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The cytotoxin α-sarcin behaves as a cyclizing ribonuclease

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Abstract The hydrolysis of adenylyl(3′→5′)adenosine (ApA) and guanylyl(3′→5′)adenosine (GpA) dinucleotides by the cytotoxic protein α-sarcin has been studied. Quantitative analysis of the reaction has been performed through reverse-phase chromatographic (HPLC) separation of the resulting products. The hydrolysis of the 3′-5′ phosphodiester bond of these substrates yields the 2′-3′ cyclic mononucleotide; this intermediate is converted into the corresponding 3′-monophosphate derivative as the final product of the reaction. The values of the apparent Michaelis constant (K_M), k_{cat} and k_{cat}/K_M have also been calculated. The obtained results fit into a two-step mechanism for the enzymatic activity of α-sarcin and allow to consider this protein as a cyclizing RNase.

Key words: Dinucleotide; Ribonucleolytic activity; Ribosome-inactivating protein; Ribotoxin

1. Introduction

α-Sarcin is a cytotoxic protein produced by the mould *Aspergillus giganteus* MDH 18894 [1,2]. It cleaves a unique phosphodiester bond of the 28S ribosomal RNA [3], inactivating ribosomes and inhibiting protein biosynthesis [4,5]. This bond is located at the named sarcin/ricin loop, the longest conserved ribosomal RNA sequence [6], thoroughly characterized [7,8], including the three-dimensional structure of a 29-base oligonucleotide comprising such a sequence [9]. However, there is a limited amount of available information regarding the enzymatic mechanism of α-sarcin. Several residues of this protein have been proposed to be involved in the catalytic process [10–12], based on the sequence similarity between this toxin and ribonuclease T1 from *Aspergillus oryzae* (RNase T1). The geometry of the four β-strands where the catalytic residues of RNase T1 are located, as well as the nature and orientation of the residues themselves, are conserved in α-sarcin [13]. Accordingly, this cytotoxin might behave as a cyclizing ribonuclease, although direct experimental evidence has not been reported. In this work, we present evidence revealing that α-sarcin would catalyze the hydrolysis of RNA according to such a mechanism (Scheme 1).

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Abbreviations: 2′-AMP, adenosine 2′-monophosphate; 3′-AMP, adenosine 3′-monophosphate; 2′(3′)-AMP, adenosine 2′- and 3′-monophosphate (mixed isomers); ApA, adenylyl(3′→5′)adenosine; 2′,3′-cAMP, adenosine 2′,3′ cyclic monophosphate; 3′-GMP, guanosine 3′-monophosphate; 2′,3′-cGMP, guanosine 2′,3′ cyclic monophosphate; GpA, guanylyl(3′→5′)adenosine; poly(A), polyadenylic acid; RNase A, bovine pancreatic ribonuclease A; RNase T1, ribonuclease T1 from *Aspergillus oryzae*

2. Materials and methods

Bovine pancreatic ribonuclease A (RNase A) (type XII-A), RNase T1 (grade V), adenylyl(3′→5′)adenosine (ApA), guanylyl(3′→5′)adenosine (GpA), adenosine 2′,3′ cyclic monophosphate (2′,3′-cAMP), guanosine 2′,3′ cyclic monophosphate (2′,3′-cGMP), adenosine 3′-monophosphate (3′-AMP), adenosine 2′- and 3′-monophosphate (mixed isomers) (2′(3′)-AMP), guanosine 3′-monophosphate (3′-GMP) and adenosine were purchased from Sigma. α-Sarcin was purified to homogeneity from *Aspergillus giganteus* MDH 18894 cultures according to [1], as described in [14] and it was shown to be active when assayed against ribosomes [4,15].

Enzymatic activity towards dinucleotides was measured by analysis of the reaction products by high-performance liquid chromatography (HPLC), as described in [16]. The incubation mixtures contained different amounts of dinucleotide and enzyme in either 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl and 5 mM EDTA, or 50 mM sodium phosphate, pH 7.0, containing 0.1 M NaCl and 5 mM EDTA. Control mixtures without enzyme were always included. Concentrations of substrates and enzyme were determined according to their absorption extinction coefficients [11,17]. Reactions were carried out at room temperature for different periods of time. Samples were then injected onto a Kromasil 100 C18 column (5 μm, 0.46×15 cm). Elution was achieved with a 35-min linear gradient of 100 mM potassium phosphate buffer, pH 7.0, to 90 mM potassium phosphate buffer, pH 7.0, in 32.5% (v/v) methanol at a flow rate of 1.0 ml/min at room temperature. The absorbance was monitored at 254 nm with a Beckman detector module 166. Integration of the peaks and analysis of the results were accomplished with the Beckman System Gold software. Calibration curves were constructed after injection of known amounts of convenient standards.

