Kinetic Study of the Aggregation and Lipid Mixing Produced by α-Sarcin on Phosphatidylglycerol and Phosphatidylserine Vesicles: Stopped-Flow Light Scattering and Fluorescence Energy Transfer Measurements

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ABSTRACT α-Sarcin is a fungal cytotoxic protein that inactivates the eukaryotic ribosomes. A kinetic study of the aggregation and lipid mixing promoted by this protein on phosphatidylglycerol (PG) and phosphatidylserine (PS) vesicles has been performed. Egg yolk PG, bovine brain PS, dimyristoyl-PG (DMPG) and dimyristoyl-PS (DMPS) vesicles have been considered. The initial rates of the vesicle aggregation induced by the protein have been measured by stopped-flow 90° light scattering. The formation of a vesicle dimer as the initial step of this process was deduced from the second-order dependence of the initial rates on phospholipid concentration. The highest α-sarcin concentration studied did not inhibit the vesicle aggregation, indicating that many protein molecules are involved in the vesicle cross-linking. These are common characteristics of the initial steps of the aggregation produced by α-sarcin in the four types of phospholipid vesicles considered. However, the kinetics of the scattering values revealed that more complex changes occurred in the later steps of the aggregation process of egg PG and brain PS vesicles than in those of their synthetic counterparts. α-Sarcin produced lipid mixing in vesicles composed of DMPG or DMPS, which was measured by fluorescence resonance energy transfer assays. A delay in the onset of the process, dependent on the protein concentration, was observed. Measurement of the rates of lipid mixing revealed that the process is first order on phospholipid concentration. Egg PG and brain PS vesicles did not show lipid mixing, although they avidly aggregated. However, α-sarcin was able to promote lipid mixing in heterogeneous systems composed of egg PG + DMPG or brain PS + DMPS vesicles. The dilution of the fluorescence probes was faster when these were incorporated into the bilayers made of synthetic phospholipids than were present in those made of natural phospholipids. The bilayer destabilization produced by the protein in the vesicles composed of the dimyristoyl-phospholipids should be transmitted to the more stable ones made of natural phospholipids. The obtained results are interpreted in terms of lipid mixing occurring within vesicle aggregates larger than dimer.

INTRODUCTION

α-Sarcin is a single polypeptide chain cytotoxin secreted by the mold *Aspergillus giganteus*. This protein exhibits a well characterized ribonuclease activity that inactivates the ribosomes by cleaving a single phosphodiester bond in the 28S ribosomal RNA (see Wool et al., 1992 for a review). Accordingly, α-sarcin is considered a type 1 (single-chain) ribosome-inactivating protein. Although this activity of α-sarcin is thought to be ultimately responsible for its cytotoxicity, the intracellular nature of its target molecule makes protein translocation across the cell membrane the first required step for the overall action of α-sarcin. This raises a fundamental question that can be extended to most protein toxins: how do water-soluble proteins insert into biological membranes? α-Sarcin is cytotoxic for a variety of human tumor cell lines, producing inhibition of the protein biosynthesis in the absence of any permeabilizing treatment (Turnay et al., 1993). An easier penetration of α-sarcin through the more permeable membrane of virus-infected cells has been observed (Fernández-Puentes and Carrasco, 1980). No protein membrane receptors have been so far described for this molecule. In regard to this, α-sarcin strongly interacts with lipid model membranes, which may suggest internalization into endocytic vesicles (Turnay et al., 1993). The protein promotes aggregation and fusion of acidic phospholipid vesicles (Gasset et al., 1989, 1990). Studies performed at the protein-vesicle interaction equilibrium revealed that α-sarcin modified the thermotropic behavior of the phospholipid vesicles (Gasset et al., 1991a) whereas these vesicles produced conformational changes on the protein (Gasset et al., 1991b). A penetration of α-sarcin into the hydrophobic core of these bilayers was also concluded from these studies (Gasset et al., 1991a). The protein was able to degrade tRNA trapped inside asolectin vesicles, which was itself degraded by liposome-encapsulated trypsin in the presence of a large excess of external trypsin inhibitor protein (Oñaderra et al., 1993). These results were interpreted in terms of an operative translocation of α-sarcin across those bilayers.

Translocation of α-sarcin across membranes may be related to the ability of the protein to induce membrane destabilization, which also happens to cause membrane fusion in a liposome system. Although membrane fusion is a known ubiquitous biological process, the involved molecular events are not yet well understood. Fusion of liposomes has been modeled as a process involving two essential and operative steps: 1) aggregation and close apposition of membranes, and 2) a transitional destabilization of the bilayers (Bentz et al.,

Received for publication 29 March 1994 and in final form 28 June 1994.
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0006-3495/94/09/1117/09 $2.00

1983). The main potential barriers for the aggregation of lipid vesicles are the charge repulsion of the bilayers and their surface hydration (Feigenson 1986; Rand and Parsegian, 1989; Leikin et al., 1987). Neutralization of the bilayer charge would overcome the charge repulsion. This is apparently clear from the results obtained from the study of the divalent cation-induced fusion of lipid vesicles (e.g., Hong et al., 1991; Nir, 1991; Wilschut, 1991; Leckband et al., 1993; Walter and Siegel, 1993), the modification of the hydration surface being also deduced from these studies. By extrapolation, fusogenic proteins must be able to promote charge neutralization and dehydration of the lipid surface at least locally. The amino acid sequence of α-sarcin would potentially meet the requirements expected for charge neutralization and modification of the hydration surface of the vesicles. It is a basic and highly polar polypeptide (24 basic and 17 acid residues; 34 Asn + Gln + Thr + Ser; 26 Pro + Gly, among the 150 amino acids of the protein) (Sacco et al., 1983). However, α-sarcin lacks long enough hydrophobic stretches to be easily ascribed to some kind of destabilization of the membranes, the second step required for a fusion of bilayers. Nevertheless, four protein segments are predicted to be arranged in a highly hydrophobic β-sheet (unpublished results) that may represent a membrane-perturbant locus. The bilayer destabilization is the least understood step in the overall fusion process. As summarized by Hong et al. (1991), lipid-packing defects, liquid-crystalline phase transitions, bilayers to hexagonal phase transitions, formation of inverted micelles, protein penetration in the hydrophobic region of the membrane, and lipid dehydration have been proposed to be involved in such a step. In some cases, Ca^{2+} ions are required in the fusion promoted by proteins, but α-sarcin does not bind calcium ions (Martínez del Pozo et al., 1989). Thus, the effects produced by α-sarcin should be ascribed to the protein itself.

