results

Interaction of α-thionin with model membranes

Carrasco et al. (1981) observed that thionins modified the membrane permeability of cultured mammalian cells. More recently, Caaveiro et al. (1997) showed that the interaction of wheat α-thionin with model membranes resulted in both the release of encapsulated fluorophores and the aggregation of the vesicles, and that these effects were largely dependent on the liposome lipid composition. Therefore, we decided to further explore the interaction of α-thionin with model membranes.

Experimental conditions were set up as described in Materials and methods to analyze different aspects of the protein-lipid interaction, i.e., lipid mixing, liposome fusion, release of encapsulated fluorophores, and vesicle aggregation. The final lipid concentration in the cuvette was 0.1 mM, and the protein to lipid molar ratio was 0.033.

Figure 1 shows the results obtained with two different liposome compositions: egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG). It is clearly observed that whereas the thionin hardly exerted any effect on liposomes made of PC, it readily interacted with liposomes composed of PG, and induced vesicle aggregation (Fig. 1A) lipid mixing (Fig. 1B) and the release of the vesicle contents (Fig. 1C). However, it failed to induce vesicle fusion, i.e., the vesicle contents did not mix (Fig. 1D).

All these effects were dose-dependent (data not shown) and occurred within the first minutes of the interaction. Liposome aggregation was immediate as evidenced by the sudden increase in the scattering signal after peptide addition. This sudden increase was followed by a small and slow decrease in the light scattering, which can be attributed to the fact that some of the aggregates grew to a size larger that the incident light wavelength, as will be shown below.

The formation of aggregates was accompanied by the mixing of the lipids and the release of the vesicle contents. Both effects showed similar trends. There was a large increase in the fluorescence signal within the first minute, followed by a small and gradual increase until a stable value was reached. In both cases, addition of Triton X-100 to a final concentration of 0.1% (w/w) indicated the maximum fluorescence that could be observed after complete release of the fluorophores or complete mixing of the lipids.

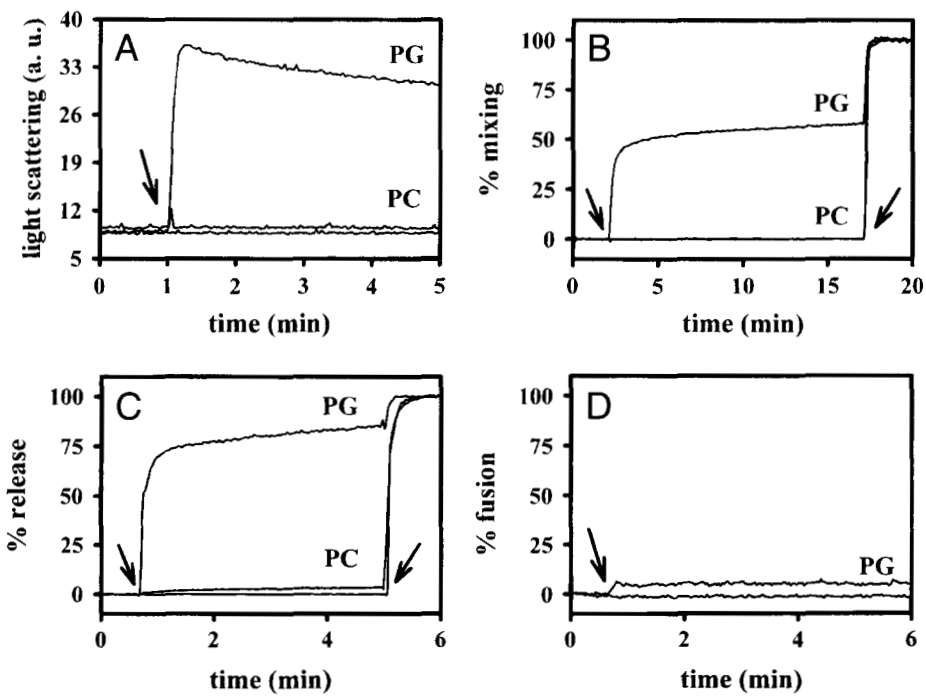

Fig. 1. Effects of wheat α-thionin on two LUV populations. Lipid compositions were 100% PG and 100% PC. **A:** Vesicle aggregation. **B:** Lipid mixing. **C:** ANTS leakage. **D:** Vesicle fusion. Lipid concentration was 0.1 mM. The first arrow indicates the addition of the peptide (final protein to lipid molar ratio = 0.033). The second arrow indicates the addition of Triton X-100 (final concentration = 0.1% w/w). Experimental conditions are described in Materials and methods. Note that in **A**, **B** and **C**, the PC trace and the controls (no protein added) are very close.

Effect of LUV composition on peptide binding

The results in Figure 1 suggest that the interaction of the positively charged thionin with liposomes composed of negatively charged phospholipids is, at least during the initial steps, mainly electrostatic. Therefore, we decided to investigate the effect of LUV composition on peptide binding.

A fixed amount of wheat α-thionin was incubated with LUV made of PG at different lipid/protein molar ratios, and after centrifugation the concentration of free protein in the supernatants was measured. As Figure 2 shows, maximum binding (98%) takes place when there are approximately 25 lipid molecules per peptide. This is the minimum amount of lipid that ensures complete peptide binding under the most favorable conditions, i.e., 100% PG.

If we change the lipid composition and decrease the PG content within the LUV, the amount of peptide bound to the liposomes decreases drastically, as can be seen in Table 1. However, it must be pointed out that even in the absence of negative charge, 25% of the peptide binds to the vesicle. This value is clearly insufficient to alter the bilayer architecture and bring about any measurable effect (Fig. 1).

Effect of the negative charge surface density

To further evaluate the relevance of the electrostatic component in this interaction, we prepared a series of phosphatidylcholine liposomes with increasing amounts of phosphatidylglycerol, and studied the effect of different negative charge surface densities on both, the amount of ANTS released and the size of the aggregates.

