Study of the interaction between the antitumour protein α-sarcin and phospholipid vesicles

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α-Sarcin is a single polypeptide chain protein which exhibits antitumour activity by degrading the larger ribosomal RNA of tumour cells. We describe the interaction of a α-sarcin with lipid model systems. The protein specifically interacts with negatively-charged phospholipid vesicles, resulting in protein–lipid complexes which can be isolated by ultracentrifugation in a sucrose gradient. α-Sarcin causes aggregation of such vesicles. The extent of this interaction progressively decreases when the molar ratio of phosphatidylcholine increases in acidic vesicles. The kinetics of the vesicle aggregation induced by the protein have been measured. This process is dependent on the ratio of α-sarcin present in the protein–lipid system. A saturation plot is observed from phospholipid vesicles–protein titrations. The saturating protein/lipid molar ratio is 1:50. The effect produced by the antitumour protein on the lipid vesicles is dependent on neither the length nor the degree of unsaturation of the phospholipid acyl chain. However, the aggregation is dependent on temperature, being many times higher above the phase transition temperature of the corresponding phospholipid than below it. The effects of pH and ionic strength have also been considered. An increase in the ionic strength does not abolish the protein–lipid interaction. The effect of pH may be related to conformational changes of the protein. Binding experiments reveal a strong interaction between α-sarcin and acidic vesicles, with $K_d = 0.06\ \mu M$. The peptide bonds of the protein are protected against trypsin hydrolysis upon binding to acidic vesicles. The interaction of the protein with phosphatidylglycerol vesicles does not modify the phase transition temperature of the lipid, although it decreases the amplitude of the change of fluorescence anisotropy associated to the co-operative melting of 1,6-diphenyl-1,3,5-hexatriene (DPH)-labelled vesicles. The results are interpreted in terms of the existence of both electrostatic and hydrophobic components for the interaction between phospholipid vesicles and the antitumour protein.

INTRODUCTION

α-Sarcin, a protein produced and secreted into the culture medium by the mould *Aspergillus giganteus* MDH 18894, exhibits antitumour activity (Olson *et al.*, 1965). Such an activity was found when culture filtrates of this mould were assayed as antitumour agents during an antibiotic screening programme of the Michigan Department of Health (Olson & Goerner, 1965). The cytotoxic effect of this protein has been observed against mouse sarcoma 180 and carcinoma 755 among other tumours (Olson *et al.*, 1965). Studies *in vitro* revealed that α-sarcin inhibits protein synthesis by cleaving one single phosphodiester bond from the larger ribosomal RNA of eukaryotic cells at a region with a highly conserved nucleotide sequence (see Wool, 1984, for a review). According to these studies, the specific antitumour effect of α-sarcin is based on its selective passage across some cell membranes. In fact, α-sarcin is a powerful inhibitor of protein synthesis in picornavirus-infected cells, but only after the cell infection (Fernández-Puentes & Carrasco, 1980). The ribonucleolytic effect of α-sarcin is explained by its sequence similarity with ribonucleases U2 and T1 (about 35% and 20% similarity respectively). The residues at the active site of the ribonucleases are among those also present in the

antitumour protein (Takahashi, 1971; Sato & Uchida, 1975). However, these two ribonucleases do not show antitumour activity.

The selective passage across some cell membranes and consequently the selective antitumour activity of α-sarcin may be related to its recognition by some membrane protein receptor. But so far no results revealing such a possibility have been reported. Translocation of proteins across cell membranes can occur through different processes primarily involving membrane surface and hydrophobic interactions. Although the molecular mechanism of this event remains elusive, the decisive involvement of membrane phospholipids seems to be clear. For example, the interaction of some positively charged proteins with negatively charged phospholipid vesicles results in passage through bilayers (Nesmeyanova, 1982; Rietveld *et al.*, 1983; Batenburg *et al.*, 1985). Recently, it has been reported that phosphatidylglycerol (PG), an anionic phospholipid, is involved in the translocation of newly synthesized proteins across the inner membranes of *Escherichia coli* (de Vrije *et al.*, 1988). α-Sarcin contains a very high number of positively charged residues and hydrogen-bond donating and accepting groups (Sato & Uchida, 1975), which may be involved in interactions with the lipid surface of membranes in the events occurring during the cell penetration process. In order to

get insights into the molecular mechanism of the passage of α-sarcin across cell membranes, we have studied the potential ability of this protein to interact with lipid model systems. The results obtained demonstrate the existence of a strong interaction between α-sarcin and phospholipid vesicles.