The action of α-sarcin on phospholipid vesicles results in large structures (Gasset et al., 1990). Interpretation of the effects produced by the protein needs the consideration of the occurring events at a molecular level. We studied the mechanism of the membrane destabilization induced by α-sarcin by considering the early stages of the process, given that they would tentatively be the more easily modeled and understood at such a molecular level. The aggregation process was analyzed at the time level of milliseconds by using stopped-flow light-scattering measurements. The lipid mixing has been also kinetically analyzed as a measurement of the bilayer destabilization step. These kinetic analyses would allow a better knowledge of the effects of α-sarcin on bilayers, providing new insights on the mechanism of interaction between this water-soluble protein and lipid bilayers, and consequently about its intrinsic cytotoxicity.

MATERIALS AND METHODS

Synthetic DMPG and DMPS, as well as egg yolk PG and bovine brain PS, were purchased from Avanti Polar Lipids (Alabaster, AL), and their homogeneity was assayed by thin-layer chromatography. The lipid vesicles were prepared at 1 mg/ml phospholipid concentration in 30 mM Tris/HCl or Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, by extrusion through 0.1-μm polycarbonate filters (Nucleopore, COSTAR, Cambridge, MA) in an Extruder (Lipex Biomembranes Inc., Vancouver, Canada), essentially as described by Hope et al. (1985). The average size of the vesicle population was 100 nm as determined by Coulter counting. Lysophospholipids were not detected by chromatographic analysis of the lipid component (Gavilanes et al., 1981) after the preparation of the vesicles. The lipid concentration was determined essentially as described (Bartlett, 1959).

α-Sarcin was purified from cultures of *Aspergillus giganteus* MDH 18894. The spectroscopic properties, circular dichroism, and fluorescence emission spectra of the purified protein were identical to those previously reported (Martínez del Pozo et al., 1988). The purified native protein exhibited a denaturing transition at 52°C when we analyzed the process by differential scanning calorimetry (data not shown). Therefore a fully active protein was always used in the experiments described below (performed at 37°C, or 42°C when DMPS vesicles were involved), which was routinely ascertained by using an assay based on a cell-free reticulocyte lysate (Promega, Madison, WI) by observing the production of the α-fragment resulting from the action of the active protein on the ribosomal RNA (Lamy and Davies, 1991). The protein concentration was determined by absorbance measurements at 280 nm based on the reported extinction coefficient of α-sarcin at this wavelength (Gavilanes et al., 1983).

Stopped-flow light-scattering measurements were performed on a modular Hi-Tech Canterbury SF-40 (Salisbury, U.K.) stopped-flow apparatus (dead time 3 ms) equipped with a 50-W quartz halogen light source. A 360-nm wavelength was selected. The 90° scattered light was measured on a photomultiplier PM-60. A 320-nm cutoff filter was inserted in front of the photomultiplier. A time constant of 33 ms was used for the measurements. The apparatus was operated through a Tandon PC computer (Moorpark, CA). The data were analyzed by using the software provided by the manufacturer. Lipid vesicle and protein solutions were freshly prepared at the required concentrations as described above. All the protein and phospholipid concentrations corresponding to the data from these experiments are those of the solutions in the observation chamber of the stopped-flow apparatus. Initial rates of aggregation were determined from the kinetic analysis of the variation of the scattered light intensity. The experiments were performed at a constant temperature of 37°C or 42°C as indicated. The initial rate of the scattered intensity (I) variation $[(dI/dt)_{t=0}]$ (V/s) was calculated from the initial (<0.5 s) linear portion of the average of at least six chart tracings. Control samples in the absence of protein were considered for each vesicle preparation.

The absorbance variation at 360 nm produced by the addition of α-sarcin to a lipid vesicle suspension was measured on a Beckman DU-8 spectrophotometer (Palo Alto, CA) at time intervals of 1 min, in a thermostated cell holder. Control samples in the absence of protein were always considered.

Intermixing of membrane lipids was analyzed by considering fluorescence resonance energy transfer (RET) assays (Struck et al., 1981). The RET assay monitors continuously the relief of fluorescence energy transfer between a donor/acceptor pair as the two probes dilute from labeled into unlabeled bilayers. A vesicle population containing 0.6% Rh-PE (acceptor) and 1% NBD-PE (donor) (Avanti Polar Lipids) as fluorescence lipid analogues was mixed with unlabeled vesicles at 1:1 molar ratio (Wilschut et al., 1985; Walter and Siegel, 1993). The increase of the fluorescence emission at 530 nm (fluorescence emission of the donor) upon excitation at 450 nm (4 nm slit width for both excitation and emission beams), was continuously recorded on an SLM Aminco 8000 spectrofluorometer (Urbana, IL) operated through a PC computer with the manufacturer's software. Freshly prepared vesicles and protein solutions were employed. The protein was added to the cuvette (0.2-cm optical path) under continuous gentle stirring (SLM device). The measurements were performed in a thermostated cell holder at 37°C or 42°C as indicated, monitored by an inner temperature probe. The rate of lipid mixing was calculated as fluorescence emission intensity (F_{530}) per second from the linear portion of the kinetic tracing. The percent fused dimer formation (% fd) was calculated from the fluorescence values as described (Walter and Siegel, 1993), by considering as the fluorescence intensity corresponding to the "ideal dimer formation" that intensity obtained when vesicles were prepared at 0.3% Rh-PE and 0.5% NBD-PE