The amount of ANTS released as a function of the phosphatidylglycerol content of the liposome is shown in Figure 3A. The release was moderate when the PG content was equal to or lower than 40%, but PG contents of 50% or higher resulted in the complete release of the encapsulated solute. Apparently, there is a threshold value above which the release is complete. This value probably represents the minimum negative charge density that must be present at the surface of the vesicle so as to ensure the binding of the amount of protein producing the maximal release, which in this case is 50% PG.

In Figure 3B, we observe the effect of the PG content of the liposomes on the aggregate size. Low PG contents (20% or less) did not promote vesicle aggregation, whereas higher PG contents resulted in the formation of large aggregates. The largest aggregates were obtained when the liposomes contained between 40 and 60% of PG and their average diameter was approximately 8 mi-

Table 1. *Binding of wheat α-thionin to LUV of different composition*[a]

Vesicle composition	Lipid concentration (mM)	Lipid/protein molar ratio	Percentage bound protein
PC	0.41	49	25.2
PC/PG (1/1)	0.41	49	65.3
PG	0.41	49	92.8
PG	2.46	291	89.6

[a]Duplicate experiments were carried out as described in Materials and methods. Peptide concentration was estimated from the supernatant by the bicinchoninic acid assay (Smith et al., 1985) and bound protein was calculated from the diference between total and free. Results are the mean of two separate experiments

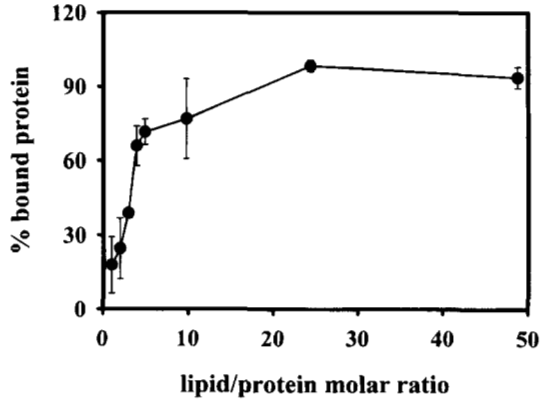

Fig. 2. Binding of wheat α-thionin to LUV made of PG. Peptide concentration was 8.4 μM. Experimental details are described in Material and methods. Points represent the mean ± standard error of two independent experiments.

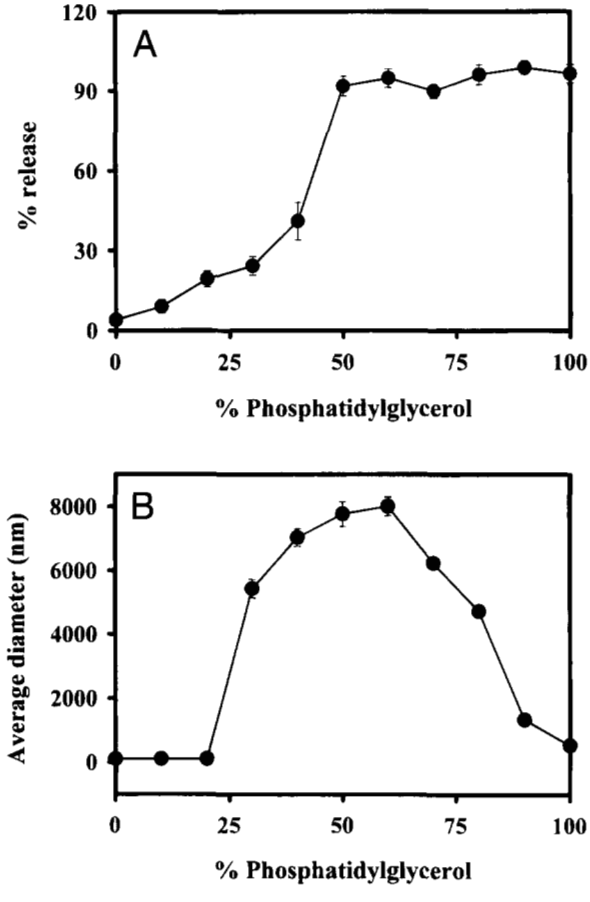

Fig. 3. Effect of the negative charge surface density within the LUV on (**A**) the amount of ANTS released by α-thionin and (**B**) the size of the aggregates. Lipid concentration was 0.1 mM. Protein to lipid molar ratio was 0.033. Points represent the mean ± standard error of three independent experiments.

crons. When the liposomes contained more than 60% PG, the mean diameter of the aggregates were considerably smaller than those observed with lower negative charge surface density, but significantly larger than the controls. This behavior could be the result of electrostatic repulsions between highly negatively charged vesicles that would counterbalance the trend to form large lipoprotein aggregates.

Effect of the ionic strength

Both the release of encapsulated fluorophore and the formation of aggregates depend largely on the negative charge surface density of the vesicles. To further confirm this hypothesis, we studied the effect of the ionic strength by decreasing the NaCl concentration of the reaction buffer from 200 to 100 mM. The results are shown in Figures 4A and 4B.

Figure 4A shows the effect of ionic strength on the amount of ANTS released from vesicles with different PG contents. The results obtained at 200 mM NaCl are replotted from Figure 3A. In both cases, the release of the encapsulated fluorophore shows the same general dependence on the negative charge surface density, but at 100 mM NaCl the curve is shifted to lower PG contents and maximal release is only observed when the PG content of the vesicles is 40% or higher. In other words, at high ionic strength there must be more negative charges at the surface of the vesicles to bind the amount of protein needed to induce complete release of the encapsulated ANTS.

Figure 4B represents the effect of a decrease in the ionic strength on the size of the aggregates formed at different negative charge surface densities. The results obtained in 200 mM NaCl are replotted from Figure 3B. In 100 mM NaCl three important effects are observed. First, no aggregates are formed when the PG content is lower than 10%, and the curve is shifted to lower PG contents by about 10%. This shift coincides with that observed in Figure 4A. Second, in 100 mM NaCl, the maximum size of the aggregates is obtained when the PG content ranges between 30 and 40%, with a mean diameter of approximately 4 microns (half the size of those formed at higher ionic strength, and shifted to lower negative charge). Third, when the PG content is higher than 50%, the mean diameter of the aggregates decreases to approximately 300 nm, probably because the electrostatic repulsions regain relevance. It must be noted that in this region of the curve, the size of the aggregates is considerably smaller than those formed in 200 mM NaCl.

