Translocation of α-sarcin across the lipid bilayer of asolectin vesicles

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α-Sarcin is a cytotoxic protein produced by the mould *Aspergillus giganteus*. Insertion of α-sarcin into asolectin membranes has been demonstrated by protein labelling with photoreactive phospholipids. α-Sarcin added externally to tRNA-containing asolectin liposomes degrades the entrapped tRNA. Trypsin-containing asolectin liposomes were also prepared. Encapsulated trypsin degrades α-sarcin, even in the presence of a large excess of external hen egg-white trypsin inhibitor to prevent any α-sarcin degradation outside the vesicles. These processes occur only with acidic phospholipids and were not observed when phosphatidylcholine vesicles were used. These results indicate that α-sarcin penetrates the lipid bilayer and becomes exposed to the lumen of negatively charged liposomes.

INTRODUCTION

α-Sarcin, a protein produced by the mould *Aspergillus giganteus*, has been described as an anti-tumour agent against two different tumour systems induced in mice [1–4]. It inhibits protein biosynthesis by cleaving the larger RNA of the 60 S ribosomal subunit [5,6], the nucleotide sequence at the cleavage site being highly conserved from yeast to rat [5,6]. This protein has also been reported to be a powerful inhibitor of *in vivo* protein synthesis in picornavirus-infected cells, which exhibit a modified membrane permeability [7–12]. Therefore α-sarcin shares with other protein toxins the common requirement of insertion into or transport across the plasma membrane of the host cell.

The structure of α-sarcin must be responsible for two functions, i.e. ribonuclease activity and the ability to enter cells. α-Sarcin might pass across some cell membranes, and consequently show its toxic activity, by recognition of some membrane protein receptor(s). However, no results supporting such a possibility have so far been reported. The lipids, which determine to a large extent the barrier function of the membrane, may play an important role in the translocation of α-sarcin. Our previous studies have shown that α-sarcin exhibits a high affinity for negatively charged phospholipids [13–16]. Binding of the protein to these model membrane lipids results in many different changes in the bilayers [13–15]. The structural reorganization of the lipids in these model membranes upon addition of the protein may suggest translocation of α-sarcin across the bilayer, although the molecular mechanism of this type of process is as yet obscure. In any event, it seems obvious that α-sarcin has to pass through the cell membrane to show the observed toxicity, and this leads to questions about its passage across the lipid bilayer. We have studied the potential translocation of α-sarcin in a well-defined model membrane system and found that the protein passes across such model membranes.

MATERIALS AND METHODS

Preparation of protein–lipid complexes

Soybean asolectin (Sigma), purified by dry acetone treatment, had the phospholipid composition previously reported [17] when analysed by t.l.c. [18]. Large unilamellar vesicles of asolectin or egg phosphatidylcholine (PC) (Avanti Polar Lipids) were prepared by freeze–thawing and extrusion [19]. A dried film of lipids was suspended in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl at a lipid concentration of 10–20 mg/ml [20]. The resulting suspension was vigorously vortexed; the liposomes were then frozen in liquid nitrogen and left to thaw at room temperature. The freezing–thawing cycle was repeated five times. Extrusion of the frozen and thawed preparations through two stacked 100 nm-pore polycarbonate filters was then performed.

α-Sarcin was purified from cultures of *Aspergillus giganteus* MDH 18894 essentially as described [1]. The purified protein sample contained a small proportion of dimer, less than 10%, which is observed in polyacrylamide gels even under reducing conditions [15,21]. The amino acid composition and the spectroscopic properties of purified α-sarcin were as previously reported [22]. Lipid-vesicle–protein complexes were obtained by adding α-sarcin to recently prepared liposomes at different lipid/protein molar ratios [13]. The protein concentration was determinated by absorbance measurements at 280 nm based on the reported absorption coefficient of $1.34\ l \cdot g^{-1} \cdot cm^{-1}$ [23].

Trypsin-containing liposomes

Trypsin-containing liposomes were prepared as described above, except that the lipid film was rehydrated in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 8–10 mg/ml Tos-Phe-CH_2Cl ('TPCK')-treated trypsin (Merck). The vesicles were separated from the non-encapsulated material in Sephadex G-75 columns packed in a 5 ml disposable syringe; vesicles were further eluted by centrifugation of the column at 500 *g* for 2 min. This procedure was repeated twice to remove all untrapped trypsin. The internal volume of the trypsin-containing asolectin vesicles was 2.2 μl/μmol of phospholipid, as determined by measuring the encapsulated trypsin activity; a chromogenic substrate (see below) was used for these assays.

