Membrane Interaction of a β-Structure-Forming Synthetic Peptide Comprising the 116–139th Sequence Region of the Cytotoxic Protein α-Sarcin

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ABSTRACT α-Sarcin is a cytotoxic protein that strongly interacts with acid phospholipid vesicles. This interaction exhibits a hydrophobic component although α-sarcin is a highly polar protein. A peptide comprising the amino acid sequence corresponding to the 116–139th segment of the α-sarcin cytotoxin has been synthesized by a standard fluoren-9-yl-methoxycarbonyl-based solid phase method. Its primary structure is: (116)-NPGPARVIYTYPNKVFCGIIAHTK-(139). Two β-strands have been predicted in this region of α-sarcin, where the less polar stretches of the protein are found. The synthetic peptide interacts with negatively charged large unilamellar vesicles of either natural or synthetic phospholipids. An apparent fragmentation of the vesicles is produced by the peptide based on electron microscopy studies. The peptide promotes leakage of the intravesicular aqueous contents and lipid mixing of bilayers. The packing of the phospholipid molecules is greatly perturbed by the peptide, as deduced from the drastic changes induced by the peptide in cooperative properties associated with the phase transition of the bilayers. At saturating peptide/phospholipid ratios, the phase transition of dimyristoylphosphatidylglycerol vesicles is abolished. All of these effects are saturated at about 0.3 peptide/lipid molar ratio. The peptide adopts a mostly random structure in aqueous solution. A conformation composed of a high proportion of antiparallel β-sheet is induced as a consequence of the interaction with the phospholipid vesicles in opposition to trifluoroethanol that promotes α-helical peptide structures, as deduced from circular dichroism measurements. The obtained results are discussed in terms of the potential involvement of the region comprising residues 116–139 of α-sarcin in the hydrophobic interactions of this cytotoxic protein with membranes.

INTRODUCTION

The α-sarcin cytotoxin, secreted by the mold *Aspergillus giganteus*, is a ribonuclease that cleaves a single phosphodiester bond in a universal sequence of the 28S rRNA molecule, promoting the inactivation of the ribosomes (see Wool et al., 1992, for a review). The protein has been revealed to be cytotoxic for many human tumor cell lines, inhibiting their protein biosynthesis (Turnay et al., 1993). The process by which α-sarcin enters cells has not been yet explained, as for most protein toxins. No membrane protein receptors have been found for α-sarcin, although it binds to the cell membrane (Turnay et al., 1993). In addition, there are many reports about the interaction of α-sarcin with phospholipid model membranes. Thus, the protein perturbs the hydrophobic core of the bilayers promoting their lipid mixing as well as leakage of the intravesicular aqueous contents (Gasset et al., 1990; Mancheño et al., 1994); indeed, the protein is labeled with photoreactive probes located at the acyl chains of the bilayer phospholipids (Gasset et al., 1991; Oñaderra et al., 1993). All these facts strongly suggest some kind of hydrophobic interaction between α-sarcin and the bilayers, although this is not easily predictable by simply considering the amino acid sequence of α-sarcin. The protein is a highly polar molecule with 150 amino acid residues (Sacco et al., 1983), without stretches of non-polar residues long enough to be apparently considered responsible for hydrophobic interactions. The hydropathy profile of α-sarcin revealed that the region exhibiting a somewhat less exposed character is located around residues 120–140 (Martínez del Pozo et al., 1988). Therefore, this protein segment could be involved in the destabilization of bilayers produced by α-sarcin. On the other hand, this protein shows a significant degree of sequence similarity with some fungal ribonucleases (RNases) of the RNase T1 subfamily (Mancheño et al., 1995), although these proteins contain about 40 amino acid residues less than α-sarcin. The three-dimensional structures of some of these ribonucleases are known and all of them are highly similar to that of RNase T1 (Pace et al., 1991). Based on that similarity, we proposed a structural model for α-sarcin, considering alignment of primary structures, three-dimensional structures of the RNase T1 subfamily proteins, and secondary structure predictive methods (Mancheño et al., 1995). A hydrophobic β-sheet was thus predicted in α-sarcin, and it was considered potentially involved in the hydrophobic interactions of the protein with membranes. The abovementioned region of α-sarcin, residues 120–140, contributes to such a β-sheet. In fact, two antiparallel β-strands were predicted at this protein portion. To gain insights into the mechanism of membrane destabilization promoted by α-sarcin, we have considered the potential participation of such a protein region in the effects caused in bilayers.

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Thus, we have synthesized a peptide containing the amino acid sequence located between positions 116 and 139 of α-sarcin. This peptide ((116) Asn-Pro-Gly-Pro-Ala-Arg-Val-Ile-Tyr-Thr-Tyr-Pro-Asn-Lys-Val-Phe-Cys-Gly-Ile-Ile-Ala-His-Thr-Lys-NH_2 (139)), the (116–139) peptide, contains the two predicted β-strands (120–125th and 130–135th, sequence positions), which are separated and flanked by potential β-turn regions (Mancheño et al., 1995). We have studied the interaction of this peptide with lipid model bilayers. The obtained results are in agreement with the potential involvement of this protein region in the destabilization of membranes promoted by α-sarcin. Although the effects of a synthetic peptide may only partially mimic the complexity of those of the parent protein, the studies performed will improve the knowledge about the molecular mechanism of the α-sarcin functionality. In addition, the obtained results would be useful for comparative purposes because there are a number of natural peptides containing from ~10 to ~30 amino acid residues, which promote a wide variety of effects at the level of membranes (see Bernheimer and Rudy, 1986; Cornut et al., 1993, for reviews), and their composition might resemble that of the (116–139) peptide.