These observations can be regarded as the result of the shielding of the phospholipid negative charges by the ions in solution. At high ionic strength (1) more negative charges must be present at the surface of the vesicles to observe any effect, and (2) the electrostatic repulsions within the aggregates are less important and do not interfere so strongly with the formation of very large aggregates.

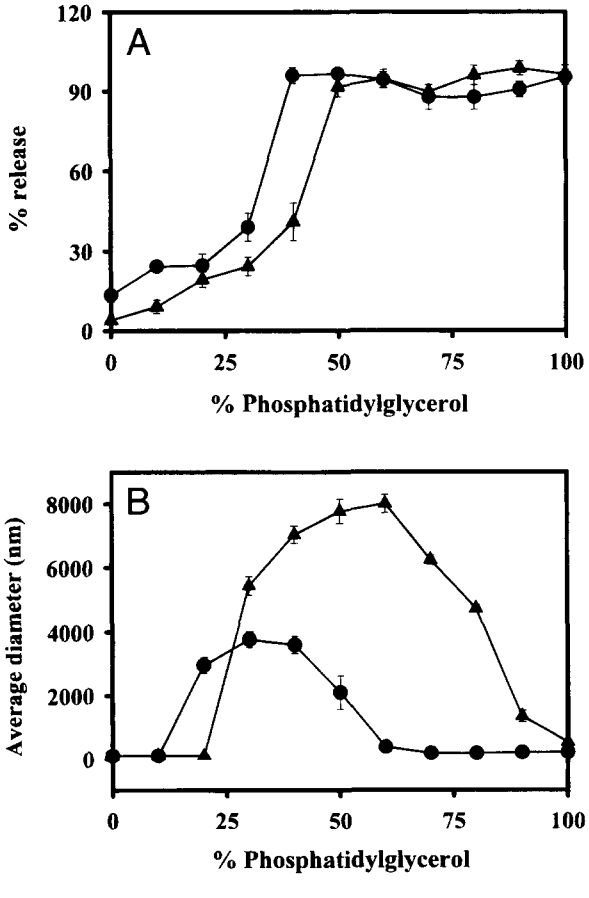

Fig. 4. Effect of the negative charge surface density within the LUV on (**A**) the amount of ANTS released by α-thionin and (**B**) the size of the aggregates at two different ionic strengths. (●) Data obtained in 10 mM HEPES, 100 mM NaCl, pH 7.5; (▲) data obtained in 10 mM HEPES, 200 mM NaCl, pH 7.5 (replotted from Fig. 3). Lipid concentration was 0.1 mM. Protein to lipid molar ratio was 0.033. Points represent the mean ± standard error of three independent experiments.

Aggregation kinetics

This interpretation of the results was further corroborated by the study of the increase in aggregate size (measured by QELS) as a function of time with three different lipid compositions in buffer containing 100 mM NaCl (Fig. 5). When the liposomes consisted of an equimolecular mixture of PC/PG, the aggregates reached a mean diameter of aproximately 2,500 nm. This increase in size was

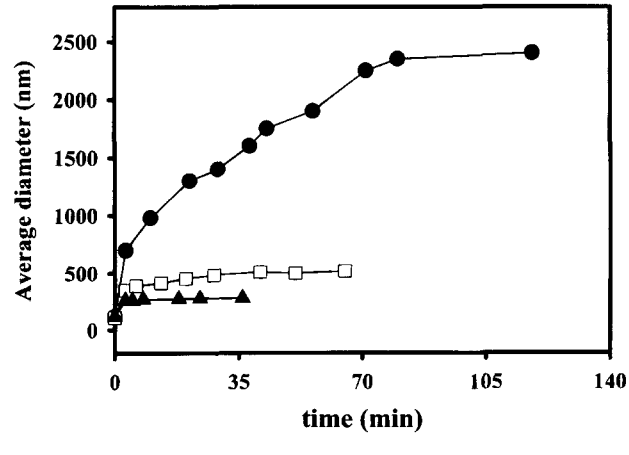

Fig. 5. Kinetics of the size increase of the aggregates formed after addition of α-thionin to LUV of different lipid composition. (●) PC/PG (50/50), (□) PC/PG (40/60), (▲) PC/PG (30/70). Assay buffer was 10 mM HEPES, 100 mM NaCl, pH 7.5. Lipid concentration was 0.1 mM. Protein to lipid molar ratio was 0.033.

not immediate, and it was not until ~2 h that the sample reached a stable value. We tried to fit the experimental results to a single exponential function and found an exponential time constant $k = 0.02\ min^{-1}$. It must be pointed out that the first point (which, for technical reasons, could not be obtained before 3 min) showed already a mean diameter of approximately 700 nm. This result provides an explanation for the decrease in the scattering signal, which in many cases follows the rapid increase after peptide addition (Figs. 1A, 8A). When the size of the aggregates exceeds that of the incident light wavelength, the Rayleigh assumption no longer applies, and the scattering of the sample cannot be measured by this method. Increasing the PG content of the vesicles to 60 and 70% produced two effects. First, the size of the aggregates decreased to 500 and 280 nm, respectively, and second, the time needed to reach the final value decreased as the PG content of the vesicle increased (30 min with 60% PG and less than 3 min with 70% PG). Both effects migth be looked at as the result of the increase in electrostatic repulsions that counterbalance the thionin-induced aggregation of the vesicles, as discussed above for the equilibrium measurements (Figs. 3B, 4B).

Effect of protein concentration

The effect of increasing protein concentration on the amount of ANTS released was studied in liposomes containing negative charge surface densities ranging from 40 to 100%, because it is in this range where the effect of the protein is more evident.

To 1 mL of a suspension of vesicles loaded with the ANTS/DPX fluorophore/quencher pair, different amounts of wheat α-thionin were added to obtain the desired protein to lipid molar ratio, and the release of ANTS was measured. In every case, the final lipid concentration in the cuvette was 0.1 mM, and the NaCl concentration of the reaction buffer was 200 mM. The results are shown in Figure 6.