fluorescence probe concentrations. The zero level of the fluorescence emission scale corresponded to the residual fluorescence of the vesicles in the absence of protein. Different experiments were performed throughout this study: 1) a homogeneous system, with fluorescence-labeled (*) vesicles + unlabeled vesicles (1:1 proportion), both vesicle populations being composed of the same phospholipid [DMPG* + DMPG; DMPS* + DMPS; egg PG* + egg PG; brain PS* + brain PS]; 2) a heterogeneous system, where the labeled vesicles were mixed with an unlabeled population (1:1 proportion), the synthetic and the natural phospholipid being either counterparts [DMPG* + egg PG; egg PG* + DMPG; DMPS* + brain PS; brain PS* + DMPS;].

Fluorescence depolarization measurements were performed on an SLM Aminco 8000 spectrofluorometer equipped with 10 mm Glan-Thompson polarizers. Cells of 0.2-cm optical path were used. The slit widths were 4 nm for both excitation and emission beams, respectively. Labeling of the vesicles with 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich, Milwaukee, WI) was performed as previously described (Gasset et al., 1989). The degree of polarization of the fluorescence emission of DPH was measured at 425 nm for excitation at 365 nm, after equilibration of the sample at the required temperature. This parameter was measured by using an inner temperature probe. Independent experiments demonstrated a negligible contribution of α-sarcin to the polarization of the fluorescence probe. Successive dilutions for each sample were performed to check the potential contribution of the sample turbidity to the polarization degree values. This contribution was negligible for the concentrations and optical paths used.

RESULTS

Measurement of the initial rates of the vesicle aggregation by stopped-flow light scattering

The addition of α-sarcin to phospholipid vesicles produces a time-dependent increase in light-scattering intensity caused by vesicle aggregation. The initial rate of this process was analyzed by stopped-flow light-scattering measurement, because the time scale of this process made necessary the use of rapid mixing techniques. An example of the obtained curves is given in Fig. 1. The initial rates have been estimated from the slope of the linear increase in light-scattering intensity (Fig. 1 *B*). For vesicles made of the natural phospholipids egg PG or brain PS, the chart tracings appear as curve *a* in Fig. 1 *A*; curve *b* in this figure corresponds to the typical tracing obtained for vesicles composed of the synthetic phospholipids DMPG or DMPS. A summary of the results obtained by considering different phospholipid/α-sarcin molar ratios is given in Fig. 2. The different initial rate values obtained for DMPG are about 4- to 6-fold higher than for PG, whereas for PS vesicles the values are similar for the synthetic and the natural phospholipid except at 60 μM phospholipid concentration, where a higher (about 1.5-fold) initial rate value is obtained for DMPS. Moreover, the values obtained for both types of natural phospholipids, egg PG and brain PS, are similar.

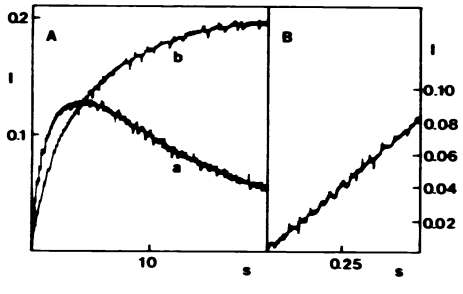

FIGURE 1 Stopped-flow measurements of the light-scattering intensity (*I*) variation produced when α-sarcin and phospholipid vesicles are mixed. (*A*) Chart tracing of the scattered intensity (V) versus time (s) for a mixture of: (*curve a*) 1 μM α-sarcin and 30 μM brain PS, and (*curve b*) 1 μM α-sarcin and 30 μM DMPS, final concentrations. (*B*) Chart tracing of the scattered intensity (V) at an expanded time (s) scale (0.8 μM α-sarcin and 30 μM DMPS, final concentrations). This latter kind of recorded data was used for the calculation of the initial rates. The experiments performed with DMPS were carried out at 42°C and with other vesicles at 37°C.

It can be observed in Fig. 2 that the initial rate values were dependent on α-sarcin concentration and became saturated as the protein concentration increased. Double logarithmic plots of the initial aggregation rates versus vesicle concentration yield lines of slope 2 for all the considered phospholipids (Fig. 2 *E*). Therefore, the aggregation process produced by α-sarcin is second order in phospholipid vesicles, in the phospholipid concentration range herein considered (10–60 μM). The initial rates measured at the time level of milliseconds would mainly account for the earliest step of the aggregation process. Thus, the above dependence on phospholipid concentration would indicate that the aggregation of phospholipid vesicles produced by α-sarcin proceeds via formation of a vesicle dimer at the initial step. These results would also indicate that the pathway for the initial aggregation events is qualitatively the same for all the considered phospholipids in the studied concentration range.