MATERIALS AND METHODS

Synthetic dimyristoylphosphatidylglycerol (DMPG), bovine brain phosphatidylserine (PS), *N*-(7-nitro-2–1, 3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *N,N'-p*-xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Buffers were prepared in Milli-RO water (Millipore, Millford, MA).

The (116–139) peptide was synthesized as amide in C-terminal on an automated multiple peptide synthesizer (AMS 422, Abimed, Langenfeld, Germany) by using the solid-phase procedure and standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry in a base of 25 μmol. The synthesis was carried out on an *N*-α-Fmoc-DMP resin (4-(2′, 4′-dimethoxyphenyl-Fmoc-amino-methyl)-phenoxy resin) (Novabiochem, La Jolla, CA), with Fmoc-protected amino acids activated in situ with benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexa-fluorophosphate in the presence of *N*-methyl morpholine and 20% piperidine/dimethylformamide for deprotection. The protecting side chain groups were as follows: Ser, Thr and Tyr (*t*-butyl-), Arg (pentamethylchroman sulfonyl-), Cys and His (triphenylmethyl-), Lys (*t*-butoxycarbonyl-). Peptide was cleaved from the resin with 2.5% ethane dithiol (King et al., 1990) as scavenger, precipitated and washed with cold methyl-tert-butyl ether, water-extracted, and lyophilized. The peptide was subjected to further purification by reverse-phase high performance liquid chromatography analysis on an Ultrasphere-ODS C_{18} column (10 × 150 mm) with a linear gradient water/35% acetonitrile in 0.1% trifluoroacetic acid. Fast atom bombardment mass spectrometry yielded a mass/charge value of 2660.0 (calculated 2659). The amino acid composition of the purified peptide exactly matches that expected. Peptide concentration was determined from amino acid analysis and by absorbance at 278 nm by using a molar extinction coefficient at pH 7.0 of 2700 M^{-1} cm^{-1} (see Results). The peptide was hydrolyzed at 108°C in evacuated and sealed tubes for 24 h, with 5.7 N HCl, containing 0.1% (w/v) phenol and norleucine as internal standard. The analyses were performed on a Beckman System 6300 (Palo Alto, CA) amino acid analyzer.

Circular dichroism (CD) spectra were obtained on a Jovin Yvon Mark III dichrograph (Longjumeau, France) fitted with a 250 W xenon lamp. The spectra were recorded at 0.2 nm/s scanning speed. Samples were analyzed in either 0.05 or 0.01 cm optical path cells. CD results were expressed in units of degree × cm^2 × $dmol^{-1}$ of amino acid residue. These values were calculated on the basis of 110 as the mean residue weight for this peptide. Fluorescence emission spectrum of the peptide was recorded at 1 nm/s scanning rate on an SLM Aminco 8000 spectrofluorimeter (Urbana, IL), for excitation at 275 nm wavelength. The optical path of the cells was 0.2 cm. The slit widths for both excitation and emission beams were 4 nm. Fluorescence emission was expressed in arbitrary units.

The different vesicles (except those for the leakage assay) were formed by hydrating a dry lipid film with Mops buffer (50 mM Mops, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA) for 60 min at 37°C. The lipid suspension was subjected to five cycles of extrusion through two stacked 0.1 μm (pore diameter) polycarbonate membranes (Nuclepore, Costar; Cambridge, MA) in an Extruder (Lipex Biomembranes Inc.; Vancouver, BC, Canada). The average size of the vesicle population was 100 nm as determined by Coulter counting. Phospholipid concentration was determined as described (Barlett, 1959)

The changes in turbidity at 360 nm of a vesicle suspension after addition of a small aliquot of peptide were measured on a Beckman DU-8 thermostated spectrophotometer (Palo Alto, CA). All vesicle suspensions (30 μM final lipid concentration) were previously thermostated at the required temperature. Control samples without peptide were also considered.

The lipid mixing was assayed according to Struck et al. (1981). A vesicle population of DMPG or brain PS containing 1% NBD-PE (donor) and 0.6% Rh-PE (acceptor) was mixed with unlabeled vesicles at 1:9 molar ratio (75 μM final lipid concentration) in the Mops buffer. At zero time, a small volume of peptide in the same buffer was added. The time course variation of the fluorescence intensity at 530 nm for excitation at 450 nm (4 nm slit width for both excitation and emission beams) was continuously monitored on an SLM Aminco 8000 spectrofluorimeter. The percentages of energy transfer (% ET) were calculated according to the expression % ET = $(1 - F/F_0) \times 100$ where F is the final fluorescence intensity at 530 nm after addition of peptide and F_0 the fluorescence intensity at 530 nm of a vesicle population of DMPG or PS (75 μM) containing 0.1% NBD-PE. To eliminate the potential contribution of the sample turbidity to the fluorescence measurements, 10 mm Glan-Thompson polarizers (SLM Aminco) (90°/0°) were used.

Leakage of vesicle aqueous contents was measured by using the ANTS/DPX assay as previously described by Ellens et al. (1985). Bovine brain PS vesicles containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, 10 mM Tris, pH 7.4, were prepared by five cycles of freeze-thawing and further extrusion through two stacked 0.1 μm (pore diameter) polycarbonate membranes (Nuclepore). Vesicles were separated from uncapsulated materials on Sephadex G-75 (Pharmacia, Uppsala, Sweden) by using Tris buffer (10 mM Tris, pH 7.4, containing 0.1 M NaCl and 1 mM EDTA) as elution buffer. In a typical assay a small volume of peptide in the Tris buffer was added to the vesicles (75 μM lipid concentration) at zero time. The variation of fluorescence intensity measured through a 3–68 cutoff filter (>530 nm) upon excitation at 386 nm was registered on an SLM Aminco 8000 spectrofluorimeter. The 0 and 100% leakage were taken as the fluorescence intensity of a PS vesicle suspension (75 μM lipid concentration) before and after addition of Triton X-100 at a final concentration of 1%, respectively. It has been recently reported that the mode of mixing vesicle and peptide solutions in the leakage assays can affect the kinetics of the process (Polozov et al., 1994). Thus, the same amount of peptide can produce different leakage curves depending on the peptide concentration of the initial solution. In the present case, a single peptide stock solution was used and the stirring conditions were not altered throughout the study.

