Deletion of the NH$_2$-terminal β-Hairpin of the Ribotoxin α-Sarcin Produces a Nontoxic but Active Ribonuclease*

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Ribotoxins are a family of highly specific fungal ribonucleases that inactivate the ribosomes by hydrolysis of a single phosphodiester bond of the 28 S rRNA. α-Sarcin, the best characterized member of this family, is a potent cytotoxin that promotes apoptosis of human tumor cells after internalization via endocytosis. This latter ability is related to its interaction with phospholipid bilayers. These proteins share a common structural core with nontoxic ribonucleases of the RNase T1 family. However, significant structural differences between these two groups of proteins are related to the presence of a long amino-terminal β-hairpin in ribotoxins and to the different length of their unstructured loops. The amino-terminal deletion mutant ∆(7–22) of α-sarcin has been produced in Escherichia coli and purified to homogeneity. It retains the same conformation as the wild-type protein as ascertained by complete spectroscopic characterization based on circular dichroism, fluorescence, and NMR techniques. This mutant exhibits ribonuclease activity against naked rRNA and synthetic substrates but lacks the specific ability of the wild-type protein to degrade rRNA in intact ribosomes. The results indicate that α-sarcin interacts with the ribosome at two regions, i.e. the well known sarcin-ricin loop of the rRNA and a different region recognized by the β-hairpin of the protein. In addition, this latter protein portion is involved in interaction with cell membranes. The mutant displays decreased interaction with lipid vesicles and shows behavior compatible with the absence of one vesicle-interacting region. In agreement with this conclusion, the deletion mutant exhibits a very low cytotoxicity on human rhabdomyosarcoma cells.

Fungal extracellular ribonucleases are a diverse group of proteins, with RNase T1 being its best known representative.

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RNase U2 displays a low specificity beyond a strong preference for 3′-linked purine nucleotide phosphodiester bonds (A > G > C > U) (29, 30). Both proteins, α-sarcin and RNase U2, are cyclizing RNases because they produce a 2′,3′-cyclic intermediate as a result of the cleavage reaction (26, 30). However, the catalytic efficiency of RNase U2 against naked RNA, homopolynucleotides, or dinucleotides is several orders of magnitude higher (26, 30, 31). Thus, α-sarcin specifically cleaves a phosphodiester bond in ribosomes, whereas RNase U2 causes extensive digestion of the RNA and is a more efficient ribonucleolytic enzyme.

It is therefore of interest to find out which portions of α-sarcin, absent in related fungal RNases, account for its ribonuclease specificity and cytotoxicity. Comparison of the three-dimensional structures of RNase U2 and α-sarcin (17, 24) revealed that the greatest differences are present in both the unstructured loops and the amino-terminal region (see Fig. 1). In α-sarcin there is a NH₂-terminal β-hairpin (residues 1–26) that forms a solvent exposed proteruberance and shows a complex topology that can be considered as two consecutive minor sheets (Asp 9

shorter/Asn 12

and Lys 8–Thr 20

connected by a type I β-turn (Pro 13–Asn 16). This structural component is absent in RNase U2 (see Fig. 1), although residues 7–14 constitute a shorter β-hairpin structure. The K11L mutant of α-sarcin shows both decreased ability to interact with lipid bilayers and reduced cytotoxicity (32). This led to the proposal that the absence of the second minor β-sheet at the NH₂-terminal of RNases U2 or T1 could explain why they are not cytotoxic (32).

We have prepared, isolated, and characterized α-sarcin Δ(7–22), a deletion mutant in which residues 7–22 were replaced by two Gly residues. Thus, the hinge region, the second minor β-sheet, and the turn in the amino-terminal hairpin of α-sarcin were replaced by the Gly–Gly turn connecting the first minor β-sheet present in RNase U2 (see Fig. 1).

EXPERIMENTAL PROCEDURES

DNA Manipulations—All of the materials and reagents were molecular biology grade. Cloning procedures and bacteria manipulations were carried out according to standard methods (33), as described previously (27, 34). Site-directed mutagenesis was used to obtain the deletion mutant as previously described (27, 34–36). The mutagenic primer used was 5′-GTGACCTGAGATGGGCTCCTCACTACA-ACCAAG-3′. The two codons that substitute the α-sarcin sequence stretch from Leu 1 to Arg 22 by Gly–Gly are underlined (Fig. 1). The Escherichia coli strains used were BW313 (ΔF KA16 pol45 ΔLys A E1—62 dut1 ung1 thi1 relA1) used to obtain the uridine-rich single-stranded DNA, DH5αF′ (′(F′) endA1 hisD17 (r c m a) supE44 thi1 recA1 gyrA (Nal 16) relA1 lacZΔ (argF) U169 deoR (800 dmol ΔlacZ M15)) for the expression constructs, and BL21(DE3) F ompT (lon) hsd (r c m a) for protein production. The thioredoxin producing plasmid (pT-Trx) (37) was a generous gift of Dr. S. Ishii (Riken Tsukuba Life Science Center).