The dimer formation may respond either to [vesicle]-protein-[vesicle] (a single protein molecule cross-linking a vesicle dimer) or [vesicle-protein]-[protein-vesicle] (several

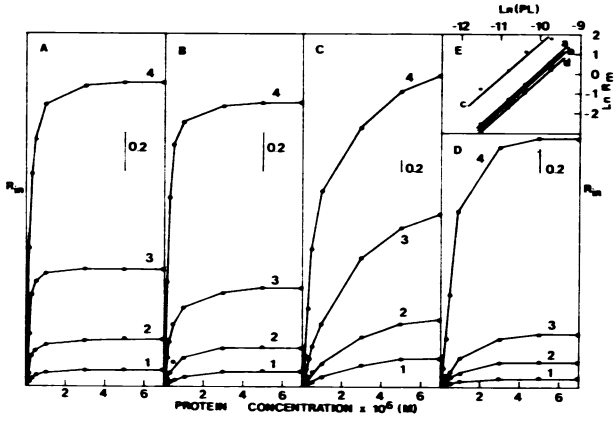

FIGURE 2 Initial rates (R_m) of the aggregation of lipid vesicles produced by α-sarcin. Plots of the calculated initial rates (V/s) of the scattered intensity variation produced when α-sarcin and phospholipid vesicles are mixed versus protein concentration (μM): (*A*) egg PG; (*B*) brain PS; (*C*) DMPG; (*D*) DMPS. Four plots are given for each type of vesicle and correspond to different phospholipid concentrations: (1) 10 μM; (2) 20 μM; (3) 30 μM; (4) 60 μM. Average molecular weights of 750 and 805 have been considered for egg PG and brain PS, respectively. Bars represent 0.2 V/s (R_m). (*E*) Double logarithmic plots of the maximum initial rate (maximum R_m values in A–D) (R_m, in units of V/s) corresponding to each type of vesicle versus phospholipid (PL) concentration (M): (a) egg PG; (b) brain PS; (c) DMPG; (d) DMPS. The experiments performed with DMPS were carried out at 42°C and with other vesicles at 37°C.

interacting protein molecules bridging the vesicle dimer) interactions. According to the first possibility, an inhibition of the initial rate of aggregation would be expected under conditions in which protein-free vesicles became rate-limiting (high protein/lipid molar ratios). However, the calculated initial aggregation rate reached a plateau, and no inhibition of the aggregation was observed even at high protein-to-lipid ratios (e.g., 10 and 7 μM phospholipid-protein concentrations, respectively) (Fig. 2). Therefore, some kind of protein-protein interactions would be involved in the initial steps of the aggregation of phospholipid vesicles produced by α-sarcin.

The pattern of light-scattering intensity variation for vesicles made of natural phospholipids (PG or PS) is clearly different from those obtained for vesicles composed of synthetic phospholipids (DMPG or DMPS) (Fig. 1 *A*). Saturating curves were obtained for DMPS and DMPG; however, an initial increase followed by a decrease in the recorded scattering intensity was observed for both egg PG and brain PS, the decrease being less pronounced as the phospholipid concentration decreased. This kind of behavior has been observed elsewhere and explained in terms of the formation of large aggregates, which reduces the number of scattering particles, thus clearing the light path (Düzgünes et al., 1981). The angular dependence of the light-scattering, which would be significant for larger aggregates although minimal for monomeric vesicles (Wei et al., 1982), should also be considered. The different kinetics observed indicate that the structures formed when synthetic and natural phospholipid vesicles interact with α-sarcin could not be similar, in spite of the identical reaction order observed for the mechanism of formation of the vesicle dimers. This different behavior between synthetic and natural phospholipids was also deduced from the results obtained by measuring the absorbance at 360 nm of vesicle-protein mixtures at the interaction equilibrium (Fig. 3). These values corresponded to the final structures formed upon the interaction. Hyperbolic plots were obtained for DMPS and DMPG with an almost linear initial portion (Gasset et al., 1989), which was not observed for the natural phospholipids considered (Fig. 3). Moreover, larger absorbance variations were observed for DMPS and DMPG than for natural PG and PS, as expected from the light-scattering plots. Increase of the ionic strength up to 0.4 M NaCl in the protein-vesicle interaction equilibrium mixtures resulted in a complete reversal of the absorbance variation for egg PG and brain PS. When DMPG and DMPS vesicles were considered, only 58 and 53% of the absorbance variations were reversed, respectively (data not shown).

Measurement of the lipid mixing rates by fluorescence RET

The lipid mixing between vesicles produced by α-sarcin was analyzed by measuring the fluorescence energy transfer variation in a system composed of NBD-PE (donor)/Rh-PE (acceptor)-labeled/unlabeled vesicles in a 1:1 molar proportion. The kinetic study was performed for phospholipid vesicles of different compositions. Figs. 4 and 5 summarize the results obtained for DMPG vesicles. Addition of α-sarcin produced lipid mixing as deduced from the increase of the fluorescence emission of NBD-PE (donor) in the RET system. Fig. 4 shows the kinetics of lipid mixing obtained for 150 μM DMPG vesicles and different α-sarcin concentrations ranging from 1 to 10 μM. All the obtained curves exhibit a sigmoidal shape resulting from a delay in the onset of the lipid mixing process. This suggests the existence of an intermediate state previous to the lipid mixing. The maximum rate of the lipid mixing (calculated from the portions of constant slope in the curves given in Fig. 4) is achieved after a period of time that varies in an α-sarcin concentration-dependent manner, so that the higher the protein concentration the less time required. This study was performed for different DMPG concentrations (from 15 to 150 μM). The calculated maximum rates measured for the different α-sarcin and DMPG vesicle concentrations are given in Fig. 5 *A*. These plots tend toward saturation at about 10 μM

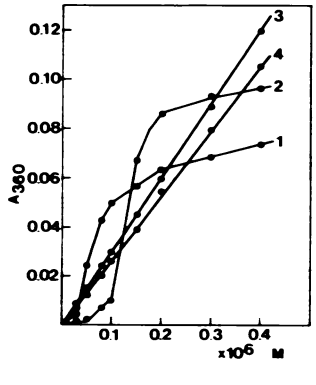

FIGURE 3 Increase in the absorbance values at 360 nm (A_{360}) produced by interaction of different α-sarcin concentrations (μM) with (1) egg PG (75 μM); (2) brain PS (75 μM); (3) DMPG (30 μM); (4) DMPS (30 μM). The time course of the absorbance variation of the reaction mixture (1 ml) was analyzed in 1-cm optical-path thermostated cells. The final absorbance values are considered for each protein concentration. Results are the average of three different determinations.

