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Role of the basic character of α-sarcin's NH_2-terminal β-hairpin in ribosome recognition and phospholipid interaction

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ABSTRACT

Ribotoxins are a family of toxic extracellular fungal RNases that first enter into the cells and then exert a highly specific ribonucleolytic activity on the larger rRNA molecule, leading to protein synthesis inhibition and cell death by apoptosis. α-Sarcin is the best characterized ribotoxin. Previous characterization of a deletion variant of this protein showed that its long NH_2-terminal β-hairpin is essential for its cytotoxicity. Docking, enzymatic, and lipid–protein interaction studies suggested that this β-hairpin establishes specific interactions with ribosomal proteins and that it is a region involved in the interaction with cell membranes. Consequently, in order to assess the influence of the basic character of this NH_2-terminal β-hairpin (there are 1 arginine and 4 lysines along its 16 residues) on the ribotoxins cytotoxic ability, five individual mutants substituting these five basic residues by glutamic acid were produced, purified to homogeneity, and characterized. Regarding ribosomal recognition, all mutants showed a diminished activity in a cell-free reticulocyte lysate, whereas the activity against an oligoribonucleotide mimicking the sarcin/ricin loop rRNA (SRL) or the homopolymer poly(A) remained unaffected, confirming that the mutated basic residues participate in electrostatic interactions with other ribosomal elements apart from this SRL. The study of the interaction with phospholipid vesicles showed that Lys 17, Arg 22, and, most importantly, Lys 14 and Lys 21, are crucial residues in the first stages of the aggregation phenomenon, where protein–vesicle and protein–protein interactions are required. The data obtained reveal that electrostatic interactions involving basic residues of the β-hairpin are required not only for establishing specific interactions with ribosomal regions other than the SRL but also to explain the ability of the protein to interact with acid phospholipid bilayers.

Introduction

α-Sarcin is the most representative member of ribotoxins, a family of fungal natural killers characterized by their exquisite ribonucleolytic specificity against ribosomes and their ability to cross cellular membranes in the absence of any known protein receptor [1,2]. These toxic proteins cleave just a single phosphodiester bond of the large rRNA fragment, located at an evolutionarily conserved loop with important roles in ribosome function [3–5]. This cleavage inhibits protein biosynthesis, leading to cell death by apoptosis [6]. This important region has become to be known as the sarcin/ricin loop (SRL)[1] because it is not only the target of α-sarcin and the rest of ribotoxins but also of the much larger group of plant ribosome-inactivating proteins (RIP), best represented by ricin [7,8].

Most ribotoxins show a high degree of sequence identity [9–12] that is also manifested in the three-dimensional structure of the two only ribotoxins studied at this level, restrictocin [13,14] and α-sarcin [15–18]. Both proteins fold into an α+β structure with a central five-stranded antiparallel β-sheet and an α-helix of almost three turns and display long and unstructured loops (Fig. 1) [15,19,20]. Residues 1–26 form a long NH_2-terminal β-hairpin that can be considered as two consecutive minor β-hairpins connected by a hinge region with its most distal part jutting out as a solvent exposed protuberance (Fig. 1). This β-hairpin is one of the regions showing the highest sequence variability among ribotoxins [9–12,21]. An α-sarcin mutant involving the deletion of this protuberance, α-sarcin Δ(7–22), retained the same conformation as the wild-type protein, as ascertained from its three-dimensional structure in solution [22]. However, functional and enzymatic studies revealed that this mutant exhibited ribonuclease activity against naked rRNA and synthetic substrates but lacked the ability to specifically cleave the SRL in intact ribosomes [23]. These results were explained by *in silico* studies that predicted how this NH_2-terminal β-hairpin could

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[1] *Abbreviations used:* SRL, sarcin/ricin loop; RIP, ribosome-inactivating proteins; CD, circular dichroism; DMPG, dimyristoylphosphatidylglycerol.

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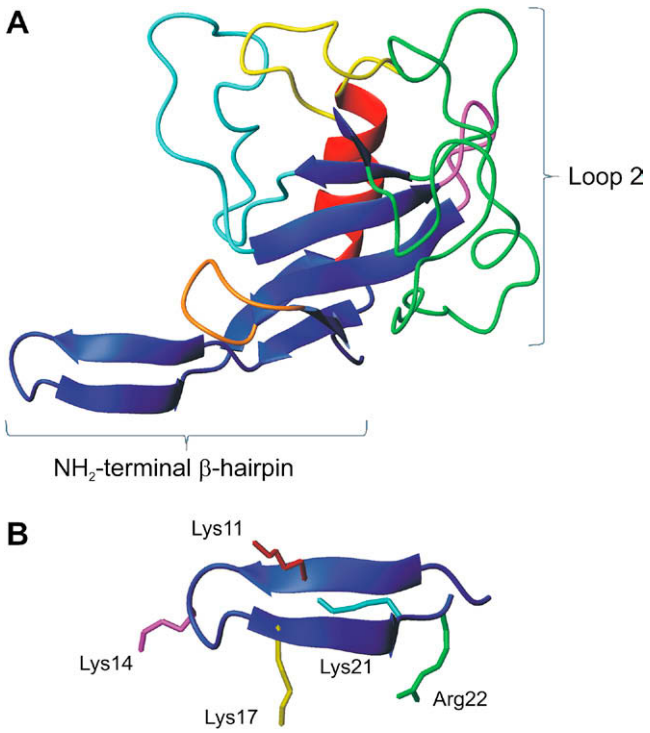

Fig. 1. Diagrams showing the three dimensional structure of α-sarcin (A) and a detail of its NH_2-terminal β-hairpin (B). The different basic residues mutated to Glu are shown.

establish essential interactions, mostly of electrostatic nature, with specific ribosomal proteins in order to direct the ribotoxin to the SRL region of the ribosome [18].