tRNA-containing liposomes

tRNA-containing liposomes were also prepared by freezing–thawing and extrusion [19]. tRNA (3–5 mg/ml) from baker's

Abbreviations used: PC, phosphatidylcholine; PC-I, 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine; PC-II, 1-myristoyl-2-{12-[(4-azido-2-nitrophenyl)amino]dodecanoyl}-*sn*-glycero-3-[^{14}C]phosphocholine; TI, hen egg-white trypsin inhibitor.

yeast (Sigma; type X-s) was added to the Mops buffer employed for the hydration of the dried lipid film. The vesicles were separated from the non-encapsulated tRNA by five 30 min centrifugation cycles at 127000 *g* in an Airfuge (Beckman). The final pellet was suspended in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl.

α-Sarcin degradation by encapsulated trypsin

α-Sarcin (10 μg) was incubated with trypsin-containing liposomes at the required lipid concentrations (see figure legends) for the desired time at 37 °C, in the presence of enough hen egg-white trypsin inhibitor (TI) (Boehringer Mannheim) to inactivate the protease present in the corresponding sample. Thus α-sarcin hydrolysis by any potential trypsin outside of the liposomes can be discounted. After the incubation, ice-cold ethanol/acetone (1:1, v/v) was added. The samples were shaken for 15 min at 4 °C and incubated overnight at −20 °C. Then the samples were microfuged for 15 min at 4 °C. The precipitated lipid-free protein was dried, redissolved in a suitable volume of electrophoresis sample buffer [24] and analysed in 15% acrylamide/1% SDS slab gels [24]. Dried Coomassie Blue-stained gels were absorbance-scanned at 500 nm on a Beckman DU 8 spectrophotometer. The area under the absorbance peak corresponding to the α-sarcin band was used to estimate the percentage of non-degraded protein. Control samples for 100% α-sarcin degradation were prepared by adding 1% Triton X-100 to the α-sarcin/trypsin-containing liposomes mixture in the absence of trypsin inhibitor. The activity of the trypsin used was previously checked by spectrophotometrical measurement (405 nm) of the initial rates for the hydrolysis of the chromogenic substrate Val-Leu-Lys-*p*-nitroanilide-2HCl.

Measurement of the degradation of encapsulated tRNA by α-sarcin

The tRNA-containing vesicles were suspended in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl. Different amounts of α-sarcin were incubated with tRNA-encapsulated vesicles for 3 h at 37 °C. Then 1 vol. of ice-cold 10% (v/v) $HClO_4$, containing 0.25% (w/v) uranyl acetate and BSA up to 0.2 mg/ml final concentration, was added. The resulting suspension was maintained for 30 min in an ice-bath and then centrifuged at 3000 *g* for 20 min at 4 °C. The absorbance at 260 nm of the supernatant was determined in a Beckman DU-7 spectrophotometer. This absorbance value is dependent on the concentration of soluble oligonucleotides produced by the ribonucleolytic activity of α-sarcin. Samples in the absence of protein were used as blanks for all assays. This analysis is based on that reported for the measurement of α-sarcin activity against purified yeast RNA [25].

Photolabelling of α-sarcin

Labelling of α-sarcin was performed in an asolectin vesicle system (1 mg/ml total phospholipid concentration) doped with the chemically synthesized photoreactive lipid probes 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycerol-3-[^{3}H]phosphocholine (PC-I) (5×10^5 d.p.m. total radioactivity; 0.006% molar ratio to total phospholipids) and 1-myristoyl-2-{12-[(4-azido-2-nitrophenyl)amino]dodecanoyl}-*sn*-glycerol-3-[^{14}C]-phosphocholine (PC-II) (1×10^5 d.p.m. total radioactivity; 0.018% molar ratio to total phospholipids) in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl, as described [26]. SDS/PAGE of the samples, Coomassie Blue staining, densitometric scanning, slicing and counting of radioactivity of the gels were performed as described [15,26].

RESULTS

Hydrophobic photolabelling of α-sarcin

In order to study the potential insertion of α-sarcin into the hydrophobic core of asolectin bilayers, we performed photolabelling experiments with asolectin vesicles containing trace amounts of photoreactive phospholipids. Figure 1 shows the labelling profiles obtained for SDS/polyacrylamide gels of α-sarcin from protein–vesicle complexes. Two radioactive peaks were observed, corresponding to the monomer and dimer fractions of α-sarcin as confirmed by the densitometric scan of the Coomassie Blue-stained gels (Figure 1c). There was significant labelling with both the PC-I and PC-II probes. These results indicate that α-sarcin not only associates superficially with asolectin vesicles (labelling with PC-I), but also penetrates the bilayer sufficiently to be labelled by the reactive group at the C-12 of the fatty acid acyl chain of PC-II. The normalized $^{14}C/^3H$ ratio for the labelled α-sarcin was 4.5, indicating greater labelling with PC-II than with PC-I. The obtained results are similar to those reported for dimyristoylphosphatidylglycerol (DMPG) vesicles, in which the $^{14}C/^3H$ ratio was 3.8 [15].