MATERIALS AND METHODS

Preparation of the lipid vesicles

Synthetic phospholipids, dipalmitoyl- and dimyristoyl-phosphatidylcholine (DPPC and DMPC respectively), dipalmitoyl- and dimyristoyl-phosphatidic acid (DPPA and DMPA respectively) and dioleoyl-, dimyristoyl- and dipalmitoyl-phosphatidylglycerol (DOPG, DMPG and DPPG respectively) were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.) and their homogeneity assayed by thin layer chromatography (Gavilanes et al., 1981). The different lipid vesicles were prepared at a phospholipid concentration of 1 mg/ml in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl, for 30 min in a water bath sonifier (Martínez del Pozo et al., 1988a). The temperature during the sonication process was maintained above the phase transition temperature of the corresponding phospholipid. Lysophospholipids were not present in the vesicles, according to the results of chromatographic analyses of the lipid component after preparation of the vesicles.

Preparation of protein–lipid complexes: ultracentrifugation studies

All the protein used for the remaining experiments corresponded to a highly purified sample (used for protein sequence studies; Rodríguez et al., 1982; Gavilanes et al., 1983) of α-sarcin. The α-sarcin–phospholipid complexes were prepared by incubation of recently obtained lipid vesicles in the presence of the protein at 37 °C for 60 min. The complexes were prepared at different protein/lipid molar ratios, and they were analysed by centrifugation in a stepwise sucrose gradient (8.5/17.0/40.0% sucrose; 4.5/4.5/2.0 ml) at 35000 rev./min ($r_{av} = 11.27$ cm for 5 h) (Beckman L5-65B, rotor SW40) (Mollenhauer & Von der Mark, 1983). Samples of 0.5 ml, in 8% sucrose, were applied to the top of the gradient. Diacyl-1-[^{14}C]-phospholipids (Amersham) (700 c.p.m./nmol) were used to prepare labelled vesicles in order to study the lipid distribution through the gradient. This pattern was determined by counting of radioactivity (Beckman LS 3801 scintillation counter) of 0.1 ml aliquots from each fraction (0.5 ml) collected from the sucrose gradient. ^{125}I-labelled α-sarcin (55000 c.p.m./nmol) was used to analyse the protein distribution which was determined by counting of radioactivity in each fraction from the gradient (Beckman 5500 gamma counter).

Labelling of α-sarcin

Iodination of α-sarcin (^{125}I, 15 mCi/μg; Amersham) was performed according to the procedure described by Tenner et al. (1981) and Gavilanes et al. (1985), to a final specific radioactivity of 10^9 c.p.m./mg after exhaustive dialysis of the sample. ^{125}I-labelled α-sarcin diluted 1:300 with unlabelled protein was used for ultra-centrifugation and binding experiments.

Aggregation studies

The study of the aggregation of the phospholipid vesicles induced by α-sarcin was performed by adding the protein to recently prepared lipid vesicles at different protein/lipid molar ratios, and measuring the kinetics of the change in the absorbance at 360 nm (thermostated cell holder in a Beckman DU-8 spectrophotometer). Controls without protein were present in all these experiments. These analyses were performed at different pH, ionic strength and temperature values (see Results and Discussion section), and for the various phospholipid vesicles studied.

Binding experiments

The protein–lipid binding experiments were performed by incubation of phospholipid vesicles with increasing amounts of α-sarcin at 37 °C for 60 min. Typically, 117 nmol of phospholipid (based on phosphorus content, determined according to Fiske & Subbarov, 1925) were incubated with ^{125}I-labelled α-sarcin (55000 c.p.m./nmol) in 0.1 M-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl. The free, unbound protein was separated from the medium by filtration through Millipore membranes (0.2 μm pore diameter). Protein and phosphorus contents in the filtrate were determined. The amount of lipid in the filtrate was negligible as demonstrated by parallel experiments with [^{14}C]-phospholipid vesicles. The protein retention in the absence of phospholipid vesicles was negligible.

Fluorescence depolarization measurements

Fluorescence measurements were carried out on a Perkin Elmer MPF-44E spectrofluorimeter operated in the Ratio mode. Cells of 0.2 cm optical-path were used. The slit widths were 7 nm and 5 nm for the excitation and emission beams respectively. Labelling of the phospholipid vesicles with 1,6-diphenyl-1,3,5-hexatriene (DPH) was performed as previously described (Gavilanes et al., 1985). Polarization of the fluorescence emission of DPH was measured with the corresponding accessory in thermostated cells. Emission of DPH was measured at 425 nm for excitation at 365 nm. The contribution of α-sarcin to the degree of polarization of DPH was negligible as deduced from the results of separated experiments. The contribution of sample turbidity to the polarization of the DPH fluorescence emission was also negligible at the protein/lipid molar ratios and optical path used. This was verified by measuring the degree of polarization of successive dilutions for each sample.