Protein Production and Purification—BL21(DE3) cotransformed with pT-Trx and the corresponding α-sarcin mutant plasmid were used to produce and purify the mutant as described for the wild-type protein (34, 38). Fungal wild-type α-sarcin was produced and purified according to methods previously reported (10, 34). Recombinant RNase U2 was purified from the extracellular medium of Pichia pastoris cultures as described (31). This protein retains the enzymatic and spectroscopic properties of the fungal natural RNase U2 (31). Polycrylamide gel electrophoresis of proteins, protein hydrolysis, and amino acid analysis were also performed according to standard procedures (34).

Spectroscopic Characterization—Absorbance measurements were carried out at room temperature in 1-cm optical path cells on a UVikon spectrophotometer (Kontron Instruments, Milan, Italy) at 100 nm/ min scanning speed. Extinction coefficients ε(0.1%, 1 cm, 280 nm) were calculated from the absorbance spectra of the proteins and amino acid analyses to determine concentration. Circular dichroism spectra were obtained on a Jasco 715 spectropolarimeter (Easton, MD) at 0.2 nm/s scanning speed; 0.1- and 1.0-cm optical path cells were used in the far and near UV, respectively. Mean residue weight ellipticities were expressed in units of degrees × cm² × dmol⁻¹. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 220 nm in the range of 25–85 °C; the temperature was continuously changed at a rate of 0.5 °C/min. Tm values (temperature at the midpoint of the thermal transition) were calculated assuming a two-state unfolding mechanism. Fluorescence emission spectra were obtained on a SLM Aminco 8000 spectrophurometer (Urbana, IL) at 25 °C in 0.2-cm path cells. All of these determinations were made as described previously (27).

NMR Experiments—Mutant Δ(7–22) was dissolved at 15 nm concentration in 0.5 ml of H2O:D2O (9:1 v/v) at pH 6.0. The data were collected at 35 °C, using sodium 3-trimethylsilyl(2,2,3,3,3–2H) propionate as internal reference. NMR experiments were performed on a Bruker Avance 800 MHz spectrometer (Karlsruhe, Germany) equipped with a triple resonance probe and three axis pulsed field gradients. 1H homonuclear total correlation spectra (39) with a mixing time of 60 ms and nuclear Overhauser effect spectra (40) with a mixing time of 50 ms were recorded by standard methods with water suppression achieved by including the WATERTAGE module (41) in the original pulse sequences. The size of the acquisition data matrix was 2048 × 512 words in f2 and f3, respectively. Before Fourier transformation, the two-dimensional data matrix was multiplied by a phase-shifted sine bell or square sine bell window function in both dimensions. The corresponding shift was optimized in every experiment. Base-line correction was applied in both dimensions. All of the spectra were processed and analyzed using the Bruker software package XWINNMR and ANSIG (42) on an IRIS Indigo work station (Silicon Graphics, Mountain View, CA). 1H NMR resonances were assigned using standard sequential assignment procedures (43). Spin systems were identified by analysis and comparison of the total correlation spectroscopy spectra with those of the wild-type protein (44). The through-space connectivities were then determined using the nuclear Overhauser effect spectra.