When the PG content of the vesicles was 40%, we could not obtain complete release of the encapsulated fluorophore, even at the highest protein concentration assayed. At a protein to lipid molar ratio of 0.033, the observed amount of ANTS release was 76%. At lower protein concentrations the percentage of release decreased. The leakage is directly proportional to the bulk protein concentration in the sample, suggesting that the binding must be noncooperative. Therefore, when the liposomes contain 40% PG and within the range of protein concentrations tested, α-thionin partitions into the lipid phase with low affinity because the amount of protein bound to the vesicle is always too low to induce complete release of the fluorophore.

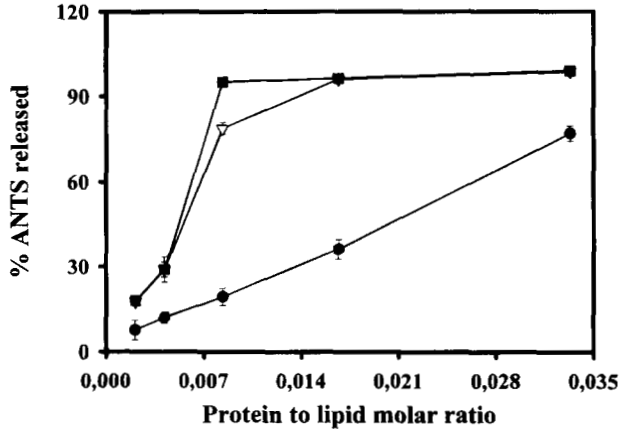

Fig. 6. Effect of protein concentration on the amount of ANTS released from LUV of different lipid composition. (●) PC/PG (60/40), (▽) PC/PG (40/60), (■) PC/PG (10/90). Lipid concentration was 0.1 mM. Assay buffer was 10 mM HEPES, 200 mM NaCl, pH 7.5. Points represent the mean ± standard error of three independent experiments.

When the PG content of the vesicles was higher than 40% complete release of ANTS could be obtained. For the sake of clarity, Figure 6 only shows the results obtained at 60 and 90% PG. The larger the PG content of the vesicle, the lower the concentration of protein inducing 100% release. For example, when the PG content of the vesicles ranged between 60 and 70%, complete ANTS release was observed at a protein to lipid molar ratio of 0.0167, and when the PG content was 80% or higher, a protein to lipid molar ratio of 0.0083 was enough to induce complete release of the encapsulated fluorophore.

We found that under these conditions (80% PG or more) practically all the protein was bound to the vesicles (Fig. 2; Table 1). If we consider that a vesicle with a mean diameter of 100 nm consists of approximately 83,000 lipid molecules (Butko et al., 1996), a protein to lipid molar ratio of 0.0083 represents an average value of about 700 α-thionin molecules per vesicle. This seems to be the minimum amount of peptide that must be bound to the vesicle in order to obtain 100% release.

Mechanism of leakage

A crucial point for understanding the interaction of the peptide with model membranes is the determination of the mechanism of leakage. The release of encapsulated solutes can take place through discrete channels formed by the reorganization of the membrane-bound peptide molecules, or be the result of the peptide-induced overall disorganization of the membrane bilayer due to the formation of transient pores.

One of the possible ways to answer this question is to study the release of encapsulated molecules that are by far too large to cross the membrane barrier through protein channels. Self-quenching fluorescent dextrans with high molecular weight can be easily encapsulated into liposomes. The release of the fluorescent dextrans into the medium results in an increase in the fluorescence signal, which is no longer self-quenched. Therefore, we used this molecule to test the mechanism of leakage.

The thionin-induced release of FITC-dextran (MW = 20,000) encapsulated into PC liposomes containing 50% PG was assayed at different lipid/protein molar ratios (Fig. 7A), and compared with that of ANTS/DPX (Fig. 7B). The results in Figure 7A show that in all cases the fluorescent dextran partially leaked from the vesicle. The release was very fast and rapidly stabilized as in the case of ANTS/DPX (Fig. 1A). This result argues against a mechanism involving the formation of discrete channels, and suggests that the thionin-induced release of the encapsulated solutes is the result of the overall disorganization of the membrane bilayer which can no longer function as a permeability barrier.

This hypothesis is strengthened by comparing the release of FITC-dextran and ANTS/DPX as a function of the lipid to protein molar ratio. Figure 7B shows that in both cases the release was dose-dependent, and that at all the protein concentrations tested, the extent of release was similar for both encapsulated solutes.

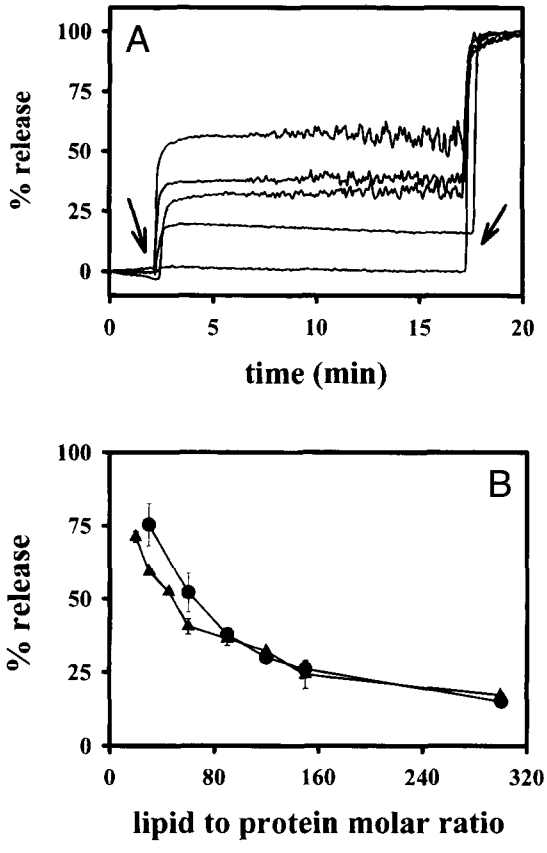

Fig. 7. **A:** α-Thionin-induced release of FITC-dextran from LUV made of PC/PG (50/50). Lipid concentration was 0.1 mM. Assay buffer was 10 mM HEPES, 200 mM NaCl, pH 7.5. Lipid to protein molar ratios were (from the bottom to the top of the figure): ∞, 300, 90, 60, and 30, respectively. The first arrow indicates the addition of the peptide. The second arrow indicates the addition of Triton X-100 (final concentration = 0.1% w/w). **B:** Comparison between the amount of (▲) FITC-dextran and (●) ANTS released from LUV made of PC/PG (50/50) at different lipid to protein molar ratios. Lipid concentration was 0.1 mM. Assay buffer was 10 mM HEPES, 200 mM NaCl, pH 7.5. Points represent the mean ± standard error of three independent experiments.