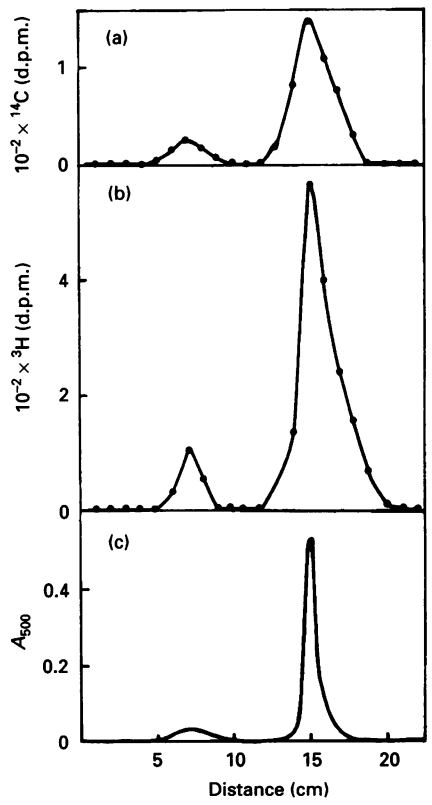

Figure 1 Hydrophobic photolabelling of α-sarcin with photoreactive phospholipids

Soybean asolectin vesicles tagged with PC-I (**b**) and PC-II (**a**) were incubated with the protein in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl (see the Materials and methods section). Protein components have been analysed by SDS/PAGE. The gel distribution of ^{3}H radioactivity (**b**; PC-I probe) and ^{14}C radioactivity (**a**, PC-II probe) are shown. Radioactivity values are given for each 2 mm slice from the corresponding gel. Panel (**c**) shows the densitometric scan of the Coomassie Blue-stained gel. Minor peaks correspond to the dimer fraction of α-sarcin. The lipid/α-sarcin molar ratio used in the photolabelling experiments was 300:1.

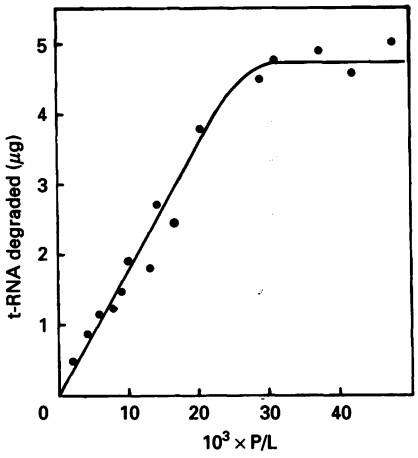

Figure 2 Hydrolysis of trapped tRNA by externally added α-sarcin

Assays were performed in 0.2 ml of 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl, by using tRNA encapsulated in asolectin vesicles. The final lipid concentration was 1 mM and aliquots of an α-sarcin stock solution (0.17 mM) were added. After incubation, tRNA degradation was determined (see the Materials and methods section). Each value represents the average of four different determinations. P/L, protein/lipid molar ratio.

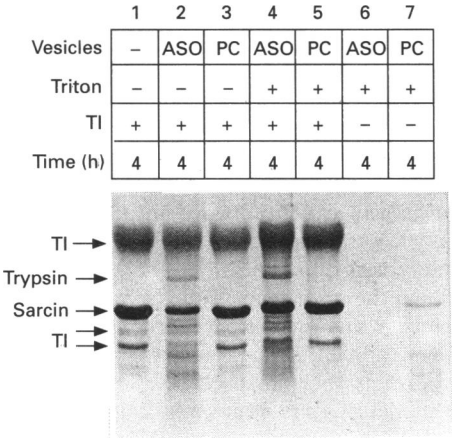

Figure 3 SDS/PAGE analysis of α-sarcin degradation by encapsulated trypsin

Trypsin-containing egg PC (lanes 3, 5 and 7) and asolectin (ASO) vesicles (lanes 2, 4 and 6) were employed at a 3.2 mM phospholipid concentration. α-Sarcin (1.2 nmol; lipid/protein molar ratio of 260:1) was added externally in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl. TI (lanes 1–5) (13.6 μM) and/or Triton X-100 (lanes 4–7) (1%) was added. Samples were electrophoretically analysed after 4 h of incubation.