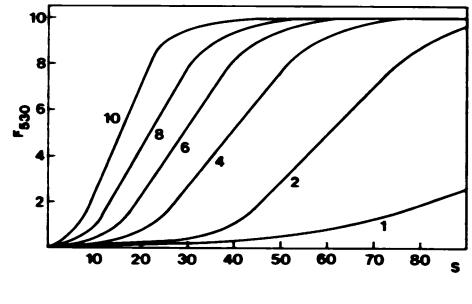

FIGURE 4 Kinetic study of the fluorescence resonance energy transfer variation produced by α-sarcin in a lipid mixing DMPG vesicle system. The fluorescence emission at 530 nm (donor fluorescence), upon excitation at 450 nm (F_{530}), expressed in arbitrary units, is given versus time (s). All the chart tracings correspond to 150 μM total DMPG concentration, with the number given at each curve being the α-sarcin concentration (μM). (1% NBD/0.6% Rh)-labeled and unlabeled DMPG vesicles were mixed in a 1:1 proportion. The experiments were performed at 37°C.

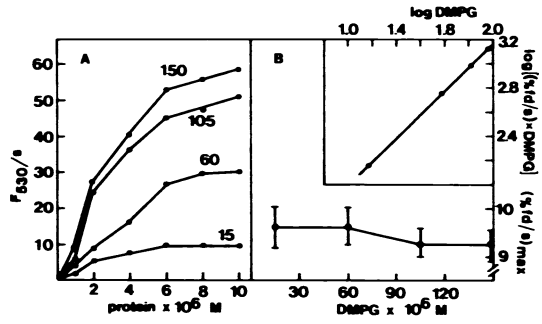

FIGURE 5 (*A*) Rates of the lipid mixing produced by α-sarcin in DMPG vesicles (expressed as fluorescence emission at 530 nm, for excitation at 450 nm, per s; the fluorescence emission is expressed in arbitrary units; these are the same as in Fig. 4. Rates (F_{530}/s) versus protein concentration (M). The total phospholipid concentration (15, 60, 105, and 150 μM) is also given for each plot. These rates have been calculated from the linear portion of the chart tracing of the corresponding kinetics (as those given in Fig. 4). (*B*) Maximum value of the lipid mixing rates given in (*A*) and expressed as (% *fd*/s)$_{max}$ ± SD (see Materials and Methods) versus phospholipid concentration (M). (*Inset*) Double logarithmic plot of [(% *fd*/s)$_{max}$ × (DMPG concentration μM)] versus DMPG concentration (μM). The experiments were performed at 37°C.

α-sarcin concentration. Therefore, the lipid mixing rates at this protein concentration were considered as saturating values at each DMPG concentration. These saturating lipid mixing rates, in units of fluorescence intensity per second, were converted to values in units of (% *fd*/s) (see Materials and Methods) to allow a direct comparison in spite of the obvious different concentration of the fluorescence probes at each particular total lipid concentration. The obtained results are given in Fig. 5 *B*. It is clear that the saturating lipid mixing rates correspond to an almost constant (% *fd*/s) value over the DMPG concentration range considered (15–150 μM). To calculate the order of the process of lipid mixing with respect to the vesicle concentration, the data in Fig. 5 *B* have been plotted as (% *fd*/s × concentration) versus concentration in a double logarithmic plot. This results in a line of slope 1.0 (Fig. 5 *B*, *inset*) indicating that the lipid mixing is first order on phospholipid concentration. This would be expected for an event that occurs within vesicle aggregates rather than between free vesicles that must collide.

The above-mentioned sigmoidal shape of the lipid mixing kinetics is dependent on the protein/lipid molar ratios. Fig. 6 *A* shows the kinetics of the (NBD) donor fluorescence emission variation corresponding to different protein/DMPG molar ratios. At the highest protein/lipid ratio considered a nearly hyperbolic kinetics is observed, and the sigmoidal shape appears as the above ratio decreases. This suggests that the potential intermediate state responsible for the sigmoidal behavior would be related to protein-protein interactions. To provide information about this possibility, the time at which 50% of the maximum fluorescence variation is produced, defined as $t_{1/2}$ s, has been measured. The values of $t_{1/2}$ plotted versus the reciprocal of the corresponding α-sarcin concentration give a straight line (Fig. 6 *B*), which suggests that the process is second order with respect to the protein concentration.

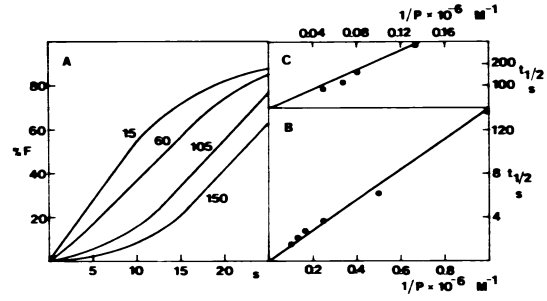

FIGURE 6 (*A*) Kinetics of the fluorescence emission at 530 nm (upon excitation at 450 nm). The fluorescence intensity, in arbitrary units (the same as those in Fig. 4), is expressed as % *F* referred to the maximum emission corresponding to each phospholipid concentration, to allow a direct comparison of the different curves. % *F* is given versus time (s). The four chart tracings given correspond to different DMPG concentrations (μM, numbers given for each curve) and a constant (10 μM) α-sarcin concentration. (*B*) calculated $t_{1/2}$ values (s) versus the reciprocal α-sarcin concentration ($1/P\ M^{-1}$) for a constant (150 μM) DMPG concentration. (*C*) As in (*B*), for a constant (70 μM) DMPS concentration. The experiments were performed at 37 and 42°C for DMPG and DMPS vesicles, respectively.