Trypsin treatment

Tryptic hydrolysis of α-sarcin in the presence of lipid vesicles (1:50 protein/lipid molar ratio) was performed by incubation of TPCK-treated trypsin (1:50 enzyme/substrate weight ratio) in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl, at 37 °C. Aliquots from the incubation mixtures were taken at different hydrolysis times and analysed by SDS/polyacrylamide-slab-gel electrophoresis (4% and 15% acrylamide for the stacking and running gels respectively; 0.1% SDS). Samples were previously reduced with 3% 2-mercapto-ethanol. Vacuum-dried gels were scanned by measuring absorbance at 550 nm on a Beckman DU-8 spectro-photometer equipped with the corresponding accessory.

Protein concentration

The u.v.-spectrum of the protein was routinely used to calculate the concentration of α-sarcin. An extinction coefficient (0.1 % w/v, 1 cm, 280 nm) of 1.34 (Gavilanes et al., 1983) was used for this purpose.

RESULTS AND DISCUSSION

The studies described have been performed with native α-sarcin (without any previous denaturing treatment), at neutral pH and moderate ionic strength (0.1 M-NaCl). Thus, all the possible effects that this protein might produce on lipid model systems will be related to a molecule exhibiting its normal antitumour activity as well as the overall conformational properties previously described (Martínez del Pozo et al., 1988b).
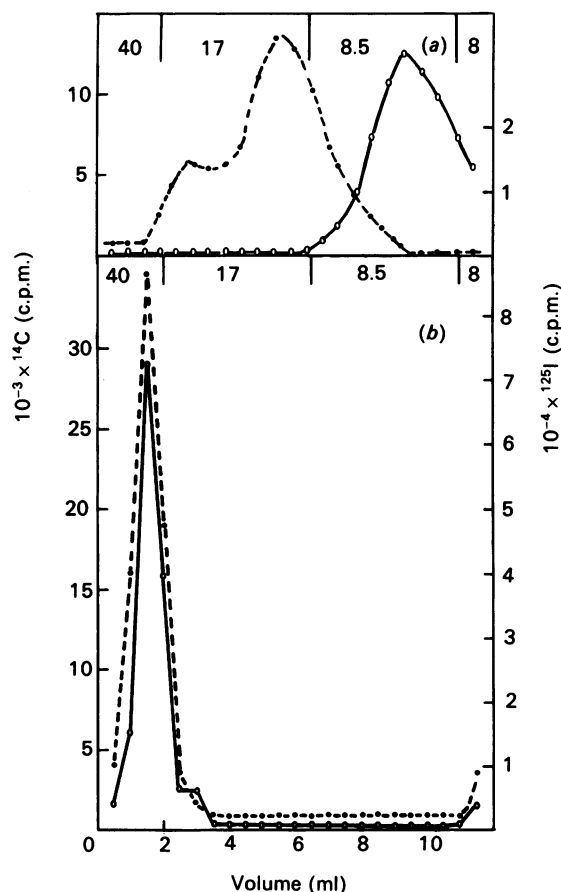
Isolation of α-sarcin–phospholipid complex

The interaction between the antitumour protein α-sarcin and phospholipid model vesicles was first studied by centrifugation in a stepwise sucrose gradient. The aim of this experiment was the isolation of a possible protein–lipid complex. This would demonstrate the existence of protein–lipid interactions which could be involved in the antitumour activity observed for α-sarcin. An α-sarcin–phospholipid complex cannot be obtained when PC vesicles are used at different protein/lipid molar ratios. However, a complex was isolated when PG vesicles were employed in the centrifugation study.

Under the conditions used, DMPG vesicles mainly banded at the 17 % sucrose step (about 80 % of the total lipid present), whereas α-sarcin only slightly penetrates in the 8.5 % sucrose zone (Fig. 1a). However, when a mixture of α-sarcin and DMPG vesicles, after incubation for 1 h at 37 °C, is centrifuged (at 1:50 protein–lipid molar ratio in the experiment given in Fig. 1b) both lipid and protein banded at the same position, in the 40 % sucrose zone (Fig. 1b). These results clearly demonstrated the existence of an interaction between α-sarcin and PG vesicles. Also, at the protein/lipid ratio used in the experiments given in Fig. 1, all the protein present must interact with the phospholipid vesicles since no significant amount of free protein is observed (Fig. 1b). Moreover, the hydrodynamic properties of the protein–lipid complex must differ from that of the lipid vesicles alone as deduced from the increased penetration of the complex through the sucrose gradient (Fig. 1). In fact, addition of the protein to a preparation of PG vesicles produces a large increase in the turbidity of the lipid dispersion, which can be easily visualized. This must be related to a size increase of the particles present in the interaction medium. This effect produced by α-sarcin allows us to study the interaction by measuring the absorbance increase in the visible region of the spectrum, due to the significant light-scattering of the resulting complexes.