Ribonucleolytic Activity—The specific ribonucleolytic activity of α-sarcin was followed by detecting the release of the 400-nucleotide α-fragment (5, 6) from a cell-free reticulocyte lysate (Promega, Madison, WI) (34, 35), which was visualized by ethidium bromide staining after electrophoresis on 2.4% (w/v) agarose. The activity of α-sarcin was also analyzed on naked RNA extracted from E. coli with acidic phenol–guanidinium thiocyanate–chloroform (33). The culture was homoge-
nized by sonication in a water bath after addition of the above denaturant solution. The integrity of the purified RNA was verified by electrophoresis, which confirmed the predominance of the 23 and 16 S rRNA species. The activity assay was performed with this RNA preparation under conditions identical to those described for the reticulocyte lysate. The reaction was stopped by addition of SDS to a final concentration of 1% (w/v), and the products were analyzed by electrophoresis on agarose gels. The specific cleavage of a synthetic 35-mer RNA by α-sarcin was also studied. The synthesis of this synthetic 35-mer RNA was carried out as described (7) by using synthetic and urea-PAGE purified DNA templates: (T7-promoter) 5′-TTCTAAATAGCATTCAAATAG-3′, and the AmpliScribe T7 transcription kit (Epitentre Technologies; Madison, WI). The resulting product was purified by electrophoresis on 8% (w/v) polyacrylamide gel containing 7 M urea in 45 mM Tris-borate buffer, pH 8.3, containing 1 mM EDTA (33). The assay was performed with 2 μM synthetic 35-mer RNA and a protein concentration (wild-type α-sarcin or deletion mutant) in the 6 nM to 6 μM range, after incubation for 20 min at 37 °C in 10 mM Tris-HCl buffer, pH 7.4 (7). The reaction products were detected by ethidium bromide staining after electrophoretic separation on a denaturing 19% (w/v) polyacrylamide gel. The specific activity of α-sarcin produces both 21- and 14-mer fragments.

The activity of the purified proteins against poly(A) was assayed in 15% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 0.3% methyl green of the homopolynucleotide. This zymogram method (34, 35, 45) was based on one previously described (46). After electrophoresis, the gel was incubated at 37 °C for 3 h and then stained with 0.2% (w/v) toluidine blue. The proteins exhibiting ribonuclease activity appear as colorless bands, because of degradation of the polynucleotide, after appropriate destaining. This assay, which was performed at two different pH values (4.5 and 7.0), is useful to detect the presence of other RNA degrading activities in the protein samples. Zymograms of these bands (based on integrating all of the pixel intensities composing the spot) were obtained with the photo documentation system UVI-Tec (Cambridge, UK) and the software facility UVisoft UVI band Windows Application V97.04. These data were used to quantify the activity.

The activity of the proteins against dinucleotides (ApA/G and ApG) was measured at pH 5.0 as described elsewhere (26) by analysis of the reaction products (ApA/G, adenosine or guanosine, 3′-AMP, and 2′,3′-cAMP) resolved by HPLC (26). RNase U2 was assayed at lower protein substrate ratio and shorter incubation time than α-sarcin because of its considerably higher enzyme activity. All of the assays were performed with controls to test potential nonspecific degradation of the substrates, which does not occur under the conditions used.

**Protein-Lipid Interaction**—All of the phospholipids used were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The vesicles were prepared by hydrating a dry lipid film with Tris buffer (15 mM Tris, pH 7.0, containing 0.1 mM NaCl and 1 mM EDTA) for 60 min at 37 °C. The lipid suspension was subjected to five cycles of extrusion through two stacked 0.1-μm (pore diameter) polycarbonate membranes (14). The average diameter of the vesicle population was 100 nm (85% of the vesicles in the range 75–125 nm), as determined by electron microscopy studies (14). Phospholipid concentration was determined as described (47).

Aggregation of phospholipid vesicles, monitored as described before (12), was measured by the increase in absorbance at 360 nm of a suspension of phosphatidylglycerol vesicles in Tris buffer (final lipid concentration, 30 μM) after addition of a small aliquot of a freshly prepared protein solution. Intermixing of membrane lipids was analyzed by fluorescence energy transfer assays as described (13, 14). A decrease in the donor-to-acceptor fluorescence energy transfer indicates lipid mixing between membranes. Leakage of vesicle aqueous contents was measured by using the 8-aminonaphthalene-1,3,6-trisulfonic acid/lipid mixing between membranes. Leakage of vesicle aqueous contents decreases in the donor-to-acceptor fluorescence energy transfer indicates decrease in the donor-to-acceptor fluorescence energy transfer as described (13, 14). A decrease in the donor-to-acceptor fluorescence energy transfer indicates lipid mixing between membranes. Leakage of vesicle aqueous contents decreases in the donor-to-acceptor fluorescence energy transfer as described (13, 14). A decrease in the donor-to-acceptor fluorescence energy transfer indicates lipid mixing between membranes.