Measurements of the fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich, Milwaukee, WI) were made on an SLM Aminco 8000 spectrofluorimeter equipped with 10 mm Glan-Thompson polarizers. Labeling of the vesicles with DPH was carried out as previously described (Gavilanes et al., 1985). Peptide-vesicle mixtures were incubated for 1 h above the transition temperature of the phospholipid, and later cooled down. The fluorescence emission was measured at 425 nm for excitation at 365 nm, after equilibration of the samples at each required temperature.

For electron microscopy studies, samples of phospholipid vesicles (obtained by extrusion through 0.1-μm pore diameter polycarbonate filters; see

above) at 2.5 mg/ml lipid concentration, were applied to a glow-discharged 400 mesh Formvar-carbon-coated copper grid for 2 min. Excess fluid was drawn away with filter paper. Samples were then negatively stained with 2% (w/v) phosphotungstic acid at pH 7.0 and examined by using a Zeiss EM 902 (Jena, Germany) transmission electron microscope operating at 80 kV.

RESULTS

Spectroscopic characterization of the synthetic (116–139) peptide

The UV absorbance spectrum of this peptide shows typical features of the tyrosine chromophores. The absorbance maximum appears at 275 nm. The calculated extinction coefficient $E^{0.1\%}$ (278 nm, 1 cm) is 1.02, which is in agreement with the presence of two tyrosine residues as single chromophores at the aromatic region. The (116–139) peptide exhibits a single fluorescence emission band centered at 304 nm, for excitation at 275 nm, as would be expected from its amino acid composition. The CD spectrum of the synthetic peptide in the far UV (peptide bond region) is given in Fig. 1 *A* (*spectrum a*). The percentages of secondary structure calculated from this spectrum are: 11% antiparallel β-structure, 26% β-turns, and 63% random. The method employed to estimate the secondary structure contents from CD data (Perczel et al., 1991), as most of these procedures, is based on protein data, and these may not constitute the best basis set to analyze peptides. But, it is clear that random conformation is the predominant structure of the peptide by simply considering the shape of the CD spectrum. The structure of the synthetic peptide has also been analyzed under conditions of enhanced intramolecular hydrogen bonding. It is well known the effect of trifluoroethanol (TFE) in promoting intrachain hydrogen bonds in polypeptides, and it is thus considered a "membrane-mimicking" solvent. Its effect on the conformation of the (116–139) peptide has been studied. The peptide adopts helical conformation upon addition of TFE (Fig. 1, *A* and *B*). There is no change in the CD spectrum up to 10% TFE. A conformational change occurs in the 10–40% TFE concentration range (Fig. 1 *B*). The CD spectrum d in Fig. 1 *A* corresponds to the (116–139) peptide at 40–60% TFE concentration. The secondary structure deduced from this spectrum would be composed of 59% α-helix, 17% β-turns, and 24% random, with the same cautions as above. Thus, the peptide can adopt a helical structure in spite of the abundance of helix-destabilizing residues (e.g., Pro, Val, Ile, Thr, or Gly). Finally, it is also worthy of mention that the CD spectrum of the synthetic peptide in aqueous solution does not show any modification in the 0.3–3.0 mg/ml (0.11–1.13 mM) concentration range.

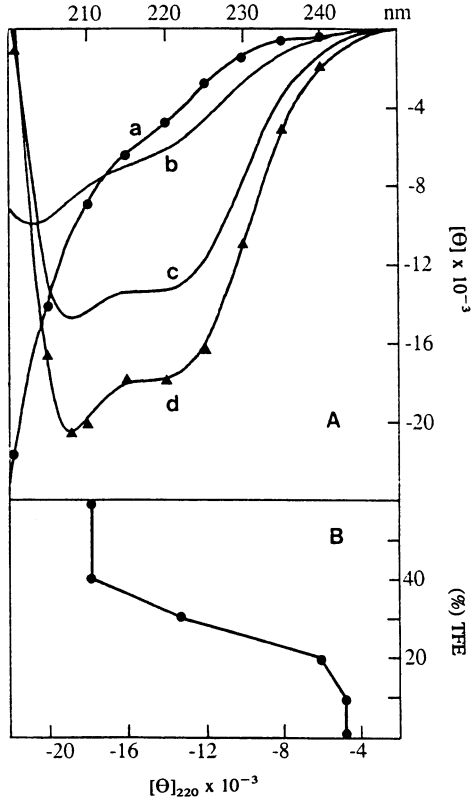

FIGURE 1 (*A*) CD spectra of the synthetic peptide in: (*a*), 0–10% TFE; (*b*), 20% TFE; (*c*), 30% TFE; (*d*), 40–60% TFE. ([Θ]) Mean residue weight ellipticities are expressed in units of degree × cm^2 × $dmol^{-1}$. Theoretical values corresponding to the secondary structures deduced from the CD spectra of the synthetic peptide in (●) aqueous solution and (▲) 40% TFE, according to Perczel et al. (1991). (*B*) Mean residue weight ellipticities at 220 nm, $[\Theta]_{220}$ in units of degree × cm^2 × $dmol^{-1}$, versus TFE concentration. The peptide samples were dissolved in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA.