Aggregation of phospholipid vesicles induced by α-sarcin

The time-course of the interaction between α-sarcin and phospholipid vesicles was studied by measuring absorbance at 360 nm. A time-dependent absorbance increase was produced when α-sarcin was added to PA or PG vesicles, although no change was detected for PC vesicles. The absorbance variation produced by α-sarcin progressively decreased when the proportion of PC present in vesicles containing both PC and PG was


Fig. 1. Isolation of α-sarcin–phospholipid complex

Centrifugation was carried out in a stepwise sucrose gradient (40.0; 17.0; 8.5 % sucrose (w/v); 4.5; 4.5; 2.0 ml) at 35000 r.p.m. for 5 h in rotor SW40 (Beckman). (a) (———) DMPG vesicles (150 nmol of phospholipid, 700 c.p.m./nmol), and (———) α-sarcin (3 nmol of protein, 55000 c.p.m./nmol), centrifuged in separate experiments. (b) As above, but for the corresponding α-sarcin–DMPG mixture. Diacyl-1-[^{14}C]-phospholipids and ^{125}I-labelled α-sarcin were used for these experiments. Radioactivity values per collected fraction (0.5 ml) are presented. Samples of 0.5 ml in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl and 8 % (w/v) sucrose were applied to the top of the gradient.

increased. The minimum PG:PC molar ratio required to observe an absorbance increase was 1:10. These results suggest the existence of an electrostatic component involved in the protein–lipid binding, which is diminished when the proportion of the zwitterionic phospholipid PC increases in the samples.

This kinetic study revealed that the longest period of time required for completion of the interaction is 40 min, depending on the amounts of both phospholipid and protein present. The value of the final absorbance increase is also dependent on the amount of protein present. In fact, when a preparation of DMPG vesicles is titrated by adding α-sarcin, a saturation plot is observed (Fig. 2a). The saturation was obtained for a 1:50 protein/lipid molar ratio, as can be deduced from Fig. 2. This result is in agreement with the centrifugation experiment at the
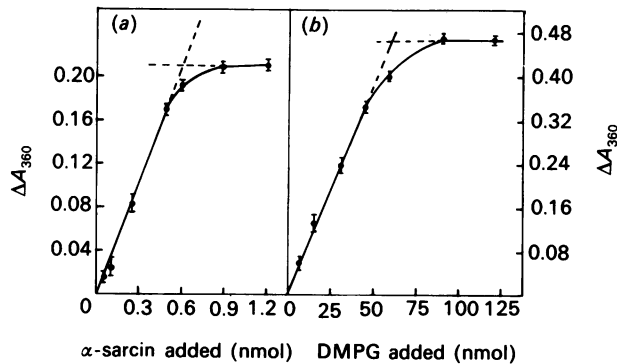
Fig. 2. Increase in the absorbance values at 360 nm produced by interaction of α-sarcin and DMPG vesicles

(a) DMPG (30 nmol of phospholipid) titrated by adding α-sarcin. (b) α-sarcin (1.2 nmol of protein) titrated with DMPG vesicles at different phospholipid concentrations. Samples of 1 ml in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl, are considered for both titrations. The intersection of the dotted lines gives the saturation point which is obtained in both cases at a protein/lipid molar ratio of 1:50. Cells of 1 cm optical path were used for these measurements. Results are given as means±S.D. ($n = 3$).
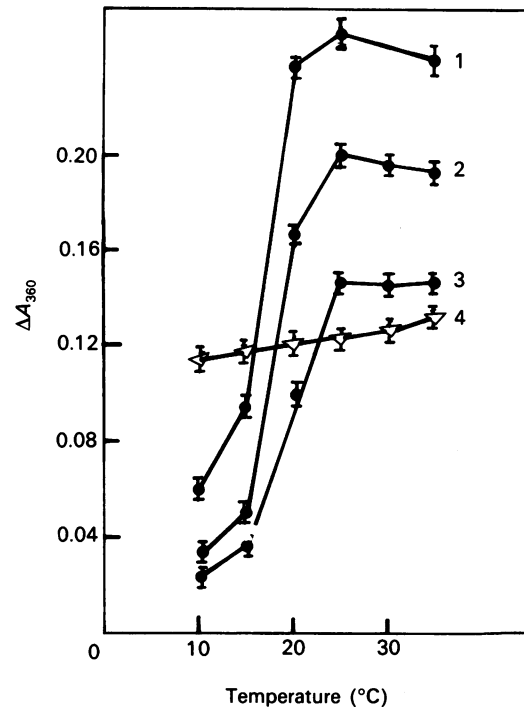