**Cytotoxicity Assay**—This assay was performed essentially as described (8, 9) by using human rhabdomyosarcoma cells. Protein synthesis was analyzed by measuring the incorporation of [1-4,5-3H]leucine (160 Ci/mmol). The radioactivity was measured on a Beckman LS 3801 liquid scintillation counter (Palo Alto, CA). The results are expressed as percentages of radioactivity incorporation in control samples. A plot of the percentage values versus toxic protein concentration in the cytoplasm toxicity assay allows the calculation of the IC50 values (protein concentration required for 50% protein synthesis inhibition). The reported values correspond to the averages of triplicate experiments.

## RESULTS

**Spectroscopic and Structural Characterization**—The deletion mutant (Δ(7–22)) of α-sarcin was purified to homogeneity as determined by its behavior on SDS-PAGE. A single immunoreactive band was found also after staining with anti-α-sarcin polyclonal antibodies in a Western-blot analysis. The amino acid composition of the purified protein was in agreement with the mutation performed. The purified recombinant mutant protein was obtained in good yield (∼13 mg of protein/liter of induced bacterial culture), which is more than twice that described for wild-type α-sarcin in the same expression system (38). The experimentally determined E0.1% 280 nm, 1 cm) value for the mutant was 1.38, which is within 5% of the value predicted from its amino acid composition.

The circulatory dichroism spectra of the mutant showed slight differences in comparison with that of WT α-sarcin (Fig. 2). 

![Image](http://www.jbc.org/)

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; SRL, sarcin-ricin loop; WT, wild-type.
CD spectrum of α-sarcin is dominated by the contribution of Trp4 (a positive CD maximum of about 100 degrees × cm² × dmol⁻¹ at 293 nm), whereas the contribution of Trp5 is about 15 degrees × cm² × dmol⁻¹ of residue in the range 285–293 nm (51). The calculated difference spectrum (wild type minus mutant; Fig. 2B) was in the ellipticity range of this latter contribution and showed the same spectral features.

The fluorescence emission of the Δ(7–22) mutant displayed characteristics similar to those of the WT protein (Fig. 2, C and D). Nevertheless, the tryptophan emission is 1.2-fold increased in the deletion mutant, whereas the tyrosine contribution is 0.2-fold decreased in comparison with the corresponding quantum yields of the WT protein. The variation in the tyrosine contribution is readily explained by the removal of Tyr25 (wild-type numbering) in the deletion mutant. The fluorescence emission of α-sarcin is dominated by the contribution of Trp5, because the other tryptophan residue of the molecule, Trp24, is strongly quenched in the mutant protein (51). Thus, the increased Trp quantum yield in the Δ(7–22) mutant could be attributed to local changes in the Trp4 microenvironment upon deletion, which would be in agreement with the variation observed in the near UV CD spectrum.

Standard two-dimensional NMR methodology was used to assign the proton spectra. The process was greatly facilitated by comparison with the previous assignment of native α-sarcin (44). At least one sequential nuclear Overhauser effect could be unambiguously assigned for all residues, including those corresponding to the mutated region Cys8-Gly7-Gly22-Leu23 (wild-type numbering). With the exception of rapidly exchanging protons, ¹H assignments were almost complete. A comparison of the backbone amide protons and Hα chemical shifts of WT α-sarcin and Δ(7–22) mutant is shown in Fig. 3. Most of the chemical shift variations were located in very restricted regions of the protein sequence, all spatially close to the deleted region. For the Hα, a large majority of chemical shifts of Δ(7–22) mutant were within 0.05 ppm of their values in WT α-sarcin. The largest variations were located at the NH₂-terminal hairpin (Trp4, Thr5, Gly7, Gly22, and Leu24) and at loop 5 (residues Lys139–Leu145). The amide proton chemical shifts differences were also within 0.05 ppm for the large majority of the residues. Significant differences were observed for the amide protons of Trp4, Cys8, Gly7, Leu23, His237, Glu143, Gly143, Glu144, and Ser149. All of these differences refer to residues surrounding the deleted portion. The observed shifts may have their origin either in conformational rearrangements of the protein groups or in field effects arising from the different distribution of anisotropic groups in the Δ(7–22) protein. In contrast, most of the protein proton resonances were virtually unaffected upon mutation. This finding strongly indicated that the substitution of the native sequence Leu2–Arg23 by Gly-Gly did not induce global conformational changes in α-sarcin.