In addition to their specific and lethal ribonucleolytic activity, ribotoxins can also cross phospholipid membranes due to their ability to interact with acid phospholipid-containing bilayers [6,24–28]. This is the basis to explain why they are especially active on transformed or virus-infected cells [6,29,30], although any ribosome could be potentially inactivated by them, given the universal conservativeness of the SRL. According to the current model accepted to explain the ability of α-sarcin to interact with phospholipid bilayers, the protein would be initially adsorbed to the charged polar head groups of the phospholipids, and then would partially penetrate the interface of the bilayer to interact with a portion of the lipid hydrocarbon chains [1,6,26,31,32]. This intercalation within the lipid matrix would promote fusion and permeability changes in the bilayers, processes that would presumably be involved in the passage of the protein across the membrane of its target cells. Two regions of the protein, located at opposite ends of the protein molecule, have been proposed to be specifically involved in vesicle aggregation [33]. These regions would be loop 2 and, again, the NH_2-terminal β-hairpin (Fig. 1), which consequently would also participate in the interaction with cell membranes. In good accordance with this proposal, the α-sarcin Δ(7–22) mutant also displayed a diminished ability to interact with phospholipid lipid vesicles showing a behavior compatible with the absence of one vesicle-interacting region [23]. In agreement with all these conclusions, the deletion mutant exhibited a very low cytotoxicity on human rhabdomyosarcoma cells [23].

α-Sarcin is a highly charged protein, with a high isoelectric point [34,35]. This high content of positively charged residues, mostly located at the loops and the NH_2-terminal β-hairpin, is probably required for recognizing and binding not only to its highly negatively charged target, the SRL rRNA, but also to ribosomal proteins and cellular membranes [18,36,37]. In this context, the role of the positively charged residues located at the NH_2-terminal β-hairpin (Table 1) in these different but closely related events has been studied in the work herein presented.

Table 1
Mutagenic primers used to construct individual mutant versions of wild-type α-sarcin where the NH_2-terminal β-hairpin basic residues had been substituted by Asp. The bases that change original codon to Asp are underlined.

α-Sarcin mutant	Oligonucleotide sequence
K11E	5′ ttg aac gac cag gag aac ccc aag acc 3′
K14E	5′ cag aag aac ccc gag acc aac aag tat 3′
K17E	5′ ccc aag acc aac gag tat gag acc aaa 3′
K21E	5′ aag tat gag acc gaa cgc ctc ctc tac 3′
R22E	5′ tat gag acc aaa gag ctc ctc tac aac 3′

Materials and methods

DNA manipulations

All materials and reagents were of molecular biology grade. Cloning procedures, oligonucleotide site-directed mutagenesis, and bacterial manipulations were carried out as previously described [28,38,39]. The mutagenic primers used to substitute the mutated residues are shown in Table 1. Presence of only the mutation expected in each case was confirmed by sequencing the complete cloned cDNA. The plasmid used as the template for mutagenesis experiments, containing the cDNA sequence coding for wild-type α-sarcin, has already been described [38,40].

Protein production and purification

Escherichia coli BL21 (DE3) cells cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding α-sarcin mutant plasmids were used to produce the different proteins studied, as previously described [38,40,41]. Fungal wild-type α-sarcin was obtained as previously reported [28]. Protein purification included ion exchange and molecular exclusion chromatographies [28]. PAGE of proteins, Western blot immunodetection, protein hydrolysis, and amino acid analysis were performed according to standard procedures [38,41].

Spectroscopic characterization

Absorbance measurements were performed on an Beckman DU640 spectrophotometer at room temperature in cells with a 1 cm optical path length at a scanning speed of 240 nm/min. Circular dichroism (CD) spectra were obtained on a Jasco 715 spectropolarimeter, equipped with a thermostated cell holder and a NesLab-111 circulating water bath, at 0.2 nm/s. The instrument was calibrated with (+)-10-camphorsulfonic acid. CD spectra were recorded in cylindrical cells with an optical path length of 0.1 and 1.0 cm. Mean residue weight ellipticity is expressed in units of deg cm^2 $dmol^{-1}$. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 220 nm in the range 25–80 °C; the temperature was continuously changed at a rate of 0.5 °C/min. Fluorescence emission spectra were recorded on an SLM Aminco 8000 spectrofluorimeter at 25 °C using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission beams, respectively, were used. The temperature was controlled using a circulating water bath. All these experiments were made with the proteins