Fig. 3. Effect of temperature on the PG–α-sarcin interaction

Increases in the absorbance value at 360 nm (means±S.D., $n = 3$) are shown for α-sarcin–DMPG at 1:15, 1:47 and 1:82 protein/lipid molar ratios (plots 1–3), and α-sarcin–DOPG at 1:25 protein/lipid molar ratio (plot 4). The phospholipid content in all of these experiments was 30 nmol in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl. 1 cm optical path cells were used.

same protein/lipid molar ratio, where no significant free protein was detected.

The α-sarcin–acid phospholipid interaction results in a size increase of the particles present in the lipid dispersion, as the variation in A_{360} demonstrates, but the protein does not act as simple inductor of the size increase, since this only occurs through protein binding as observed from the titration of α-sarcin with DMPG vesicles (Fig. 2b). In fact, a new hyperbolic plot is observed, reaching saturation at a protein/lipid molar ratio of 1:50 (Fig. 2b) as was also obtained for the titration of DMPG vesicles with α-sarcin (Fig. 2a).

The experiments summarized in Fig. 2 have been performed with DMPG vesicles, but the same saturation value was obtained for DPPG (results not shown) indicating that the effect of the antitumour protein is not highly dependent on the acyl chain length of the phospholipid.

Effect of temperature on the aggregation induced by α-sarcin

The effect of temperature on the interaction between α-sarcin and PG vesicles has also been considered. This study revealed that the process is temperature-dependent (Fig. 3). Different behaviour is observed above and below the phase transition temperature of the phospholipid (23 °C for DMPG). This can be deduced from the plot of the increase of A_{360} vs. temperature (Fig. 3). Thus, this absorbance increase is higher at temperatures above the thermal transition of the phospholipid, and this is observed for different protein/lipid molar ratios (plots 1–3 in Fig. 3). When DPPG vesicles are used, the absorbance increase is also higher above the phase transition temperature (41 °C for DPPG). Plot 4 in Fig. 3 shows the effect of temperature on the interaction between α-sarcin and DOPG vesicles, the transition temperature of which is well below the first temperature value considered in this set of experiments. This plot reveals that α-sarcin also increases the A_{360} value,

indicating an interaction of the protein with DOPG vesicles, but it also shows that the effect of temperature is critical only at the phase transition, since the final state of DOPG is not essentially modified upon a temperature increase above the phase transition of the phospholipid. This result also indicates that the effect of α-sarcin is not significantly dependent on the saturation of the acyl chain of the phospholipid, based on the similar results obtained for DOPG and DMPG or DPPG. The observed results on the influence of the phase state of the lipid suggests an involvement of the hydrophobic core of the vesicle on the α-sarcin–lipid interaction.

Effect of pH and ionic strength on the α-sarcin–lipid interaction

The effect of both pH and ionic strength on the interaction between α-sarcin and PG vesicles has also been studied. The results obtained are given in Fig. 4, expressed as percentages of the A_{360} variation observed at the standard interaction conditions (pH 7 and 0.1 M-ionic strength). Interaction between α-sarcin and PG vesicles occurs even at 1.0 M ionic strength, but the extent of the interaction, deduced from the A_{360} increase, is lowered as ionic strength is increased from 0.1 M. Thus, at 0.3 M ionic strength, the absorbance variation is 20 % of that observed at the standard conditions. This value then remained constant up to 1.0 M ionic strength (Fig. 4a).