Ribonucleolytic Activity—The Δ(7–22) mutant variant of α-sarcin did not show the high ribonuclease activity of the WT protein on ribosomes (Fig. 5A). The α-fragment characteristic of the α-sarcin activity was not detected when the mutant was assayed, although a nonspecific degradation of rRNA was observed when large amounts of the mutant variant (200 ng) were tested. However, both proteins hydrolyzed naked bacterial RNA specifically to produce the α-fragment, and nonspecific degradation of this substrate occurred when large amounts of both proteins were tested, with the Δ(7–22) mutant exhibiting higher activity than the WT protein (Fig. 5B). The deletion mutant, as well as WT α-sarcin, also degraded a synthetic 35-mer oligoribonucleotide that mimics the sarcin-ricin loop of 28 S rRNA producing the 21- and 14-mer fragments (Fig. 5C). This information has been used to study substrate specificity of ribotoxins (7). The Δ(7–22) mutant also degraded the polymeric nonspecific substrate poly(A). Its activity on this substrate was higher than that of the WT protein (6- and 6-fold higher at pH values of 4.5 and 7.0, respectively; Fig. 5, C and D). Dinucleotides are also low specificity substrates of α-sarcin (26, 32) with an optimum pH of 5.0 (27, 28). Therefore, the activity against the dinucleotide ApA was also assayed. WT α-sarcin and the Δ(7–22) mutant variant displayed very similar catalytic efficiencies (expressed as the $k_{cat}/K_m$ ratio), although the $k_{cat}$ and $K_m$ values were 1 order of magnitude higher for the mutant (Table 1). However, a dramatic difference was observed when the nature of the products was analyzed. As shown in Fig.
Fig. 6. Ribonuclease activity assay of WT α-sarcin and its Δ(7-22) mutant on dinucleotides. HPLC separation profiles, obtained by recording absorbance at 254 nm (λ254), of the reaction products resulting from the cleavage of ApA (three left panels) and ApG (three right panels) after incubation with WT α-sarcin, the Δ(7-22) mutant, and RNase U2 at pH 5.0. The substrate concentrations were 40 and 50 μM for ApA and ApG, respectively. An enzyme concentration of 2 μM and an incubation time of 14.5 h at room temperature were employed for the wild-type protein and the Δ(7-22) mutant. The reaction for RNase U2, a much less specific enzyme, was incubated for only 5 min, and the enzyme concentration was 145 μM.

**Interaction with Phospholipid Vesicles**—It is well documented that α-sarcin interacts with lipid vesicles through electrostatic and hydrophobic interactions (12–16). In particular, this ribotoxin promotes aggregation of vesicles, intermixing of phospholipids from different vesicles (lipid mixing), and leakage of their aqueous contents. These effects were also promoted by the Δ(7-22) mutant. However, there were clear differences between both proteins in terms of the kinetics and completion of these processes (Fig. 7 and Table II). Thus, the extent of vesicle aggregation was lower in the case of the mutant variant and the kinetic traces displayed a biphasic behavior (Fig. 7, A and B). Also, the observed initial rates of the aggregation were lower for the mutant than for WT α-sarcin (Table II). The initial rates of the lipid mixing induced by the mutant variant were lower than those of the WT protein (Fig. 7C) and Table II), and the kinetics of the process promoted by the mutant variant

| Protein          | $K_m$ μM | $k_{cat}$ μM s$^{-1}$ | $k_{cat}/K_m$ s$^{-1}$ |
|------------------|----------|-----------------------|------------------------|
| Wild type$^a$    | 40 ± 4   | (8.6 ± 1.1) x 10$^{-9}$| 2.6 ± 0.1              |
| Δ(7-22)          | 500 ± 55 | (10.5 ± 1.2) x 10$^{-1}$| 3.2 ± 0.1              |
| RNase U2$^b$     | 200 ± 20 | (5.8 ± 0.1) x 10$^{-1}$| 2.9 (0.4) x 10$^{-4}$  |

$^a$ Kinetic parameters (± S.D.) determined from the transesterification of ApA by linear regression analysis of double reciprocal plots from three different determinations (26, 27).

$^b$ Recombinant RNase U2 (31).