Effect of PEG-PE

To analyze with greater detail the aggregation of the vesicles and the mixing of their lipids, we incorporated moderate amounts of PEG-PE, a lipid derived from phosphatidylethanolamine with a bulky poly (ethylene glycol) chain covalently attached to its polar head group, into liposomes made up of an equimolecular mixture of PC and PG. This bulky head group is nonionic, highly hydrophilic, and chemically inert (Kuhl et al., 1994), but acts as a steric barrier that interferes with those processes in which the membrane bilayers must come into close contact (Basáñez et al., 1997). The molecular mass of the PEG moiety covalently bound to the PE was 2,000. This corresponds to a polymer chain built up of ca. 45 ethylene oxide monomers protruding 35 Å from the surface of the liposome (Kuhl et al., 1994).

We can modulate the accessibility of foreign molecules to the surface of the liposomes by changing the PEG-PE concentration within the membrane. When the vesicles contain 1% PEG-PE, the polymer chains are not perturbed by their neighbors and adopt the so-called "mushroom" conformation. When the surface concentration of the lipid is raised to 5%, the polymer completely covers the surface of the vesicle, and the polymer chains weakly overlap each other (Kuhl et al., 1994).

Figure 8 shows the effects of the addition of α-thionin to PC/PG vesicles containing 0, 1, and 5% PEG-PE. The presence of the polymer affected the aggregation of the vesicles (Fig. 8A), the mixing of the lipids (Fig. 8B), and the release of ANTS/DPX (Fig. 8C). When the PEG-PE content of the vesicles was 1%, the vesicles formed aggregates (Fig. 8A), although to a lesser extent than that of the controls (the scattering did not increase as much as in the absence of PEG-PE) and at a slower rate. When the PEG-PE content of the vesicles was 5%, the amount of scattered light hardly changed after the addition of the protein, suggesting that the aggregation was very limited or absent.

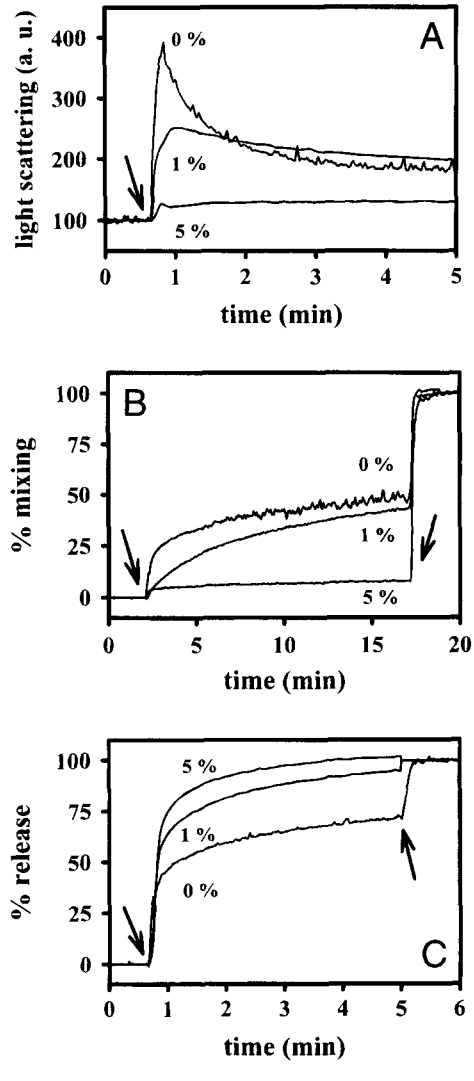

Fig. 8. Effect of PEG-PE at different concentrations on the α-thionin-induced (**A**) aggregation of the vesicles, (**B**) lipid mixing, and (**C**) ANTS release. LUV were made of PC/PG (50/50), where PG was substituted by the corresponding amount of negatively charged PEG-PE. Lipid concentration was 0.1 mM. Assay buffer was 10 mM HEPES, 200 mM NaCl, pH 7.5. The first arrow indicates the addition of the peptide (final protein to lipid molar ratio = 0.033). The second arrow indicates the addition of Triton X-100 (final concentration = 0.1% w/w).

The mixing of the lipids exhibits a similar behavior (Fig. 8B). Whereas 1% PEG-PE significantly reduced the rate at which the lipids could interchange within the vesicles without altering the final extent of the mixing, 5% PEG-PE completely abolished this process.

One could expect that the presence of PEG-PE would also inhibit the release of entrapped solutes. However, this was not the case, as evidenced by the results shown in Figure 8C. Surprisingly, the presence of PEG-PE enhanced both the rate and the amount of the release of ANTS. This means that even though the PEG-PE acted as a steric barrier that prevented the vesicles from coming into close contact, it did not affect the binding of the protein to the vesicles and the disorganization of the bilayer architecture.

Discussion

Electrostatic interactions are essential

The positively charged residues of thionin play an essential role in the binding to the target cells. This was demonstrated by Wada et al. (1982), who blocked all the amino groups of purothionin by acetyl or succinyl groups. This modification diminished the positive charges of the molecule and led to the loss of toxicity to mice or yeast. In fact, all thionins exhibiting antimicrobial activity have a net positive charge at neutral pH (Florack & Stiekema, 1994).