The effect of the ionic strength, at least up to 0.5 M,
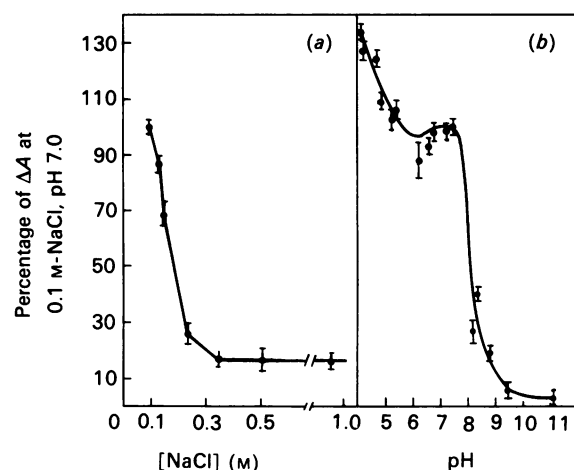
Fig. 4. Effect of (a) ionic strength and (b) pH on the interaction between α-sarcin and DMPG vesicles

A protein/lipid molar ratio of 1:25 was used with 30 nmol of phospholipid in 1 ml samples. Values (means±s.d.; $n = 3$) are expressed as percentages of the absorbance increase at 360 nm in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl. Ionic strength was increased by adding NaCl; the experiments in part (b) were performed in 30 mM-Tris/HCl and citrate buffers.

on the α-sarcin–phospholipid interaction cannot be attributed to any conformational change in the protein, as indicated by conformational controls (far- and near-u.v. circular dichroism and fluorescence emission spectra) (data not shown). In addition, the gel-filtration behaviour of the protein is not modified in the ionic strength range considered. Thus, the results in Fig. 4 are not due to protein aggregation.

These facts are interpreted in terms of the existence of an electrostatic component in the protein–lipid interaction, as also deduced from the interaction experiments with PC–PG vesicles. However, a hydrophobic component may also be involved since the interaction is not abolished even at 1.0 M ionic strength (20 % absorbance variation at 360 nm, in Fig. 4a). This would also be in agreement with the results obtained indicating the dependence of the interaction with temperature.

The existence of electrostatic and hydrophobic components has been reported for several protein–lipid systems. In this sense, it has been described that myelin basic protein exhibits both electrostatic and hydrophobic components on its interaction with PG vesicles. This last has been attributed to the existence of some segments of hydrophobic and/or uncharged residues, despite the fact that the protein is highly basic (Boggs et al., 1980; Stollery et al., 1980). This also occurs for α-sarcin. The protein contains eight segments, each containing from 5 to 13 residues, but lacking charged residues (Sato & Uchida, 1975). Three of these segments (residues at positions 1–8, 97–101 and 130–138 in the protein molecule) present hydropathy values (Martínez del Pozo et al., 1988b) reflecting regions potentially involved in hydrophobic interactions.

The aggregation caused by α-sarcin in a PG vesicle preparation is abolished at basic pH (approx. pH 9) (Fig. 4b), revealing a transition at pH 8.0. This cannot be

explained by simple deprotonation of side chain amino groups of the protein, since the transition occurred at a lower pH than that expected for such a possibility. This indicates that electrostatic effects are not the only ones involved in the protein–lipid interaction. In this case, the observed effect may be attributed to conformational changes of the protein. Indeed, α-sarcin exhibits many conformational transitions induced by pH variations (Martínez del Pozo et al., 1988b). Two of these occur at basic pH values, 8.0 and 10.2, the latter being a denaturing transition (Martínez del Pozo et al., 1988b). However, at this highest pH value there is no variation in the A_{360} value, as the results in Fig. 4b indicate. The conformational transition at pH 8.0 only affects the microenvironment of the tryptophan residues of the protein, but it is not a denaturing change (Martínez del Pozo et al., 1988b). Such a transition was attributed to the deprotonation of the α-NH_2 group of the protein. On the other hand, the phospholipid vesicles are not expected to be modified at this pH value (Watts et al., 1978). Thus, this pH-induced transition on the aggregation of acid phospholipids produced by α-sarcin (Fig. 4b) should be related to the protein itself.

Considering all these facts, the observed effect of pH on the protein–lipid interaction probably involves the N-terminal region of α-sarcin, which would be of specific importance for the events produced. Related to this, it should be noted that one of the two disulphide bridges of the protein is established between cysteine residues at positions 6 and 148 (the third position from the C-terminus), bringing close together the two extremes of the protein, which contain segments lacking charged residues (segments at positions 1–8 and 130–138) (Sato & Uchida, 1975; Martínez del Pozo et al., 1988b).

Finally, the effect observed at acid pH (Fig. 4b) may be attributed to the pK of the PG (3.0–3.5). An aggregation of the PG vesicles produced by the negative charge neutralization due to the positive protein charges would be increased by the synergistic effect of the protons. This would be in agreement with the observed result, and suggests that once vesicle aggregation occurs due to α-sarcin, the effect is enhanced if charge neutralization is also produced by protons. It is important to note that control experiments without protein reveal that protons alone are not able to produce modification in the absorbance value at 360 nm.