6, α-sarcin behaved as the typical cyclizing RNase (26), i.e. it first catalyzes the transphosphorylation of ApA, producing adenosine and 2',3'-cAMP, and then it cleaves the cyclic intermediate to 3'-AMP (Fig. 6). Under identical conditions, only the first reaction was performed by the mutant (Fig. 6). Neither was the 3'-AMP product produced when recombinant native (31) RNase U2 was employed (Fig. 6), although in this case a much shorter incubation and a much lower enzyme concentration were used. The specificity of native RNase U2 against dinucleotides showed that ApA is one of the poorest dinucleotides substrates for this enzyme (30). To rule out the possibility that the absence of the 3'-nucleotide product was due to an inefficient reaction, the same experiment was performed using saturating concentrations (50 μM) of ApG, the best dinucleotide substrate for RNase U2 (30); qualitatively identical results were obtained for the three proteins studied (Fig. 6). Thus, cleavage of ApG by WT α-sarcin produced guanosine, 2',3'-cAMP, and 3'-AMP, whereas the latter 3'-nucleotide product did not appear when this dinucleotide was assayed against the Δ(7-22) mutant variant of α-sarcin or recombinant RNase U2 (Fig. 6).

**Fig. 5.** Ribonuclease activity assays of WT α-sarcin and its Δ(7-22) mutant. A, ribosome inactivating activity assay of WT α-sarcin and the Δ(7-22) mutant. The high specific activity of α-sarcin is shown by the release of the 400-nucleotide α-fragment (arrow) from the 28 S rRNA of eukaryotic ribosomes. Cell-free reticulocyte lysates (lane 1) were incubated in the presence of 50 ng (lane 2), and 100 ng (lane 3) of WT protein, as well as 50 ng and 100 ng of Δ(7-22) (lanes 3 and 5, respectively). The reaction mixture was analyzed by agaroose gels and stained with ethidium bromide. B, E. coli ribosomal RNA (lane 3) was incubated in the presence of 110 ng (lane 1) and 250 ng (lane 2) of WT protein, as well as 1 ng and 110 ng of Δ(7-22) (lanes 4 and 5, respectively). The reaction mixture was analyzed by agaroose gels and stained with ethidium bromide. C, 35-mer oligoribonucleotide (lane 1) was incubated in the presence of 65 ng (lane 2) and 650 ng (lane 3) of WT α-sarcin and 55 ng (lane 4) and 550 ng (lane 5) of Δ(7-22). The reaction mixture was analyzed by polyacrylamide gels and stained with ethidium bromide. D and E, Coomassie Blue-stained SDS-PAGE (D) and zymogram assay (E) of the ribonuclease activity against poly(A), at pH 4.5, of 500 ng of each wild-type α-sarcin or Δ(7-22) mutant. When the zymogram was performed at pH 7.0, qualitatively identical results were obtained.
Fig. 7. Effect of WT α-sarcin and its Δ(7–22) mutant on phosphatidylglycerol (PG) vesicles. A, aggregation of vesicles measured from the increase of absorbance at 360 nm induced by the protein on a vesicle sample (relative ΔAbs, referred to that of WT α-sarcin considered as unit) versus protein/lipid molar ratio. B, kinetic traces corresponding to samples at 0.052 protein/phosphatidylglycerol molar ratio, as an example. C, mixing of phospholipids from different bilayers measured as the decrease of resonance energy transfer (relative RET, referred to that of WT α-sarcin considered as unit) versus protein/lipid molar ratio. D, kinetic traces corresponding to samples at 0.030 protein/PG molar ratio as an example. E, leakage of intravesicular aqueous contents (relative leakage considering that produced by WT α-sarcin as unit) versus protein/lipid molar ratio. F, kinetic traces corresponding to samples at 0.032 protein/phosphatidylglycerol molar ratio as example. Trace 1, WT protein; trace 2, Δ(7–22) mutant.

displayed a lag phase (Fig. 7D). The results from the leakage measurements also revealed less leakage and a lower initial rate for the mutant (Fig. 7, E and F, and Table II).

Cytotoxic Activity—α-Sarcin has been reported to be cytotoxic for different human tumor cell lines including human rhabdomyosarcoma cells (8, 9). These cells were used to assay the cytotoxic activity of WT α-sarcin and its Δ(7–22) variant, which exhibited very different behavior (Fig. 8). The former displayed an IC50 value of 0.6 μM, whereas the corresponding value for the Δ(7–22) mutant was more than 40-fold higher.