Effects of the (116–139) peptide on model phospholipid vesicles

The (116–139) peptide interacts with negatively charged large unilamellar vesicles. This is clearly deduced by observing the transmission electron micrographs of peptide-vesicle mixtures. Fig. 2, *B* and *C* show a preparation of large unilamellar PS vesicles (Fig.2 *A*) treated with the peptide. Two types of predominant structures are observed. Negatively stained particles of <50 nm in diameter appear upon the peptide treatment as predominant structures (aggregates of these small particles can be also visualized). These particles can be related to small vesicles, because they are apparently formed by bilayer structures, although non-bilayer-type ordination cannot be discarded based on these results. In this regard, this type of particle may be related to the disk-shaped micelles produced by the lytic peptide melittin (Dufourcq et al., 1986a, b). Larger structures also appear (aggregates of those particles of about 50 nm in diameter). An increase in the peptide to lipid molar ratio results in the disappearance of the individual particles. At 60:1 lipid/peptide molar ratio, an amorphous network of aggregated small particles is the most prominent feature of the micrographs (Fig. 2 *C*). Thus, based on the morphological changes, the peptide produces vesicle fragmentation.

The interaction of the peptide with phospholipid vesicles is also concluded by analyzing its effect on the thermotropic

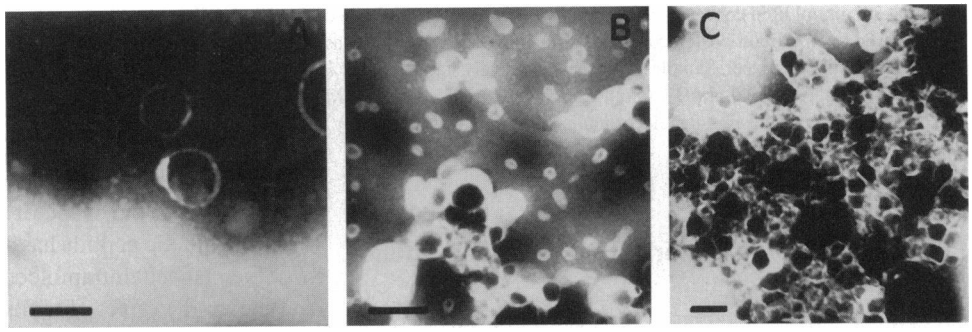

FIGURE 2 Negative stain transmission electron micrographs of phosphatidylserine vesicles (*A*), and peptide-PS vesicles at 120:1 (*B*) and 60:1 (*C*) lipid to peptide molar ratio. Bars correspond to: 100 nm in *A* and 150 nm in *B* and *C*.

behavior of DMPG vesicles. The synthetic peptide greatly modifies the phase transition profile of the vesicles. Fig. 3 shows the anisotropy variation of DPH-labeled DMPG vesicles upon addition of different amounts of the (116–139) peptide. This study has been extended up to 60°C and no other transitions are detected. The fluorescence anisotropy of DPH monotonically decreases from the corresponding values obtained at 35°C. The main transition for the pure phospholipid bilayers is observed at 23–24°C. There is no apparent change in the transition temperature in the presence of the peptide although it produces a progressive decrease of the amplitude of the observed transition as the peptide to lipid molar ratio increases. The anisotropy of DPH is slightly decreased below the transition temperature of the pure lipid bilayers, whereas it is greatly increased above the transition. This is interpreted as a decreased amplitude of a wobbling-in-cone motion of the acyl chains. The values of the DPH anisotropy (r) at 35°C can be used to monitor the effect of the peptide on the thermotropic behavior of the lipid (Fig. 3, *inset*). The effect of the peptide is maximum at about 3:1 lipid/peptide molar ratio ($R = 0.3$, peptide/lipid molar ratio), the transition being totally abolished. α-Sarcin also decreases the amplitude of the thermal transition of DMPG vesicles, although to a lower extent than the (116–139) peptide. Its effect was saturated at 50:1 lipid to protein molar ratio and the phase transition was not abolished (Gasset et al., 1989; 1991).

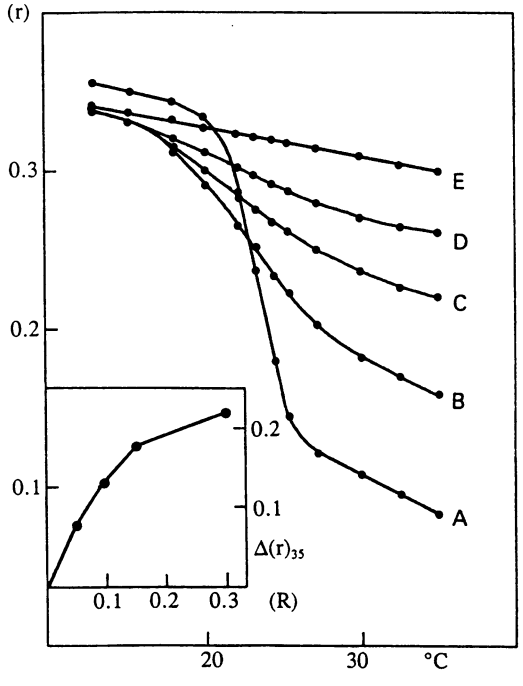

FIGURE 3 Effect of the (116–139) peptide on the thermotropic behavior of DMPG vesicles. (r) Anisotropy of DPH was measured at different temperatures. Samples were in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA; 120 nmol of DMPG were used; the different plots correspond to: (*A*) 0, (*B*) 0.05, (*C*) 0.10, (*D*) 0.15, and (*E*) 0.30 peptide/DMPG molar ratios. (*Inset*) $\Delta(r)_{35}$ measured at 35°C of the DPH-labeled DMPG vesicles as a function of the (R) peptide to lipid molar ratio; $\Delta(r)_{35}$ values are calculated from the anisotropy at 35°C of the pure lipid.