Our results clearly show that α-thionin binds to model membranes via electrostatic interactions. The addition of the peptide to liposomes made of PC did not result in any measurable effect. However, in liposomes composed of PG, protein addition resulted in the release of encapsulated ANTS/DPX, lipid mixing, and vesicle aggregation (Fig. 1).

We failed to observe the fusion of the vesicles, i.e., the aggregation of the vesicles did not result in the mixing of their contents. This observation would appear to contradict the results of Gasanov et al. (1993) with the thionin from *Pyrularia pubera*. The addition of *Pyrularia* thionin to liposomes made of PC and 20% phosphatidylserine (PS) gave rise to the broadening of the ^{1}H-NMR signal and its decrease in amplitude. The authors attributed these effects to the fusion of the liposomes, although they recognize that these effects might as well be the result of the aggregation of the vesicles. More recently, Huang et al. (1997) have postulated a mechanism for the interaction of *Pyrularia* thionin with dipalmitoylphosphatidylglycerol (DPPG) large unilamellar vesicles in which the thionin-vesicle complexes aggregate without fusion, a result in agreement with our observations.

The release of ANTS depends largely on the PG content of the vesicles (Figs. 3A, 4A). Apparently, there is a threshold value above which all the fluorophore is released. This is probably the minimum negative charge surface density that ensures the binding of the amount of protein needed to achieve maximal effect. This value depends on the ionic strength of the medium (Fig. 4A).

The electrostatic nature of the interaction is not only limited to the initial binding of the α-thionin to the surface of the vesicle. The subsequent formation of large aggregates is also governed by electrostatic interactions, although more indirectly. The protein-induced propensity to form aggregates is counterbalanced by the electrostatic repulsions among the negatively-charged vesicles. When the PG content of the vesicles is low or moderate, the tendency to aggregate predominates and conversely, vesicles with high PG content do not form large aggregates (Fig. 3B). This equilibrium of forces is also sensitive to the ionic strength of the medium (Fig. 4B).

Membrane permeabilization and vesicle aggregation can be separated

The presence of poly(ethylene glycol)-phosphatidylethanolamine (PEG-PE) conjugates within the surface of the liposome provides a steric barrier against the approach and adsorption of other macromolecules (Woodle & Lasic, 1992). This property has found an immediate application in liposome-mediated drug delivery for it dramatically enhanced the circulation times of the vesicles (Blume & Cevc, 1990; Papahadjopoulos et al., 1991), but has also been very useful in the study of lipid-protein interactions, as in the case of phospholipase C-induced liposome fusion (Basáñez et al., 1997).

When the PEG chains are in the "mushroom" regime, the aggregation of the vesicles and the mixing of the lipids are impaired, but the steric barrier imposed by the polymer chain is not enough to completely inhibit these processes. Vesicle-vesicle contacts must take place and account for the observed rates of aggregation and lipid mixing. These contacts are probably absent when the PEG-PE concentration is 5% and therefore neither aggregation nor mixing of the lipids are observed.

A similar result was obtained by Basáñez et al. (1997). These authors observed that 1% PEG-PE in the liposomes did not affect the phospholipase C activity but significantly reduced the rates of aggregation, lipid mixing, and fusion of the vesicles. When the PEG-PE concentration was 6%, the phospholipase C activity was reduced to 28% of the control, and the aggregation, lipid mixing and fusion of the vesicles were totally inhibited, because the membranes could not interact with each other.

Our results indicate that even at 5% PEG-PE α-thionin can gain access to the surface of the vesicles and breach the lipid bilayer (Fig. 7C). This result is striking because one might expect that the presence of the grafted polymer at the surface of the liposome would interfere with the binding of the peptide.

Jeon et al. (1991) found that full protection against protein adsorption is obtained when the degree of polymerization of PEG on the solid substrate is about 100. In our case, the grafted polymer consists of 45 monomers, and it could be insufficient to prevent the accessibility of the thionin. Moreover, the PEG-PE-induced inhibition of aggregation implies that there is more membrane surface available for the peptide to bind, and therefore the observed rate and extent of fluorophore release are higher. Hence, the presence of PEG-PE at the surface of the membrane separates two processes that otherwise would be taking place simultaneously, i.e., membrane permeabilization and vesicle aggregation.

The effects depend largely on the amount of bound protein

The interaction of wheat α-thionin with liposomes depends primarily on the electrostatic binding, although the fact that α-thionin weakly binds to neutral vesicles (Table 1) might be indicative of the existence of other type of interactions, which per se are not sufficient to destabilize the bilayer. There is a threshold value of negative charge surface density, which is very sensitive to the experimental conditions (Fig. 4). At this or lower values, the release of ANTS is not complete and depends linearly on the bulk protein concentration (Fig. 6), suggesting that a significant fraction of the protein is not associated to the vesicles (Table 1). Above this threshold value, most of the peptide is associated to the vesicles (Table 1).

In conditions where practically all the protein binds to the vesicles, an increase in the protein concentration leads to an increase in the amount of released fluorophore (Fig. 6) and a decrease in the

halftime of the process (data not shown). When the protein to lipid molar ratio is greater than 0.0083, the liposomes release most if not all of their contents, varying the halftime for this process between a few seconds and 2 min. Values lower than 0.0083 result in a dramatic slow down of the process and a decrease in the amount of ANTS released. This protein to lipid molar ratio represents an average value of about 700 α-thionin molecules per vesicle and corresponds to the minimum amount of peptide that is neccesary to completely release the vesicle contents. Above this value, the leakage is faster, and below this value, only a fraction of the encapsulated ANTS is released with longer halftimes.

An analogous result was obtained by Thevissen et al. (1996) with α-hordothionin. At a concentration of 0.6 μg/mL (which did not inhibit fungal growth), they failed to observe any effect on planar lipid bilayers. At 1 μg/mL the peptide induced a membrane current, but this effect depended largely on the membrane potential, and at 4 μg/mL the activity was observed even when the membrane potential was very low. Whenever a membrane current was detected the membrane collapsed, and the time at which the disruption of the membrane took place was inversely proportional to the peptide concentration.