DISCUSSION

Ribotoxins are an intriguing group of proteins in terms of structure-function relationships. Their three-dimensional pattern closely resembles that of nonspecific ribonucleases, suggesting that both groups of proteins may have descended from a common ancestor (2, 45). However, ribotoxins are highly specific ribonucleases in degrading rRNA, which makes them a useful tool in determining tertiary structures of rRNA and in studying RNA-protein interactions (53). This high specificity converts ribotoxins into potent inhibitors of translation, and immunoconjugates of ribotoxins with monoclonal antibodies directed against cancer cell targets are currently employed (54–56). Moreover, ribotoxins can be involved in allergic reactions. Thus, Asp f 1, the ribotoxin produced by Aspergillus fumigatus (the etiological agent isolated in about 80% of the Aspergillus infections in humans) is associated with IgE-mediated allergic asthma and bronchopulmonary aspergillosis and severe allergic pulmonary complications in immunocompromised patients (57). Because this is the major allergen of this microorganism (7, 58) and displays >85% sequence identity with other ribotoxins, cross-reactivity at the T and B cell level would be expected. Therefore, elucidation of structural clues responsible for the differences between nontoxic and cytotoxic RNases would be very useful for designing engineered proteins with the desired properties.

With this aim in mind, we have prepared the deletion mutant Δ(7–22) variant of α-sarcin. Its spectroscopic characterization showed that the three-dimensional structure of the variant is highly similar to that of the WT protein. The changes observed in the far UV CD spectrum can be explained by the removal of the characteristic NH2-terminal β-hairpin of α-sarcin. The near UV CD and fluorescence emission analyses indicated minor changes in the microenvironment of Trp5, which is to be expected from the absence of the proximal hairpin in the Δ(7–22) variant. NMR data also indicate the lack of any large conformational change in the protein caused by the mutation. As expected, the largest variations in chemical shifts, reflecting changes in the magnetic environment, are found in residues next to the deleted region. All amide protons and Hα changes in residues distant in the primary structure from the mutated segment can be analyzed on the basis of the three-dimensional structure of the WT protein (17). The deletion will abolish structural interactions between the second half of the NH2-terminal hairpin and loop 5 (hydrogen bond between H8 21 of Asn18 and O of His5, salt bridge between Lys11 and Glu140, and π-cation interaction between Tyr18 and Lys39) and likely account for the observed NMR changes. Moreover, the decreased stability of the mutant variant could be also related to the abolition of the above mentioned interactions involving residues of the hairpin and loop 5 (17).

The results obtained reveal that the elimination of the amino-terminal β-hairpin of α-sarcin produces an active ribonuclease but one devoid of the ability to cleave the 28 S rRNA in the ribosomes. The Δ(7–22) mutant does not produce the α-fragment in the ribosomes, but this fragment is observed when the variant acts on naked rRNA. The Δ(7–22) variant also shows the characteristic specific activity of the WT protein on the 35-mer SRL oligoribonucleotide. Therefore, the well characterized recognition of the SRL of the rRNA is not enough for both production of the α-fragment and inactivation of the ribosomes by α-sarcin. The amino-terminal β-hairpin is involved in an additional specific interaction with ribosomes required for the cytotoxic activity. In addition, elimination of the NH2-terminal β-hairpin also somehow affects the α-sarcin active center, producing an enzyme that cannot cleave the cyclic intermediate. The role of 2′,3′-cyclic phosphodiester in the RNases catalyzed cleavage of RNA has been controversial. However, now it seems clear that they are true products of the reaction and not just intermediates (59–61). Studies performed with RNase A indicated that the initial protonation state of the active site residues of this enzyme, and perhaps other RNases, is recovered by a pathway that does not involve substrate molecules (60). This is probably the case for RNase U2 too, because its molecular activity for 2′,3′-cyclic AMP is 100 times slower than for the dinucleotide ApC (30, 62), i.e. the second step of the reaction is much slower than the first one. In fact, the 3′-monophosphate derivative was not observed in the assays performed with recombinant RNase U2 against dinucleotides (Fig. 6). Thus, in this regard, it also seems that the deletion mutant of α-sarcin would behave like RNase U2. From the structural point of view, this close relationship between both proteins can be easily understood. In the environment of the catalytic center, WT α-sarcin and RNase U2 differ in the orientation of loop 5 be-
cause of the presence or absence of specific interactions with the NH2-terminal β-hairpin (17). Because the Δ(7–22) mutant lacks these interactions, it is probable that the orientation of loop 5 and consequently the electrostatic properties of the catalytic His137 resemble those of the nonspecific RNases (His137 is one of the few residues of the mutant displaying differences in the backbone HN chemical shifts). Thus, the similarity deduced from the structure of the proteins could explain the similar substrate affinity, activity, and enzymatic mechanism of the Δ(7–22) mutant and RNase U2. The mutant retains the ability of WT α-sarcin to interact with acidic model vesicles but with a decreased efficiency. The interaction of α-sarcin with bilayers is initiated by the formation of a vesicle dimer maintained by electrostatic interactions. Studies performed with single amino acid mutants at Trp51 (51), Arg121 (63), and Lys11 (32) have suggested that loop 2 and the amino-terminal β-hairpin, located at two extremes of the protein molecule, are vesicle-interacting regions. Removal of one of these regions, as in the present deletion mutant, would result in biphasic kinetics because the large vesicle aggregates (those producing more light-scattering) would result from the accumulation of a protein-vesicle complex. On the other hand, WT protein with two vesicle-interacting regions could directly bridge lipid vesicles. This would also explain the lag phase observed in the lipid mixing of different membranes, a process that occurs among bilayers of aggregated vesicles (Fig. 7).