Addition of the peptide to a suspension of negatively charged phospholipid vesicles promotes a moderate increase in turbidity. The peptide produces a time-dependent increase in the apparent absorbance at 360 nm of large unilamellar DMPG vesicles. About 1 h is required for completion of the absorbance variation, although 80% of the change is produced within the first 10 min of reaction. A summary of the obtained results is given in Fig. 4 *A*. No turbidity variation is observed up to about 10:1 DMPG/peptide molar ratio, the maximum variation being obtained at about 3:1 lipid/peptide molar ratio. When α-sarcin is assayed under identical conditions, the maximum turbidity variation is 2.5-fold higher than that produced by the synthetic peptide and is observed at 50:1 DMPG/α-sarcin molar ratio (Gasset et al., 1989). Such a turbidity increase was related to aggregation and fusion of the large unilamellar vesicles promoted by the protein (Gasset et al., 1989, 1990).

The (116–139) peptide destabilizes the DMPG vesicles promoting lipid mixing as detected by using an energy transfer assay (probe dilution). A summary of the obtained results is given in Fig. 4 *B*. The dependence of the steady-state energy transfer (% ET) variation on the peptide concentration also shows a sigmoidal behavior as occurred for the turbidity change. The process is saturated at a ~3–4 lipid/peptide molar ratio. The variation of the (% ET) in the lipid mixing assays would be consistent with four- to fivefold dilution of the probes. Considering that the labeled to unlabeled vesicle populations ratio is 1:9, this observation indicates that the peptide promotes extensive lipid mixing which, however, is not complete, perhaps because some lipid is trapped within aggregates. α-Sarcin promoted a complete lipid-mixing based on energy transfer assays and electron microscopy

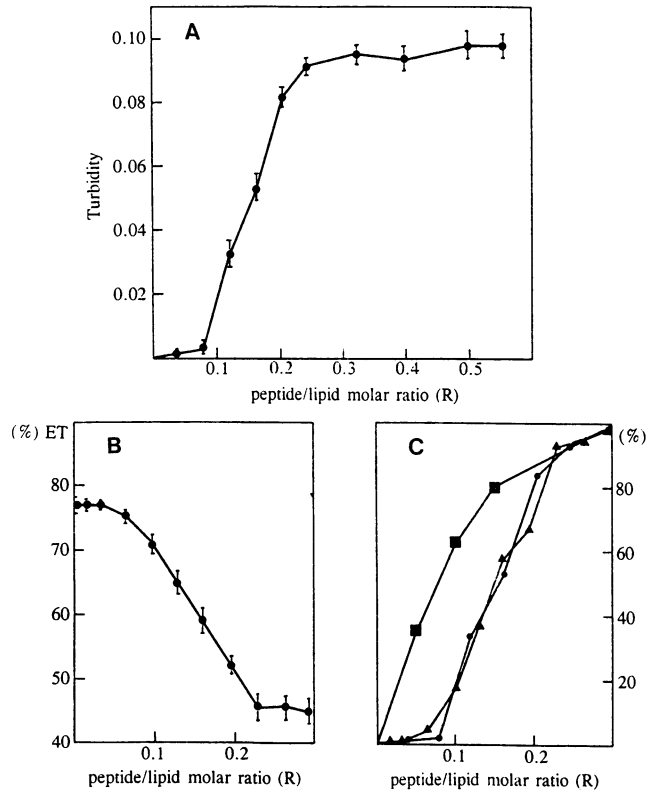

FIGURE 4 (*A*) Variation of the turbidity (measured as the apparent absorbance at 360 nm) of a large unilamellar DMPG vesicle suspension (30 nmol/ml) produced by different amounts of the synthetic (116–139) peptide. The turbidity values are plotted versus (*R*) peptide/lipid molar ratio. The experiments have been performed at 37°C by continuously recording the absorbance values in 1 cm optical path cells (1 ml total volume). The turbidity values are calculated from the final absorbance values (1 h of reaction). The peptide/vesicle system was in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA. (*B*) Lipid mixing promoted by the synthetic (116–139) peptide. Results are expressed as percentage of energy transfer (% ET), calculated from the fluorescence emission at 530 nm of the NBD-PE probe (see Materials and Methods), versus (*R*) peptide/lipid molar ratio. The assays have been performed in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, at a constant temperature of 37°C. The DMPG concentration was 75 nmol/ml, in 1 ml reaction mixture. (*C*) Percentages of the maximum turbidity (●) and % ET (▲) variation versus peptide/DMPG molar ratio. The maximum turbidity value in (*A*) and the maximum % ET variation (33%) in (*B*) have been considered as 100% for each corresponding plot. (■) Percentages of the DPH anisotropy variation at 35°C (taken from Fig. 2) versus peptide/lipid molar ratio; the maximum *r* (at 35°C) variation has been considered as 100%. Vertical bars indicate ±SD values of three experiments.

studies, the saturating lipid/protein molar ratio being 50:1 (Gasset et al., 1990).

When the turbidity and % ET values are expressed as percentages of the corresponding maximum variations observed, and the calculated values are plotted versus peptide/DMPG molar ratio (Fig. 4 *C*), it can be observed that the two processes parallel each other. It can be also observed that the peptide modifies the lipid packing, as deduced from the analysis of the lipid phase transition, at peptide/phospholipid molar ratios where changes on neither turbidity nor % ET are detected, although the three measurements reach completion at the same peptide/lipid molar ratio (Fig. 4 *C*). It is worthy of mention that the theoretical charge of the peptide at pH 7.0 would be around +4, according to its chemical composition. The variation in turbidity, lipid mixing, and DPH anisotropy become saturated at about 3:1 lipid/peptide molar ratio, which corresponds to a negative to positive charge ratio of ~1.3. These facts suggest the involvement of a charge neutralization in these processes.