Mechanism for membrane disruption

Once the protein has reached the surface of the vesicle, it destabilizes the lipid bilayer and induces the release of the fluorophore. The amount of fluorophore released depends basically on the amount of peptide bound to the membrane and is practically the same for ANTS and FITC-labeled dextran (Fig. 6). This result suggests that the entrapped solutes do not leave the vesicles through discrete channels. The two antiparallel α-helices of α-thionin consist of 10 and 6 amino acid residues, respectively (Clore et al., 1987), and are too short to span the lipid bilayer. Instead, the observed release must be the result of the overall disorganization of the membrane bilayer, which can no longer function as a permeability barrier.

At this point, we cannot say whether some part of the peptide specifically inserts into the lipid bilayer, as other authors have suggested (Huang et al., 1997). When *Pyrularia* thionin was added to DPPG unilamellar vesicles, a twofold increase in its intrinsic fluorescence and a 10 nm blue shift in its emission maximum were observed. These changes were attributed to a reduction of the water exposure of Trp8 consistent with its insertion into the bilayer. However, this conclusion is not so clear-cut. Other authors have found that although *Pyrularia* thionin binds to artificial membranes, it does not insert into them (Wall et al., 1995). This observation is not incompatible with the results published by Huang et al. (1997) because the changes in the intrinsic fluorescence could be due to vesicle aggregation. In our case, the intrinsic fluorescence signal arising from the sole tyrosine residue of wheat α-thionin is too small to carry out reliable measurements (J.M.M. Caaveiro, unpubl. obs.), but recent linear dichroism measurements on oriented DMPG films indicate that the peptide is highly oriented with the two α-helices parallel to the plane of the lipid membrane (Kelly et al., 1998).

Regardless of this putative insertion step, α-thionin binding to the liposomes results in the formation of large lipoprotein aggregates in which the bilayer organization of the membrane is disrupted, as manifested by the sudden and large release of the vesicle contents. This is also the case for *Pyrularia* thionin (Gasanov et al., 1993; Huang et al., 1997) and for α-hordothionin (Thevissen et al., 1996), where the interaction of the peptide with model membranes triggers the formation of nonbilayer structures or the collapse of planar lipid bilayers. The amphipatic nature of the surface of α-thionin (Florack & Stiekema, 1994) could be the underlying force directing these events.

This protein-induced overall disorganization of the bilayer is not an uncommon mechanism. It has been described for different peptides and proteins. Gazit et al. (1995) described a model in which the mammalian antibacterial peptide cecropin P1 covers the surface of the vesicles in a "carpet-like" fashion. This binding is directed by electrostatic interactions. When a threshold concentration of cecropin monomers is bound to the membrane, the lipid packing of the bilayer is disrupted and the membrane collapses.

In our case, about 700 α-thionin molecules must bind to each vesicle to induce total release of its contents. We can estimate the surface area of each vesicle from the number of lipid molecules in the outer monolayer and the area occupied by each lipid. If we assume that 54% of the total lipids (83,000) are in the outer monolayer and that the area per lipid is 0.7 nm^2 (Butko et al., 1996), we obtain a value of approximately 31,000 nm^2. This means that each peptide molecule would cover an area of about 45 nm^2. This value is compatible with the formation of a "carpet-like" monolayer made up of α-thionin molecules. The release of fluorescent dyes is probably a consequence of the peptide-induced disintegration of the liposome membranes. In this sense α-thionin acts like a detergent. However, unlike detergents, the peptide is not able to form stable micelles and therefore, once the bilayer architecture is disrupted, the exposed hydrophobic region of the lipids constitute the driving force for the vesicle aggregation.

This detergent-like property has been described for other small peptides such as magainins 1 and 2 (Matsuzaki et al., 1989, 1991), dermaseptin (Pouny et al., 1992), or melittin (Katsu et al., 1989; Benachir & Lafleur, 1995) and larger proteins like the bacterial toxin α-haemolysin secreted by *Escherichia coli* (Ostolaza et al., 1993) or CytA, a 27 kDa protein belonging to the family of δ-endotoxins occurring in parasporal crystals of *Bacillus thuringiensis* var. *israelensis* (Butko et al., 1996, 1997). Moreover, attempts have been made to design peptides with detergent-like properties for the solubilization of integral membrane proteins (Schafmeister et al., 1994) for which the term "peptitergents" has been coined.

In summary, our results indicate that wheat α-thionin binds to large unilamellar vesicles via electrostatic interactions. The negative charge surface density of the vesicles directs the partition equilibrium between the membrane and the aqueous phase. Binding of the peptide triggers the aggregation of the vesicles and the mixing of their lipids, disrupting the bilayer structure of the membrane and inducing the release of the vesicle contents. These effects depend largely on the amount of bound peptide. A number of about 700 α-thionin molecules per vesicle are needed to observe total release of its contents, suggesting a mechanism in which the formation of a "carpet-like" peptide monolayer on the surface of the vesicle disrupts the permeability barrier of the bilayer in a detergent-like fashion.

Materials and methods

Materials

Egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) were grade I from Lipid Products (South Nutfield, UK). RhB-PE, NBD-PE, and PEG-PE were from Avanti Polar Lipids (Alabaster,

Alabama). ANTS and DPX were from Molecular Probes (Eugene, Oregon). FITC-labeled dextran (MW = 20,000) was from Sigma (St. Louis, Missouri).

Purification of the protein

A crude thionin preparation was obtained from the endosperm of hexaploid wheat, *Triticum aestivum* L. Cv. Candeal, by petroleum-ether extraction and HCl/ethanol precipitation, as previously described (García-Olmedo et al., 1968). A mixture of genetic variants of wheat thionins was obtained from the crude extract by preparative electrophoresis on 10% polyacrylamide colums (1.5 × 10 cm) with 0.1 M acetic acid buffer, pH 2.9, at 20 V/cm, essentially as reported (Ponz et al., 1984). The yield obtained was 50 mg/kg of dry endosperm. Purification of α-thionin was achieved by RP-HPLC, on a Ultrapore C_3 column (1 × 25 cm; particle 5 μm; pore 300 Å) from Beckman, using H_2O/2-propanol gradients, 0.1% trifluoroacetic acid, with an elution rate of 0.5 mL/min (Molina et al., 1993).