Degradation of encapsulated tRNA

These experiments were performed in order to analyse the potential hydrolysis of encapsulated tRNA by externally added α-sarcin. Degradation of tRNA would produce $HClO_4$-soluble oligonucleotides (see the Materials and methods section). Previous experiments, in the absence of asolectin vesicles, demonstrated a clear correlation between absorbance at 260 nm of the acid-soluble products and degradation of free tRNA produced by α-sarcin (results not shown). When α-sarcin was added to the tRNA-containing vesicles, the tRNA was degraded (Figure 2). The amount of tRNA degraded was proportional to the ex-

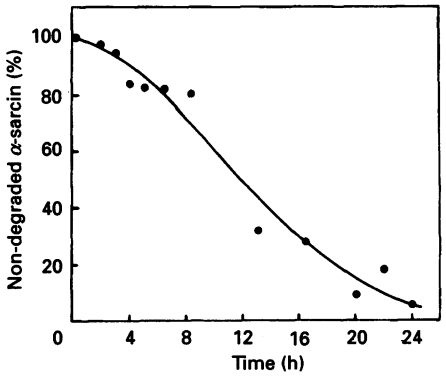

Figure 4 Time course of the degradation of α-sarcin by encapsulated trypsin

Trypsin-containing vesicles were incubated with α-sarcin (2.9 μM) in 0.2 ml of 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl, under the conditions described in the Materials and methods section. The percentage of non-degraded protein, evaluated by densitometric scanning of the corresponding PAGE gels, is given versus digestion time. The values are the means from four experiments.

ternally added α-sarcin concentration up to about a 40:1 lipid/protein molar ratio (Figure 2). Therefore it appears that some regions of α-sarcin (at least the one corresponding to the active site) gain access to the interior of the liposomes. Controls indicated that in the corresponding supernatants: (i) no free tRNA was detected after exhaustive washing of the liposomes employed; and (ii) no free tRNA was detected after exhaustive washing of α-sarcin/tRNA-containing vesicle complexes. However, we cannot exclude a potential tRNA leakage upon interaction of α-sarcin with the vesicles; if this occurs the liberated tRNA must be degraded by α-sarcin, since no free tRNA was observed.

Digestion of α-sarcin by encapsulated trypsin

When α-sarcin was incubated with trypsin-containing asolectin liposomes in the presence of a large excess of external TI, α-sarcin was digested (Figure 3). A substantial digestion of α-sarcin had occurred after 4 h of incubation (Figure 3, lane 2). PAGE analysis of the polypeptide component of the assay mixture (see the Materials and methods section) and further scanning of the gels confirmed this. This degradation was due to the encapsulated trypsin, since all potential traces of external trypsin would have been inactivated by the inhibitor. This fact has been confirmed by control experiments: (i) a 2:1 (w/w) inhibitor/trypsin ratio was enough to inactivate the total protease present in these assays, and a 15–20:1 (w/w) inhibitor/trypsin ratio was employed; (ii) incubation of the supernatants obtained from washing the trypsin-containing liposomes with α-sarcin did not result in protein degradation; and (iii) no α-sarcin digestion was observed upon addition of Triton X-100 to the assay mixture (Figure 3, lane 4). When the vesicles were disrupted with Triton X-100 in the absence of TI, complete degradation of α-sarcin was observed (Figure 3, lane 6). Experiments with trypsin-containing PC vesicles, which do not interact with α-sarcin [12,13], showed no degradation of α-sarcin (Figure 3, lane 3, and controls in lanes 5 and 7). Egg PC vesicles have smaller trapped volumes than asolectin vesicles [27]; this would explain why the amount of encapsulated trypsin was not enough to completely digest α-sarcin in lysed vesicles in the absence of TI (Figure 3, lane 7). In this regard, we have also reported that α-sarcin in solution, in the

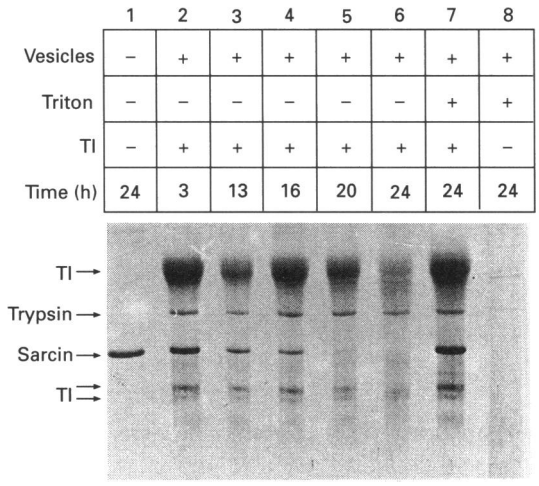

Figure 5 Electrophoretic analysis of the time course for the degradation of α-sarcin by encapsulated trypsin