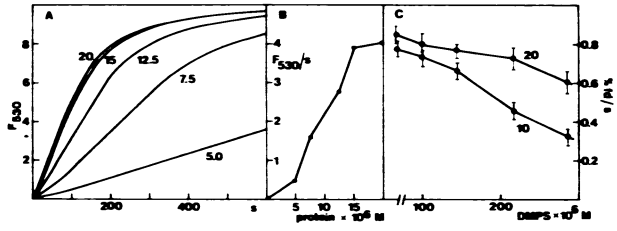

FIGURE 7 (*A*) Kinetics of the fluorescence emission at 530 nm (upon excitation at 450 nm). Fluorescence intensity, expressed in arbitrary units (but the same as in Fig. 4) versus time (s). All the chart tracings correspond to 70 μM DMPS concentration, the number given at each curve being the α-sarcin concentration (μM). (1% NBD/0.6% Rh)-labeled and unlabeled DMPS vesicles were mixed in 1:1 proportion. (*B*) Rates of the lipid mixing produced by α-sarcin in DMPS vesicles (expressed as fluorescence emission at 530 nm for excitation at 450 nm/s; the same units as in Fig. 4) versus protein concentration (M). The total phospholipid concentration is 70 μM. These rates were calculated from the linear portion of the chart tracing of the corresponding kinetics in (*A*). (*C*) Maximum value of the lipid mixing rates corresponding to two α-sarcin concentrations (10 and 20 μM, indicated in the figure), expressed as % *fd*/s (see Materials and Methods) versus DMPS concentration (M). The experiments were performed at 42°C.

The above study was also performed for DMPS vesicles. Fig. 7 *A* shows the kinetics of the lipid mixing obtained for 70 μM DMPS and different α-sarcin concentrations (5–20 μM). The process was visibly slower than that observed for DMPG vesicles (Fig. 4). A plot of the measured maximum rates versus protein concentration is given in Fig. 7 *B*. The rate tended toward saturation at about 20 μM α-sarcin concentration. However, higher protein concentrations were required for saturation of the lipid mixing rate when the phospholipid concentration was increased. Fig. 7 *C* shows a summary of the results obtained at 10 and 20 μM α-sarcin concentration in the 70–285 μM DMPS concentration range. The (% *fd*/s) values decreased as the phospholipid/protein molar ratio increased. This result is in agreement with the

α-sarcin binding data corresponding to this phospholipid (unpublished results). At concentrations higher than 100 μM DMPS, the vesicles were not saturated by α-sarcin in the considered concentration range because of the lower affinity that exhibits this phospholipid in comparison with DMPG. The dependence of $t_{1/2}$ with the reciprocal of the α-sarcin concentration was linear for protein/lipid molar ratios higher than 0.10 (Fig. 6 *C*). This suggests that the process, as occurred for DMPG vesicles, is second order with respect to the protein.

The lipid mixing induced by α-sarcin was also studied for vesicles made of the natural phospholipids egg PG and brain PS. No lipid mixing was detected up to 15 min for PS vesicles under experimental conditions identical to those employed for DMPG. In the same assay, egg PG vesicles show some fluorescence emission variation upon addition of α-sarcin. But the rate of the process is ~3000-fold lower (data not shown) than that obtained for DMPG at the same concentration of lipid.

The lipid mixing produced by α-sarcin was also studied in heterogeneous systems composed of either (egg PG + DMPG vesicles) or (brain PS + DMPS vesicles), both types of vesicles in each system in 1:1 molar proportion. The assay was performed in two different ways: by using NBD-PE-labeled egg PG + unlabeled DMPG vesicles and (Rh-PE)-labeled DMPG + unlabeled egg PG. The same approach was also considered for brain PS and DMPS. In all four considered cases a significant lipid mixing was observed, which necessarily implies that both types of phospholipids participate in the lipid mixing process. A summary of the lipid mixing rates obtained is given in Table 1. All the values obtained are lower than those measured for the corresponding synthetic phospholipid in the homogeneous system assay mentioned above. It can be observed that the lipid mixing rates were about five- to sixfold higher when the fluorescence probes were incorporated into the corresponding synthetic phospholipid vesicle population. The fluorescence anisotropy values at 37°C of DPH-labeled vesicles composed of either DMPG or egg PG are 0.070 and 0.064, respectively. The value of such a parameter at 42°C for DMPS and brain PS was 0.092 and 0.066, respectively. This parameter can be related to the fluidity of the bilayer. Thus, the bilayers of the vesicles made of natural phospholipids would be more fluid than those formed with the corresponding synthetic phospholipid at the considered temperature. It can be observed that the measured lipid mixing rates are higher when the fluorescence probes must diffuse to a more fluid bilayer (from the synthetic to the natural phospholipid bilayers).