The (116–139) peptide promotes leakage of intravesicular aqueous contents. Addition of the peptide to a suspension of large unilamellar PS vesicles containing entrapped ANTS/DPX results in a time-dependent ANTS fluorescence increase, consistent with the dequenching promoted by dilution of both probe (ANTS) and quencher (DPX) into the extravesicular medium. The maximum leakage observed represents 70% of the ANTS fluorescence increase produced upon disruption of the vesicles with Triton X-100, considered as control for 100% leakage. The observed effect is dependent on peptide concentration (Fig. 5 *A*). Both initial leakage rates and extent of leakage show a similar dependence on peptide

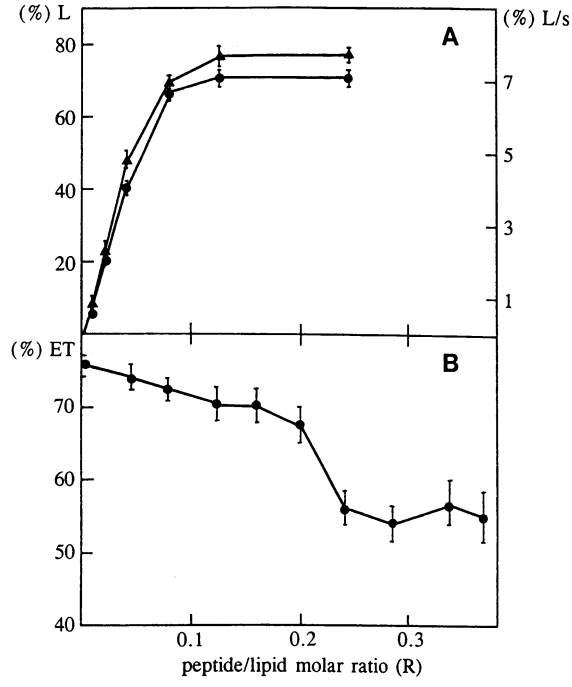

FIGURE 5 (*A*) Leakage of aqueous contents promoted by the (116–139) peptide. Bovine brain PS large unilamellar vesicles containing both ANTS and DPX (see Materials and Methods) at 60 nmol/ml phospholipid concentration were treated with different amounts of peptide (total volume 1 ml). Experiments were performed at 37°C. (●) Values are expressed as % leakage (% L) at 5 min after the addition of the corresponding amount of peptide; 100% leakage was considered as the fluorescence emission value obtained after addition of Triton X-100 (0.1% final concentration). (▲) Initial rates of leakage (% L/s) calculated from the kinetics plots of the ANTS fluorescence variation. (*R*) Peptide to lipid molar ratio. (*B*) Lipid mixing expressed in units of % ET of bovine brain PS large unilamellar vesicles promoted by the (116–139) peptide. The assays were performed in 1 ml, containing 60 nmol phospholipid at a constant temperature of 37°C. Other experimental details as in the legend to Fig. 4 *B*. (R) Peptide to lipid molar ratio. Vertical bars indicate ±SD values of three experiments.

concentration and are saturated at about 10:1 lipid/peptide molar ratio. α-Sarcin also promoted leakage of intravesicular contents, but this effect was saturated at 50:1 lipid/protein molar ratio (Gasset et al., 1990).

The synthetic peptide also produces lipid mixing of PS vesicles. A summary of the obtained results is given in Fig. 5 *B*. Increasing amounts of the (116–139) peptide produce a slight increase on the observed lipid mixing up to a lipid/peptide molar ratio of 5:1. In the 5–3 lipid/peptide molar ratio range, an abrupt increase in the lipid mixing promoted is observed. It can be observed that both processes, lipid mixing and leakage, do not parallel (Fig. 5, *A* and *B*). This can also be deduced from the corresponding kinetic analyses (Fig. 6). The leakage is a faster process than the lipid mixing (~20-fold in terms of initial rates). This suggests that the vesicles leak first and the bilayers mix later.

Conformational changes of the (116–139) peptide upon vesicle interaction

The conformation of the synthetic peptide is modified upon interaction with the phospholipid vesicles. Fig. 7 shows the CD spectra of vesicle-peptide mixtures. It is known that sample turbidity might result in a potential distortion of the CD signal. However, at the concentration of peptide and vesicles and optical path (0.01 cm) herein used, no significant alteration of the CD spectra is produced. In fact, controls with ribonuclease A, a protein that does not interact with bilayers, in the presence of vesicle dispersions at the same effective absorbance as that of the peptide-vesicle samples, show no significant alteration of the CD spectra in the considered range (data not shown). The study performed indicates that β-structures are induced in the peptide upon vesicle interaction. The effect of the phospholipids on the peptide conformation becomes saturated at a ~0.3 peptide/lipid molar ratio, the same saturating ratio as that observed for the different actions promoted by the peptide on the vesicles. The secondary structure calculated from the CD spectrum at 3:1 lipid/peptide molar ratio (Fig. 7 *f*) would be composed of 10% α-helix, 55% β-structure, 8% β-turns and 27% random. As indicated for the peptide in aqueous solution, these values should be cautiously considered, as they are determined from a protein data basis. In addition, in this case the significant ellipticity values used for the estimation of secondary structure have been considered in the 210–250 nm range, which diminishes the reliability of the estimation. Nevertheless, the increased β-structure content is clearly deduced by simply observing the shape of the CD spectra. This increase in the β-structure content promoted by the phospholipids is in opposition to the α-helical conformation promoted by TFE (Fig. 1, *A* and *B*).