Preparation of liposomes

The appropriate lipids were mixed in organic solvent and evaporated thoroughly. LUV were prepared by the extrusion method (Hope et al., 1985), using polycarbonate filters with a pore size of 0.1 μm (Nuclepore, Pleasanton, California). For vesicle aggregation or lipid mixing assays, liposomes were routinely prepared in 10 mM HEPES, 200 mM NaCl, pH 7.5 buffer. In those experiments where the effect of the ionic strength was tested, the concentration of NaCl was varied. For assays of vesicle leakage, the buffer contained in addition 25 mM ANTS and 90 mM DPX. For the assays of fusion, the buffer contained either 50 mM of ANTS or 180 mM of DPX (Ellens et al., 1985). Nonencapsulated fluorescent probes were separated from the vesicle suspension through a Sephadex G-75 gel filtration column (Pharmacia, Uppsala, Sweden) eluted with buffer. Solution osmolarities were checked with an Osmomat 030 instrument (Gonotec, Berlin, Germany). Phospholipid concentration was measured according to Bartlett (1959).

Binding experiments

A fixed amount of wheat α-thionin was incubated for 1 h with LUV at different lipid/protein molar ratios. Bound and free protein were separated by ultracentrifugation in a Beckman Optima TLX ultracentrifuge using a TLA-120.2 rotor at 120,000 rpm (500,000 × *g*) at room temperature for 2 h. In a control experiment the protein was centrifuged in the absence of lipid and we observed that under those conditions the protein did not pellet. However, a significant fraction of the protein adsorbed to the tube walls. Therefore, for all calculations, we considered that total protein concentration was that of the control tube after centrifugation. The concentration of free protein was estimated from the supernatants by the bicinchoninic acid assay (Smith et al., 1985), and bound protein was calculated from the difference between total and free.

Liposome aggregation assay

Vesicle aggregation was routinely followed as an increase in light scattering at 90° in a Perkin Elmer LS-50 fluorometer with both monochromators at 520 nm. Occasionally, aggregation was measured as the increase in average particle size, measured by QELS in a Zetasizer 4 spectrometer (Malvern, UK). For QELS measurements the buffer had been previously filtered trough 0.22 μm filters.

Assay for leakage of liposomal contents

At high concentrations (i.e., inside the vesicle), ANTS and DPX form a low-fluorescence complex. Breakdown of the vesicle membrane leads to contents leakage, complex decomposition, and high ANTS fluorescence. Thus, leakage was assayed by treating the probe-loaded liposomes (final lipid concentration = 0.1 mM) with the appropriate amounts of peptide in a fluorometer cuvette, at room temperature and under constant stirring. Changes in fluorescence intensity were recorded in a Perkin Elmer LS-50 spectrofluorometer with excitation and emission wavelengths set at 350 and 510 nm, respectively. One hundred percent release was achieved by adding to the cuvette Triton X-100 to a final concentration of 0.1% (w/w). Leakage was quantified on a percentage basis according to the equation:

$$\%\text{Release} = \left(\frac{F_f - F_0}{F_{100} - F_0}\right) \times 100, \qquad (1)$$

F_f being the equilibrium value of fluorescence after peptide addition, F_0 the initial fluorescence of the vesicle suspension, and F_{100} the fluorescence value after addition of Triton X-100. In some experiments, the release of FITC-labeled dextran from PC/PG (1:1) liposomes was tested. The liposomes were prepared in 10 mM HEPES, 200 mM NaCl, pH 7.5 buffer. The molecular weight of the fluorescent dextran was 20 kDa, and was encapsulated at a self-quenching concentration of 5 mM. Non-encapsulated dextrans were separated from the liposome suspension through a Sephadex HR-300 gel filtration column eluted with a buffer containing the same concentration of unlabeled dextran to keep the osmotic conditions identical. The excitation and emission wavelengths were set at 462 and 517 nm, respectively. An interference filter with a nominal cut off value of 470 nm was placed in the emission light path to minimize the contribution of the aggregation of the vesicles to the fluorescence signal.

Lipid mixing assay

Peptide-induced vesicle lipid mixing was measured as in Struck et al. (1981). This assay is based on the decrease in resonance energy transfer between two probes (NBD-PE and RhB-PE) when the lipids of the probe-containing vesicles are allowed to mix with lipids from vesicles lacking the probes. This occurs because the average distance between the donor (NBD-PE) and the acceptor (RhB-PE) increases when the lipids mix. The concentration of each of the fluorescent probes within the liposome membrane was 0.6%. Liposomes were prepared as described above. Labeled and unlabeled vesicles in a proportion 1:4 were placed in a fluorometer cuvette at a final lipid concentration of 0.1 mM. The experiment was performed at room temperature and under constant stirring. The fluorescence was measured with a Perkin-Elmer LS-50 fluorimeter, exciting the sample at 460 nm and recording the emission at 536 nm. Excitation and emission slits were set at 10 nm, and a 515 nm cut off filter was placed between the sample and the

photomultiplier. One hundred percent mixing was estimated by adding the peptide to a liposome preparation in which the membrane concentration of each probe was 0.12%. Mixing was quantified on a percentage basis using the equation described above.

Vesicle fusion assay

Fusion of the vesicles (mixing of aqueous contents) was assayed as described by Ellens et al. (1985). Briefly, liposomes containing 50 mM ANTS were mixed with liposomes containing 180 mM DPX in a 1:1 proportion. Final lipid concentration was 0.1 mM. Fluorescence conditions were as in the leakage assay. In a control experiment, ANTS-containing liposomes were mixed with liposomes lacking DPX in a 1:1 proportion. Percentage of fusion was calculated by using the following equation:

$$\%\text{fusion} = \left(\frac{F_0 - F_f}{F_0}\right) \times 100 \qquad (2)$$

where F_f is the fluorescence recorded after the addition of the peptide and F_0 is the fluorescence measured in the control experiment.

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