The Action Mode of the Ribosome-Inactivating Protein α-Sarcin

Luen Hwu a, Kuan-Chun Huang b, Dow-Tien Chen b, Alan Lin b
Institutes of aMicrobiology and Immunology and bGenetics, National Yang-Ming University, Taipei, Taiwan, ROC

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Abstract
Based on the tertiary structure of the ribosome-inactivating protein α-sarcin, domains that are responsible for hydrolyzing ribosomes and naked RNA have been dissected. In this study, we found that the head-to-tail interaction between the first amino β-strand and the last carboxyl β-strand is not involved in catalyzing the hydrolysis of ribosomes or ribonucleic acids. Instead, a four-strand pleated β-sheet is indispensable for catalyzing both substrates, suggesting that α-sarcin and ribonuclease T1 (RNase T1) share a similar catalytic center. The integrity of an amino β-hairpin and that of the loop L3 in α-sarcin are crucial for recognizing and hydrolyzing ribosomes in vitro and in vivo. However, a mutant protein without the β-hairpin structure, or with a disrupted loop L3, is still capable of digesting ribonucleic acids. The functional involvement of the β-hairpin and the loop L3 in the sarcin stem/loop RNA of ribosomes is demonstrated by a docking model, suggesting that the two structures are in essence naturally designed to distinguish ribosome-inactivating proteins from RNase T1 to inactivate ribosomes.

Introduction
The ribosome-inactivating protein α-sarcin, secreted by filamentous fungi (Aspergillus giganteus), has been known to inhibit the protein synthesis of all kinds of cells. The inhibition is the result of an endonucleolytic cleavage of an RNA domain (the sarcin domain) at 23-28S rRNA when ribosome is the substrate [1, 8, 37]. The protein is also a known ribonuclease which hydrolyzes adjacent to purine bases in single- or double-stranded ribonucleic acids [9].

The sarcin domain is a universally conserved purine-rich stem-loop structure of the large subunit rRNA [43]. The tetranucleotides GAGA, at the apex of the single-stranded loop of the sarcin domain, form the site at which the ribosome-inactivating protein acts [4, 10, 11, 32, 43, 44]. On the other hand, despite several genes of ribosome-inactivating proteins having been determined [15, 18, 24,
33, 42], the structure of the ribosome-inactivating protein that participates in catalyzing the hydrolysis of ribosomes or ribonucleic acids has not been completely revealed yet. Elucidation of the action of ribosome-inactivating protein on its substrates has been attempted using genetic manipulation, with limited success, however, because of the lethality of the protein-inactivating protein to the host cells [19, 22, 23, 45]. The structures of two ribosome-inactivating proteins, restrictocin and α-sarcin, have recently been disclosed by X-ray crystallography [46] and nuclear magnetic resonance (NMR) studies [2, 3], respectively. Based on molecular docking, a cooperative effort of several peripheral loops in recognizing the sarcin domain of ribosomal RNA has been proposed [46]. Similarly, according to the tertiary structure, residues His19, Glu96, Arg121 and His137 from the pleated β-sheet of α-sarcin are tentatively aligned with catalytic residues from ribonuclease T1 (RNase T1) [14, 35, 38, 39, 43, 46], suggesting that α-sarcin and RNase T1 are related. However, only a few active residues from α-sarcin have been experimentally identified [18, 19, 20, 23, 45]. In this study, we supplemented the biochemical data to detail the mode of action of α-sarcin through genetic cloning. The important domains that govern the catalysis of ribosome inactivation and the hydrolysis of ribonucleic acids are reported below.

Materials and Methods

Construction of the α-Sarcin Gene and its Mutants by PCR Amplification

Total genomic DNA from A. giganteus was prepared according to standard procedures described elsewhere [7]. The genomic DNA was used as the template. Cloning of the genomic α-sarcin gene (pGEM-T/FL-α plasmid) was performed using PCR amplification with two primers (N and C primers) that were derived from the published cDNA sequence of the α-sarcin gene [33]. The N primer (5'-CCATC-GAATTCAATGTTGCA-3') is located at the 3' end of the sequence of the mature α-sarcin gene, with a stop codon plus a noncoding sequence of 22 nucleotides. Both primers were designed to introduce an EcoRI site in the desired region. Carboxy end deletion mutants (ΔC10, ΔC17 and ΔC37) were prepared from the pGEM(T)/α plasmid (NdeI clone) using the same method. Additionally, four internal deletion mutants (Δ61–66, Δ86–91, Δ103–109 and Δ115–125) were created using the same PCR-mediated strategy with one slight modification. Briefly, two PCR products were generated separately at the region of deletion by two sets of primers that carried NeoI/BamH1 sites (the amino half-segment) and BamH1/NdeI sites (the carboxy half-segment). They were separately cloned into pGEM(T) plasmids to become pGEM(T)/α-N and pGEM(T)/α-C, respectively. DNA fragments that carried NeoI/BamH1 sites were purified and ligated into a α-sarcin-encoding cDNA that was digested with the same restriction enzymes (NeoI/BamHI). A new pGEM(T)/α variant plasmid that contained internally deleted α-sarcin protein genes was therefore created. The plasmid was subsequently subcloned into a cytosolic expression pET28a vector by NdeI, Sall restriction cutting.

All recombinant plasmids of pET28a were propagated in Escherichia coli strain B12.1 (DE3) cells. The cells were grown at 37 °C in the presence of kanamycin (50 μg/ml) in M9ZB broth. The expression of recombinant protein was carried out by adding IPTG (final concentration of 1 mM) and fresh M9 broth containing 30 μg/ml of kanamycin when the growth of the cells had reached an OD600 value of 0.3. After a period of growth in the presence of IPTG, the cells were harvested by centrifugation. The expression of recombinant proteins was examined from total cell lysate using Western blot with an anti-α-sarcin antibody (preparation from our laboratory). Recombinant proteins were purified from the expression of pET28a plasmids that contained different cloned genes by standard Ni2+ chromatography followed by treatment with thrombin. The purity of each recombinant protein was analyzed by SDS-containing polyacrylamide gel electrophoresis.

Ribosome Inactivation by the Recombinant Proteins

Investigation of the in vitro ribosome inactivation by the recombinant proteins was carried out by the standard procedure of rabbit reticulocyte ribosome assay [26]. The amount of protein ranged from 0.2 to 1 μg from each purified recombinant protein. The protein was incubated with 5.7 OD260 rabbit reticulocyte lysate at 37°C for 15 min in buffer containing 50 mM Tris-HCl, pH 7.6, 50 mM KCl and 20 mM EDTA. Total RNAs were then extracted from the reaction mixture by the phenol-SDS method and analyzed on a 2% agarose gel in TBE buffer. The presence of α/fragments that derived from rabbit reticulocyte ribosomes was examined and assessed for the presence of ribosome inactivation.

The in vivo action of recombinant proteins against ribosomes was also investigated using a similar procedure, except this was done from the host cell extract. The host cells that harbored plasmids were induced with IPTG for 1 h. Total RNAs were extracted from the IPTG-induced cells and analyzed on an agarose/polyacrylamide composite gel.

Detection of Ribonuclease Activity by RNA-Impregnated Polyacrylamide Gel

RNA-impregnated SDS polyacrylamide gel electrophoresis was used to examine the ability of recombinant proteins to hydrolyze naked ribonucleic acids [25]. Purified recombinant proteins from mutants were electrophoretically separated on an SDS polyacrylamide gel slab that contained 2.5 mg/ml of large fragments of ribo-