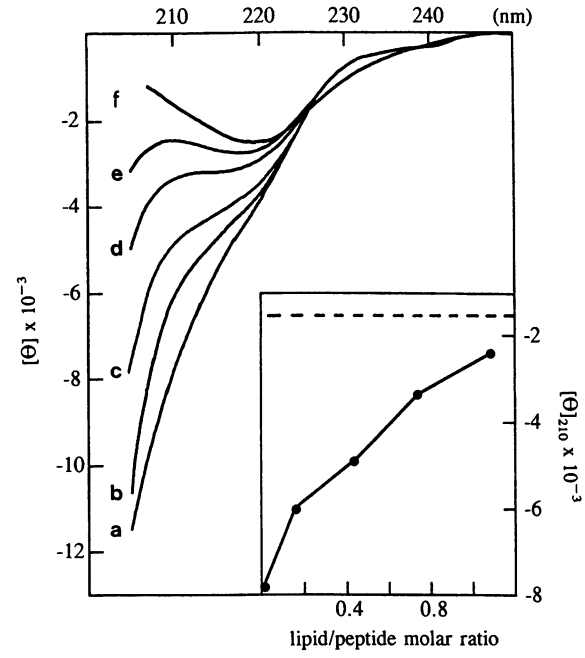

FIGURE 7 CD spectra of the (116–139) peptide in the peptide bond region, at (*a*) 0, (*b*) 0.15, (*c*), 0.43; (*d*) 0.73, (*e*) 1.07, and (*f*) 3.00 lipid to peptide molar ratios. [Θ], Mean residue weight ellipticities are expressed in units of degree × cm^2 × $dmol^{-1}$. (*Inset*) Ellipticity at 210 nm, $[\Theta]_{210}$, versus lipid/peptide molar ratio. The dotted line corresponds to the $[\Theta]_{210}$ value of the sample at 3.0 lipid to peptide molar ratio (*spectrum f*). Samples were dissolved in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA.

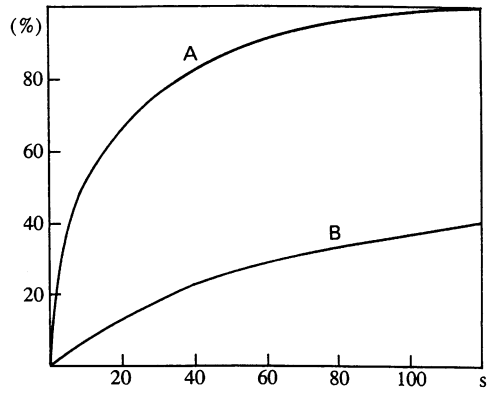

FIGURE 6 Kinetics of the (*A*) leakage and (*B*) lipid mixing induced by the (116–139) peptide in large unilamellar PS vesicles, expressed as percentages of the corresponding maximum fluorescence variation. Both kinetic traces correspond to samples containing 60 nmol of PS vesicles and 15 nmol of peptide, at 37°C.

DISCUSSION

The cytotoxicity of α-sarcin depends on its ability to enter the target cells, since it is an universal ribosome-inactivating protein. Previous studies with model membranes have demonstrated that the protein strongly interacts with bilayers, exhibiting membrane-perturbing properties, which would be related to its passage across membranes. In this context, there is strong evidence of the involvement of hydrophobic interactions in the action of the α-sarcin cytotoxin on lipid bilayers. Thus, the protein promoted large morphological changes in the lipid bilayer, which suggested strong perturbations of the phospholipid arrangement (Gasset et al.,

1990). In addition, penetration of the protein into the hydrophobic core of the bilayers was deduced from the labeling of α-sarcin observed in the presence of vesicles containing photoreactive phospholipid analogues (Gasset et al., 1991; Oñaderra et al., 1993). However, α-sarcin cannot be considered a hydrophobic protein. It only contains 31 amino acid residues displaying a hydropathy index higher than that of glycine (hydrophobic side chains) (Kyte and Doolittle, 1982) among the 150 amino acids of the protein, and they are not arranged in stretches of more than three consecutive residues (Sacco et al., 1983). Therefore, it is not easy to propose the participation of definite amino acid sequence regions of the protein in its hydrophobic interactions with bilayers. Recently, we have proposed a model for the structure of α-sarcin (Mancheño et al., 1995). It was based on the sequence similarity that α-sarcin displays with the ribonucleases of the RNase T1 subfamily. Since the three-dimensional structures of these RNases are well known, the proposed model included a folding for some of the ordered secondary structure elements of α-sarcin. A hydrophobic β-sheet composed of four β-strands is the central part of such a model, and therefore it would be a good candidate for the hydrophobic interactions involving α-sarcin. Two of these four β-strands are consecutive in the primary structure of the protein, and they are located at the 120–135 sequence region of α-sarcin. Thus, we have synthesized the (116–139) peptide to evaluate the potential participation of the corresponding α-sarcin region in the hydrophobic interactions of the protein with bilayers. The synthetic peptide comprises the two consecutive β-strands mentioned above, and also includes four residues at each extreme that are predicted to be involved in two respective β-turns. The characterization of this peptide has revealed that it is highly water-soluble, at least up to a 3 mg/ml concentration, and it is mostly random based on its low content of ordered secondary structure in aqueous solutions.