Endocytosis is the mechanism responsible for the internalization of α-sarcin via acidic vesicles (9). Subsequently, it escapes from the endosomal compartment via Golgi structures and exerts its cytotoxic effect on the cytoplasm (9). Because the ability of α-sarcin to interact with membranes is retained in the mutant, it seems safe to conclude that the absence of the NH2-terminal β-hairpin almost abolishes its cytotoxic properties by impairing its ability to specifically cleave ribosomes.

Thus, it seems clear that this structural element is involved in the interaction with the cell membranes but primarily participates in the recognition of the ribosome.

It can be concluded from our results that the ribonucleolytic machinery of α-sarcin is preserved in the Δ(7–22) mutant and even improved in terms of catalytic efficiency. However, the characteristic specificity of the cytotoxin on ribosomes was lost. This suggests some involvement of the β-hairpin of α-sarcin in substrate recognition. In this context, the crystal structures of some restrictocin-inhibitor complexes have been recently elucidated (64) and reveal that Lys110, Lys111, and Lys113 of restrictocin (Lys111, Lys112, and Lys114 are their counterparts in α-sarcin) contact the base of G4319, the only nucleotide known to be critical for recognition of the SRL. These residues are far apart from the β-hairpin (at about 30 Å), which makes the loss of specificity in the Δ(7–22) mutant intriguing. It could be argued that potential conformational changes in the protein may occur upon binding to the substrate, bringing these three residues and the β-hairpin region in close proximity and explaining its involvement in specificity. However, such an induced fit after complex formation does not make important changes in the protein structure. The Co atoms of uncomplexed and complexed restrictocin superimpose with a root mean square deviation ranging from 0.5 to 0.7 Å for the different inhibitors studied (64). We have two potential explanations for the loss of specificity yet preservation of ribonuclease catalysis. First, it has been reported (45, 53) that the sequence ^TNKYTEK_21 of α-sarcin, which is highly conserved among all of the members of this family of ribotoxins (the most dissimilar sequence is TNKWEDK), displays significant similarity (only conservative changes are observed) to a sequence found in some elongation factors that interact with the SRL. Therefore, the β-hairpin of α-sarcin, which possesses this sequence, may be an additional interacting region with the ribosome that is required for the ribonuclease specificity even though it is distant from the three lysines contacting G4319. On the other hand, a docking model has been constructed for the three-dimensional structures of α-sarcin and a 20-mer RNA substrate analog (3) and reveals that two protein regions separated by more than 11 Å display suitable geometric and electrostatic properties to potentially interact with the oligonucleotide (17). One region corresponds to the three lysine residues mentioned above. The second one is formed by two segments, residues 51–55 and loop 5 (residues 139–143). Similar results were reported for the crystal structure of restrictocin (18). Several residues of loop 5 of α-sarcin are involved in interactions with the β-hairpin: Lys139 and Tyr148 display a π-π interaction; and Glu140 is hydrogen-bonded to Asp5. The loss of these interactions could change the orientation of loop 5 and may also alternatively explain the intriguing absence of specificity in this active ribonuclease Δ(7–22) mutant of α-sarcin. The elucidation of the three-dimensional structure of this mutant is currently in progress to evaluate structure-activity relationships at an atomic level.

In summary, the NH2-terminal β-hairpin of α-sarcin is not
Deletion of the NH$_2$-terminal β-Hairpin of the Ribotoxin α-Sarcin Produces a Nontoxic but Active Ribonuclease

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