DISCUSSION

We have already reported that the cytotoxin α-sarcin produces aggregation (Gasset et al., 1989) as well as fusion (Gasset et al., 1990) of acidic phospholipid vesicles. Freeze-fracture electron micrographs have revealed that unilamellar DMPG vesicles fuse into large multilamellar vesicular structures at low protein/lipid molar ratios, whereas the lipid is organized in planar sheets at high α-sarcin concentrations. Such structures represent final states of interaction inasmuch as they correspond to protein-vesicle mixtures at equilibrium. These results indicate that the protein promotes a significant rearrangement of the lipid structures. The first steps of the protein-vesicle interaction occurs in a very short time, and their study thus requires the analysis of the process by stopped-flow-based techniques. The addition of α-sarcin to a suspension of acidic phospholipid vesicles produces an increase in light-scattering intensity at the time level of milliseconds (Fig. 1). Measurement of the initial rates of this variation reveals that the analyzed process is second order on phospholipid concentration. This is interpreted in terms of formation of a vesicle dimer as initial step of the aggregation of vesicles promoted by the protein.

The rate of dimer formation (Fig. 2) is not inhibited at very high protein/lipid molar ratios (protein-coated vesicles) when protein-free vesicles would become rate-limiting of any process that could involve them. This would indicate that the vesicle dimer is not cross-linked by a single protein mol-

TABLE 1 Initial rates of the lipid-mixing induced by α-sarcin in PG/DMPG and PS/DMPS vesicles

α-Sarcin concentrations (nmol/ml)	Total phospholipid concentration (μg/ml)					
	200		100		50	
	A*	B‡	A	B	A	B
1) PG vesicles						
20	1.290	7.070	1.005	5.380	0.763	2.660
15	0.825	4.570	0.698	4.400	0.493	2.470
2) PS vesicles						
20	0.260	1.640	0.180	1.050	0.103	0.610
15	0.190	1.040	0.160	0.780	0.085	0.450

Values are expressed as F_{530}/s (the fluorescence emission in arbitrary units, but the same as in Fig. 4).

* Type A assays: the fluorescence energy transfer system is composed of (NBD-PE/Rh-PE)-labeled PG and unlabeled DMPG (Part 1) or labeled PS and unlabeled DMPS (Part 2), respectively (1:1 proportion labeled/unlabeled vesicles).

‡ Type B assays: the fluorescence energy transfer is composed of (NBD-PE/Rh-PE)-labeled either DMPG and unlabeled PG (Part 1) or labeled-DMPS and unlabeled PS (Part 2), respectively (1:1 proportion labeled/unlabeled vesicles).

Both types of assays correspond to a heterogeneous system (see Materials and Methods).

ecule as occurred in the vesicle aggregation produced by poly-L-lysine, where the aggregation rate reached a maximum and then decreased at polypeptide concentrations at which the vesicles would be coated by protein monomers (Lampe et al., 1983). The rate of the vesicle dimer formation was saturable with respect to the α-sarcin concentration, which indicates that the dimer is formed between vesicles cross-linked by several protein molecules as previously described for the aggregation produced by the myelin basic protein (Lampe et al., 1983). However, in that case the dependence of the aggregation on protein concentration was highly cooperative, and a delay in the onset of aggregation was observed at low protein/lipid molar ratio. Lampe et al. (1983) gave a possible explanation for the observed sigmoidal shape, related to an insufficient stability of the protein-protein cross-linked vesicle dimer at low protein density. This stability would be increased when multiple associations between adjacent vesicles were formed, thus explaining the existence of the observed delay. However, the dependence of the vesicle dimer formation on α-sarcin concentration does not exhibit a sigmoidal shape. Accordingly, it can be thought that the vesicle dimer promoted by α-sarcin would have enough stability, and multiple associations between vesicles would not be required for the formation of a stable dimer; consequently, a hyperbolic dependence would be observed.

These results also indicate that, although more than a single α-sarcin molecule is involved in maintaining the vesicle dimer, protein-protein interactions are not rate-limiting. In fact, this possibility would imply a delay in the onset of aggregation and an inhibition of the process at high phospholipid/protein molar ratios. This is not observed in the α-sarcin-vesicle interaction. In addition, the obtained results also exclude the possibility of a vesicle dimer maintained by a protein aggregate formed before to the α-sarcin-vesicle interaction. This is in agreement with the nondetection of protein aggregates in α-sarcin solutions even in the presence of chemical cross-linking reagents at the protein concentrations examined herein (data not shown).

After the vesicle aggregation, a membrane destabilization is produced by α-sarcin. In this regard, the protein penetrates deeply enough into the hydrophobic core of the bilayer to be labeled with a photoactivatable probe located at the C-12 of the fatty acid of the phospholipid (Gasset et al., 1991a). The addition of α-sarcin to the phospholipid vesicles produces a relief in the fluorescence resonance energy transfer in a classical RET assay (Struck et al., 1981). This result is interpreted in terms of lipid mixing between bilayers promoted by the protein. This process is slower than that reflected by the 90° light-scattering intensity variation. The kinetics of the fluorescence emission variation exhibits a sigmoidal shape, a delay in the onset of the process appearing (Fig. 4). Measurement of the maximum rates revealed that the process is first order on phospholipid concentration as would be expected for a lipid mixing in an ideal system modeled as vesicle → vesicle dimer → fused vesicle. But according to this mechanism, the observed lipid mixing would exhibit an apparent second-order dependence on phospholipid concentration under conditions of vesicle dimer formation as rate-limiting step (low vesicle concentration) (Wilschut et al., 1980; Nir et al., 1982). Such a second-order dependence is not observed for the lipid mixing promoted by α-sarcin. So, the dimer formation is not a rate-limiting step even at low vesicle concentrations. This intrinsically means that the rate constant of the vesicle aggregation process is higher than that of the lipid mixing. However, even considering this fact, the first-order dependence observed in the long range of phospholipid concentration studied would indicate that the lipid mixing predominantly occurs within vesicle aggregates more than in a vesicle dimer. This would explain the observed delay in the onset of the lipid mixing process. The time length of the above delay depends on protein concentration (Figs. 4 and 6). Indeed, at the time at which measurable lipid mixing occurs, vesicle aggregates larger than a dimer are expected to be formed. Accordingly, the lipid mixing promoted by α-sarcin should not be modeled as a vesicle dimer-to-fused vesicle process. A vesicle aggregate of a higher order than dimer is required to observe lipid mixing. This may be related to the above-mentioned involvement of many protein molecules in the cross-linking of vesicles, as deduced from the results obtained from the stopped-flow light-scattering measurements. The observed second-order dependence of the lipid mixing on α-sarcin concentration suggests that the protein acts as an aggregate at least at some stage of this process. In fact, a vesicle aggregate involves many α-sarcin molecules, and the lipid mixing would occur within the vesicle aggregate.