α-Sarcin (0.6 nmol) was added to trypsin-containing soybean asolectin vesicles (260:1 lipid/protein molar ratio; 0.2 ml total volume). TI (13.6 μM) was added (except in lanes 1 and 8). Triton X-100 at a 1% final concentration was employed for the experiments in lanes 7 and 8. Digestion times (3, 13, 16, 20 or 24 h) are indicated for each sample.

absence of lipids, requires an approx. 24 h incubation with trypsin (50:1, w/w) for complete hydrolysis [28]. This is in spite of its high lysine-plus-arginine content (15% of the total amino acid residues), which suggest that it is potentially a good substrate for trypsin. On the other hand, in the presence of lipids, α-sarcin is not completely degraded by trypsin [28].

A time course of α-sarcin digestion by encapsulated trypsin in asolectin liposomes is given in Figure 4. Most of the protein was digested within 24 h. A summary of the results from SDS/PAGE analysis of a typical experiment is shown in Figure 5. The α-sarcin digestion is clearly seen as a decrease in the intensity of the α-sarcin band. These results indicate that α-sarcin can penetrate the vesicle membrane and become exposed to the internal medium. Finally, equivalent results were obtained with different asolectin and α-sarcin batches.

DISCUSSION

In previous papers we have demonstrated that the anti-tumour protein α-sarcin interacts with acid phospholipid vesicles at neutral pH [13,14]. The interaction produces conformational changes in the protein molecule [16] that were explained in terms of electrostatic and hydrophobic interactions. These were suggested to be involved in the passage of the protein across membrane cells. In general, cytotoxins are thought to enter mammalian cells by one of two mechanisms: endocytosis, with subsequent entry into the cytoplasm from an intracellular vesicle, or direct penetration through the plasma membrane. Both modes of entry require that the toxin eventually crosses a membrane barrier [29]. The experiments herein reported were designed to study the ability of α-sarcin to translocate across a lipid bilayer in a model system. Hydrophobic photolabelling can be considered to be an assay of membrane penetration [30,31]. The results obtained for α-sarcin in asolectin vesicles provide strong evidence of its insertion into the bilayer. The data presented indicate a deep penetration into the hydrophobic environment of the lipid acyl chain. In addition, the ability of the protein to pass through the lipid barrier of the vesicles has been shown in two sets of studies. In the first set, translocation was analysed by measuring the ribonuclease activity of α-sarcin as a marker. Trapped tRNA would be accessible to and degraded by externally added α-sarcin only if the protein enters into liposomes, and this has been clearly observed. However, uncertainty arises from the possibility of tRNA leakage as a consequence of the α-sarcin–asolectin vesicle interaction. This would also lead to tRNA degradation, and this possibility cannot be ruled out under our experimental conditions. In a second set of experiments, trypsin activity was the selected marker. α-Sarcin, initially added outside the vesicles, would become exposed to trapped trypsin if passage of the protein across the lipid bilayer occurs. The observed degradation of externally added α-sarcin by encapsulated trypsin thus also indicates that translocation of the protein across the lipid bilayer occurs. In this regard, previous results have demonstrated that α-sarcin is protected from externally added trypsin on α-sarcin–vesicle interaction [28], which supports the translocation of α-sarcin.

Similar principles to those herein considered have been also used to study the translocation across membranes of the enzymic moiety of diphtheria toxin, fragment A [32], and of apocytochrome *c* [33–35].

The ability of α-sarcin to gain access to the inside of the vesicle is a lipid-specific process. In fact, α-sarcin is not digested when added to trypsin-containing PC vesicles, nor is it labelled in photoreactive PC vesicles [15], in agreement with the absence of an interaction with this phospholipid [13]. A lipid-dependence for the translocation of apocytochrome *c* across model membranes has also been reported [36], and different toxins appear to have different requirements for optimal interaction with lipid membranes [37,38].

The mechanism of cell intoxication by protein toxins with intracellular targets can be divided into three steps: (a) cell binding, (b) membrane penetration and in some cases translocation, and (c) target modification. The second step is the least understood and the most remarkable from the point of view of protein chemistry and membrane topology [39]. The data presented here reveal that α-sarcin is able to pass a lipid barrier and that this process shows lipid specificity. The translocation of α-sarcin into model bilayers could mimic its penetration in cell membranes *in vivo*.

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