Effect of α-sarcin on the thermotropic behaviour of phospholipid vesicles

The effect of α-sarcin on the gel-to-fluid phase transition of phospholipid vesicles was also studied. This was performed by measuring the depolarization of DPH-labelled vesicles. A change in the transition profile would indicate a modification of the environment of the acyl chains of the phospholipids, either at a local level or due to long range factors.

The presence of α-sarcin modifies the thermotropic behaviour of DMPG vesicles (Fig. 5), whereas it does not affect that of DMPC vesicles. First-derivative analysis of the phase transition profiles in Fig. 5 indicates that α-sarcin does not modify the melting temperature of PG vesicles. However, a significant decrease in the amplitude of the fluorescence anisotropy change of the process is produced by the protein, as deduced from the first-derivative plots (Fig. 5). This decrease is about 50 % for the protein/lipid molar ratio used in the experiments
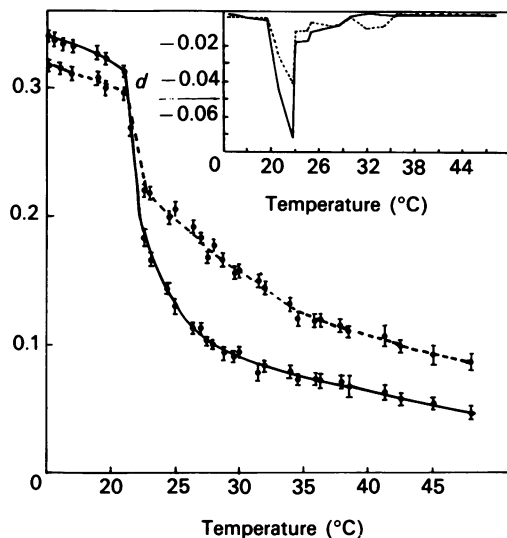
Fig. 5. Phase transition temperature profiles of DPH-labelled DMPG vesicles.

Anisotropy (r) values are the means ± s.d. corresponding to two different determinations. (———) indicates DMPG vesicles and (———) indicates α-sarcin–DMPG vesicles at 1:50 protein/lipid molar ratio. The analyses were performed in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl. Anisotropy was determined after equilibration of the sample for 10 min at each temperature. Inset: First-derivative (d) analysis of the melting profiles, expressed in arbitrary units. Calculations were carried out on a IBM computer. The position of the minimum corresponds to the transition temperature of the melting process.

Fig. 6. Binding of α-sarcin to DMPG vesicles

(a) bound protein vs. total protein added to 117 nmol of phospholipid. In (b) P_b = bound protein; P_t = total protein and L_t = total phospholipid (see Results and Discussion section). These studies were performed in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl. Means ± s.d. ($n = 3$) are given.

given in this Fig., which corresponds to the saturation value. Moreover, the fluorescence anisotropy change induced by α-sarcin is higher for the liquid-crystalline than for the gel-crystalline phospholipid phase.

These results are interpreted in terms of penetration of α-sarcin into the lipid bilayer through hydrophobic interactions, although the protein does not perturb the acyl chain packing of the bulk lipid since no modification in the phase transition temperature is detected. The degree of non-polar association would depend on the fluidity of the lipid, and thus the interaction is preferentially with the liquid-crystalline phase. The phospholipid molecules around the protein would be relatively immobilized and consequently they would not participate in the co-operative melting of the bulk lipid, as deduced from the 50% decrease in the amplitude of the phase transition.

These experiments corroborate the existence of a non-polar (hydrophobic) component for the interaction between α-sarcin and phospholipid vesicles, as previously indicated. However, they also show a charge specificity since PC vesicles are not affected by the presence of the antitumour protein.