No lipid mixing is detected when natural phospholipids egg PG and brain PS are considered, although α-sarcin promotes aggregation of these vesicles. This may be thought to be related to differences in bilayer fluidity, given that both types of phospholipids, synthetic and natural, would differ only in terms of their fatty acid composition. However, the measured differences in fluidity determined by fluorescence anisotropy of DPH-labeled vesicles do not seem to be large enough to explain the absence of lipid mixing. This apparent discrepancy between results obtained for different phospholipids but exhibiting the same polar headgroup has been also described. Thus, Walter and Siegel (1993) have reported that palmitoyl-oleoyl-PS, although aggregated avidly in the presence of divalent cations, did not exhibit lipid mixing, whereas brain PS showed lipid mixing under identical experimental conditions. This different behavior was related to the composition and distribution of the acyl chains of these phospholipids. These authors concluded that lipid defect formation rates for PS systems can be controlled by factors other than headgroup chemistry or phase transition of the phospholipid. Eklund (1990) observed that acidic phospholipid vesicles with fully saturated fatty acids (DMPG and dipalmitoyl-PS) fuse in the presence of monovalent cations, whereas those containing unsaturated fatty acids (egg PG and brain PS) do not. These experiments were performed with vesicles prepared in the absence of Na^+, and the further addition of the monovalent cation represented a significant concentration gradient across the bilayer. In this regard, it has been

suggested that the protonation of the internal surface of the bilayer of sonicated PS vesicles may be responsible for the thermodynamic stability of the vesicles (Demel et al., 1987). Therefore, fusion could be produced in the less stable vesicles under the stress conditions created by the Na^+ gradient. It has also been proposed that increased phospholipid unsaturation in membranes perturbs the interlipid hydrogen bonding involving water and that the process is independent of effects on lipid order (Slater et al., 1993). Then it can be speculated that α-sarcin promotes lipid mixing within aggregates of vesicles composed of fully saturated fatty acids because of their different hydrogen bonding network.

α-Sarcin produces lipid mixing when vesicles composed of natural phospholipids are mixed with others made of synthetic phospholipids. This should therefore imply that the destabilization promoted by the protein in the dimyristoyl-phospholipid vesicles is transmitted to the natural phospholipid vesicle. Considering that the formation of a vesicle dimer involves many α-sarcin molecules and that a protein-protein interaction may underlie the lipid mixing, such an interaction could be the pathway for the transmission of the membrane destabilization. In this regard, a change of the α-sarcin conformation is produced upon interaction with DMPG vesicles (Gasset et al., 1991b). A potential explanation for the observed lipid mixing in a heterogeneous system may be as follows. As a consequence of the membrane destabilization produced by the protein in the vesicles of synthetic phospholipids, a conformational change may occur in the polypeptide. This change would subsequently modify (through protein-protein interaction) the conformation of interacting proteins bound to vesicles made of natural phospholipids, and this latter change produces destabilization of the membrane. This hypothesis would imply two different conformations for the protein bound to either natural or synthetic phospholipid vesicles. In this regard, it has been described that rhodanase bound to either cardiolipin or PS liposomes shows different conformation (Zardeneta and Horowitz, 1993). Nevertheless, other explanations could also be valid. The interaction of α-sarcin with both types of phospholipid vesicles may result in a different modification of the hydration surface of the vesicles. In the aggregated heterogeneous system, such a modification would affect all the interacting vesicles, thus allowing the lipid mixing between them. Vesicles composed of natural or synthetic phospholipids could exhibit a different hydration surface in spite of the coincidence in polar headgroups, because as indicated above it has been suggested that unsaturation may involve a perturbation of the membrane surface hydrogen bonds (Slater et al., 1993). Also, the changes observed in the infrared spectra of dispersions of phosphatidylserines upon addition of cations have been interpreted in terms of changes in hydrogen bonding (Hübner et al., 1994).

In summary, α-sarcin aggregates negatively charged phospholipid-containing vesicles. This process is initiated by the formation of a vesicle dimer, which is maintained by protein-protein bridges. Once the aggregation proceeds, lipid mixing occurs between the bilayers of aggregated vesicles. This process is related to the presence of vesicles composed of phospholipids containing saturated fatty acids. These vesicles are destabilized by the protein, and the resulting defect can be transmitted to other aggregated vesicles.

This work has been supported by grant PB90-0007 from the DGICYT (Spain).

Abbreviations used: DMPG, dimyristoyl-phosphatidylglycerol; DMPS, dimyristoyl-phosphatidylserine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Mops, 3-(*N*-morpholino)propanesulfonic acid; NBD-PE, *N*-(7-nitro-2-1, 3-benzoxadiazol-4-yl)-dimyristoyl-phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; RET, resonance energy transfer; /bxRh-PE, *N*-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine.

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