The obtained results demonstrate that the (116–139) peptide interacts with negatively charged large unilamellar lipid vesicles of either natural or synthetic phospholipids, and strongly suggest the existence of peptide-bilayer hydrophobic interactions. The effect of the peptide on the phase transition of lipid vesicles is drastic, and soundly suggests an insertion of the peptide in the membrane. In fact, the peptide abolishes the phase transition of the bilayer, as expected for an integral molecule that restricts the mobility of the phospholipid acyl chains. Abolition of the phase transition of bilayers has been also reported for the interaction of cardiotoxin II, a 60-residue peptide considered as a lytic factor, with PG and PS vesicles (Faucon et al., 1983). Also, the transition of dimyristoylphosphatidylcholine liposomes can totally disappear in the presence of high amounts of δ-hemolysin, a 26-residue peptide (Bhakoo et al., 1985). The synthetic (116–139) peptide also promotes an apparent vesicle fragmentation. Disruption of phospholipid bilayers by polypeptides into micelles or small vesicles has been also reported. Thus, protein-induced fragmentation of lipid bilayers has been observed with serum apolipoproteins (Brouillette et al., 1984; Anantharamaiah et al., 1985) and myelin basic protein (Roux et al., 1994). A similar effect was also reported for the peptides glucagon (Jones et al., 1978) and melittin (Dufourc et al., 1986, 1989; Dufourcq et al., 1986a,b). In this regard, melittin is a 26-residue lytic peptide that exhibits some sequence similarity (~20%; data not shown) with the (116–139) peptide. The leakage of vesicle aqueous contents promoted by the (116–139) peptide may be related to such a morphological change of the bilayers. These observed effects can be modeled as follows. First, the positively charged peptide electrostatically interacts with the negative surface of the vesicles. The net charge of the peptide and its surrounding phospholipids becomes reduced and hydrophobic interactions can occur. Then, the peptide penetrates the bilayer and perturbs the phospholipid acyl chain core. These would be the first events, which would lead to fragmentation of the vesicles and leakage of aqueous contents. The resulting structures can be related to small vesicles, based on the obtained electron micrographs. Micelles could be also formed, but complete leakage of aqueous contents, as would be expected for a detergent-like action, is not observed, although non-vesicular peptide-lipid complexes cannot be precluded from the obtained results.

The peptide also produces a modest increase in the light scattering of the reaction mixture. This turbidity variation is only observed at higher peptide concentrations than those required for vesicle leakage. In addition, aggregation of large vesicles produces greater turbidity variations as observed for the entire protein (Gasset et al., 1989). These facts may support the notion that the absorbance variation promoted by the (116–139) peptide was related to aggregation of the small phospholipid-peptide vesicle-type structures (aggregates of these particles are observed by electron microscopy; Fig. 2). The peptide also induces lipid mixing of bilayers. Higher peptide concentrations than those required for both leakage of aqueous contents and alteration of the lipid packing are also needed for detecting lipid mixing. Thus, the observed lipid mixing of bilayers would occur within the aggregates of the small vesicles. This may be supported by the fact that no complete dilution of the fluorescence probes was detected in the lipid mixing assays. In this regard, micelle-type structures would not be involved in lipid mixing. Thus, the probe dilution would not be extended to the whole phospholipid population, and the measured decrease in energy transfer in the lipid mixing assay would be lower than the maximum expected.

The dependence of both lipid mixing and light scattering variation on peptide concentration may suggest the involvement of peptide-peptide interactions. Concerning this possibility, no changes in secondary structure are observed in concentrated aqueous solutions of the peptide, which would discard an aggregated peptide form as bilayer-interacting structure. But peptide-peptide interactions within the complex with the vesicles cannot be discarded. In this sense, the peptide contains one cysteine residue, which could be potentially involved in the formation of a disulfide bridge between two peptide monomers upon interaction

with membranes. However, carboxyamido-methylation of peptide-vesicle mixtures reveals that only free thiol are present in these complexes (data not shown).

Finally, regarding the relation of the peptide to the native α-sarcin, the synthetic peptide promotes in the vesicles qualitatively similar effects than α-sarcin. The protein also produces leakage of aqueous contents, lipid mixing of bilayers, and alteration of the lipid packing. However, peptide and protein show significant differences, mainly in terms of the morphological changes induced in the membranes. Thus, α-sarcin produces large vesicle aggregates and fused structures (Gasset et al., 1990), whereas the peptide promotes apparent vesicle fragmentation. Regarding differences between the peptide itself and the peptide as part of the protein, the corresponding region of α-sarcin is involved in a disulfide bridge, which may result in a modified behavior on interaction with vesicles. However, in spite of this, the extent and nature of the effects promoted by the (116–139) peptide allow us to propose that this protein region is a participant in the hydrophobic interactions of α-sarcin with bilayers. The CD spectrum of the (116–139) peptide in the presence of lipids reveals a high content of β-structure, as opposed to the helical conformation induced by TFE. TFE sometimes is considered a helical promoter (Merutka and Stellwagen, 1989; Nelson and Kallenbach, 1989); but it has been also described as a helix enhancer (Sönnichsen et al., 1992) because a requirement for helix propensity was considered necessary to develop this structure. However, there are also reports on stable β-strands in TFE (Narayanan et al., 1986). At any rate, TFE is considered a "membrane-mimicking" solvent because of its ability to promote intramolecular hydrogen bonding as it would occur within the membranes. However, in this case it is clear that a different conformation is observed upon interaction of the peptide with the bilayers. Thus, although the synthetic peptide can adopt a helical structure, this is not its preferred conformation when it interacts with bilayers. In this regard, synthetic peptides corresponding to the lipid-binding domains of apo B-100 were rich in β-structure when incubated with phospholipids (Lins et al., 1994). These authors considered that their observation was quite unusual, given that most peptides after insertion into the lipid bilayer have been shown to adopt mainly a helical conformation (Martin et al., 1993). Lins et al. (1994) concluded that their data suggest that hydrophobic β-sheet present in native apo B-100 is another structure that strongly interacts with lipids. The (116–139) peptide is also rich in β-structure upon interaction with bilayers, and a similar conclusion can be drawn for α-sarcin: the potential hydrophobic β-sheet of the protein would be involved in the hydrophobic interactions that characterize the overall action of this cytotoxin on phospholipid vesicles. However, there is no evidence that such a fragment of α-sarcin acts as a membrane-interactive region in vivo. In this regard, we have recently produced recombinant α-sarcin (Lacadena et al., 1994), and the potential preparation of a truncated form of α-sarcin lacking the 116–139 sequence may be of great interest.

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