Analysis of the α-sarcin binding

The interaction between α-sarcin and PG vesicles is not a simple adsorption process, since it produces changes in the thermotropic behaviour of the hydrophobic moiety of the vesicles consistent with penetration into the lipid bilayer. Moreover, simple adsorption of proteins onto phospholipid vesicles is a sufficiently weak
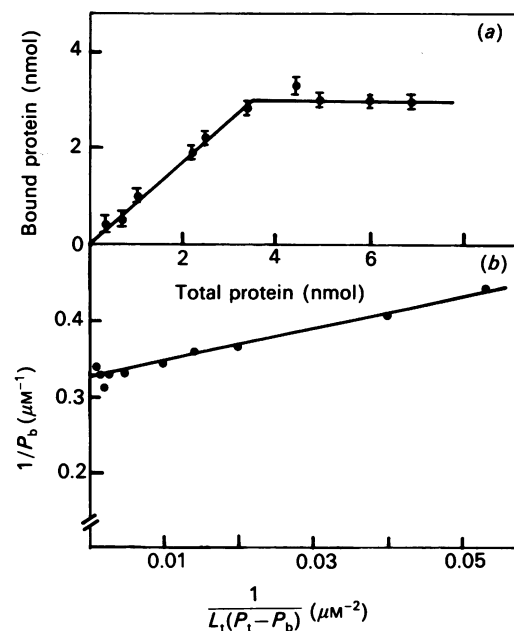
interaction that polar groups on neither the vesicle nor the protein become desolvated during the process. The strong interaction between α-sarcin and PG vesicles can be deduced from the results of binding experiments (Fig. 6). These were not performed by centrifugation, in order to avoid the possible modification of the equilibrium conditions by the sedimentation. The binding results have been analysed by considering protein (P) + 'free lipid sites' = PL_n as interaction equilibrium, n being the number of phospholipid molecules involved at each binding site. Thus, a dissociation constant, K_d, can be defined as a function of total protein (P_t), bound protein (P_b), total phospholipids (L_t), and n, and the following equation can be deduced:

$$\frac{1}{P_b} = \frac{K_d n}{L_t} \times (P_t - P_b) + \frac{n}{L_t}$$

A plot of $1/(P_b)$ vs. $1/[L_t \ (P_t - P_b)]$ (Fig. 6b) allows the calculation of both K_d and n. The α-sarcin binding involves about $n = 40$ phospholipid molecules. This value would represent the number of phospholipid molecules per 'binding site'; however, 'binding site' for protein–lipid interactions is an ambiguous concept due to the lipid diffusion movement. Thus, such a value must be interpreted only from an operative point of view as the average number of phospholipid molecules affected by the protein. The calculated dissociation constant is 6×10^{-8} M which does not correspond to a weak interaction. Relatively small dissociation constant values, as the one found for this interaction, are expected for processes occurring through several sequential steps. This would occur if the interaction between α-sarcin and

PG vesicles involves many electrostatic interactions, hydrogen bonding and hydrophobic forces. Thus, salts will produce local solvation on the protein and will compete with the lipid vesicles for interaction with the protein, affecting the electrostatic component but not the hydrophobic one, as it occurs for the present system.

Proteolysis of α-sarcin in protein–lipid complexes

The binding of α-sarcin to negatively-charged phospholipid vesicles produces an effective protection for the antitumour protein against proteolysis. For this analysis, trypsin was selected because of the relatively high number of basic residues in α-sarcin (about 15 % of the total amino acids). After 24 h at 20 °C, the protein is completely hydrolysed in the absence of phospholipid vesicles. However, when this study is performed for the α-sarcin–PG vesicles system (at 1:50 protein/lipid molar ratio, the saturation ratio), only 30 % protein hydrolysis is produced after 24 h hydrolysis time, as determined from the corresponding densitometric scans of SDS/polyacrylamide-gel electrophoresis. The lipid component does not affect the proteolytic enzyme as indicated by experiments with synthetic substrates of trypsin (results not shown). This protective effect was not observed in the presence of PC vesicles; in this case, the obtained result was coincident to that in the absence of lipid. Since these experiments were carried out by adding trypsin to preformed α-sarcin–phospholipid complexes, the results obtained must be interpreted in terms of protection of the lysine residues of α-sarcin against the external medium due to its interaction with the PG vesicles.

The results herein presented provide clear evidence of the existence of a strong interaction between the antitumour protein α-sarcin and lipid vesicles, specifically requiring acid phospholipids. This interaction produces aggregation of the vesicles with bound protein molecules, which penetrate into the hydrophobic moiety of the bilayer. In spite of the existence of receptor-mediated interactions (not described), these results show that α-sarcin is able to strongly ($K_d = 6 \times 10^{-8}$ M) interact with specific lipid components of membranes (acid phospholipids). This may be involved directly in the molecular mechanism by which α-sarcin enters cell membranes, as described for protein translocation across *E. coli* inner membranes (de Vrije *et al.*, 1988). Conditions promoting spontaneous insertion of proteins into preformed vesicles, as in the present case, are well correlated with those producing fusion between vesicles (Downer *et al.*, 1976; Eytan & Broza, 1978; Eytan, 1982). Initial ionic inter-actions could allow hydrophobic interactions, which in turn could lead to translocation of α-sarcin.

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