3. Results and discussion

Quantitative analysis of the α-sarcin activity can be performed by using ^{32}P-labelled oligoribonucleotides as substrates [7,8]. However, this procedure is expensive and time consuming since it involves the synthesis of the radiolabelled substrates, as well as the electrophoretic separation of the reaction products and further densitometric analysis of the corresponding autoradiographic plates. Indeed, information about the intermediate steps conforming the reaction mechanism cannot be obtained by this procedure.

On the other hand, hydrolysis of dinucleotides followed by HPLC separation of the reaction products is a widely used method to measure the activity of ribonucleases [16–18]. Within this idea, we used two different dinucleotides to study the catalytic mechanism of α-sarcin. ApA was chosen because it is well known that this protein is especially active against the homopolynucleotide polyadenylic acid (poly(A)) [3]. GpA was selected in order to mimic the real substrate of α-sarcin, since this protein cleaves the phosphodiester bond between G-4325 and A-4326 in the 28S RNA [7–9].

The HPLC fractionation procedure employed allows the quantitative separation of all the reaction products resulting from the incubation of ApA or GpA with different amounts of α-sarcin (Fig. 1), the lower limit of detection being around

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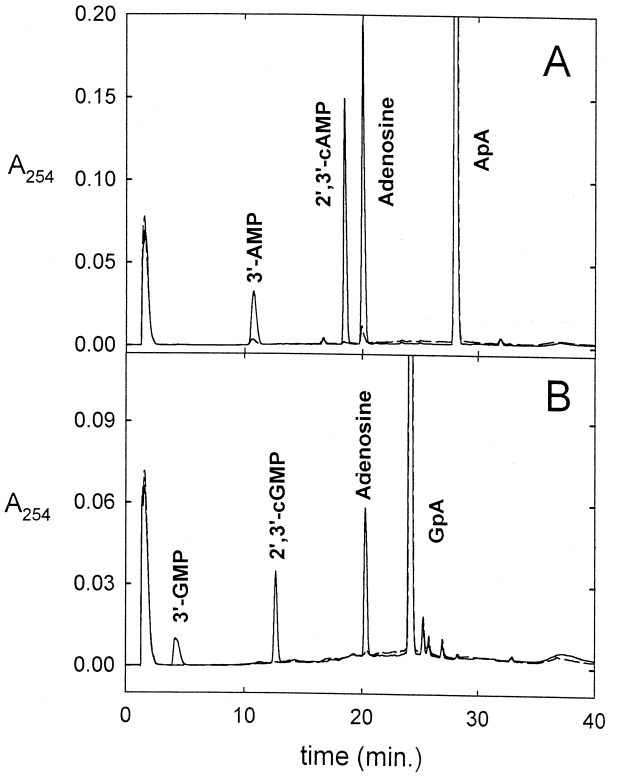


Fig. 1. HPLC separation of the reaction products resulting from the incubation of (A) 180 μM ApA and (B) 270 μM GpA in the presence of 3.8 μM α-sarcin, for 16 h at room temperature in 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 5 mM EDTA. The dashed lines correspond to the elution profile resulting when the incubation was performed in the absence of α-sarcin, thus revealing the absence of non-enzymatic hydrolysis of the dinucleotides.

10–20 pmol of product. The α-sarcin-catalyzed hydrolysis of ApA yielded adenosine, 2′,3′-cAMP and 3′-AMP (Fig. 1A),

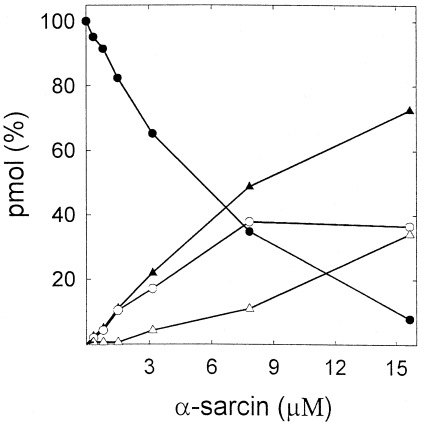
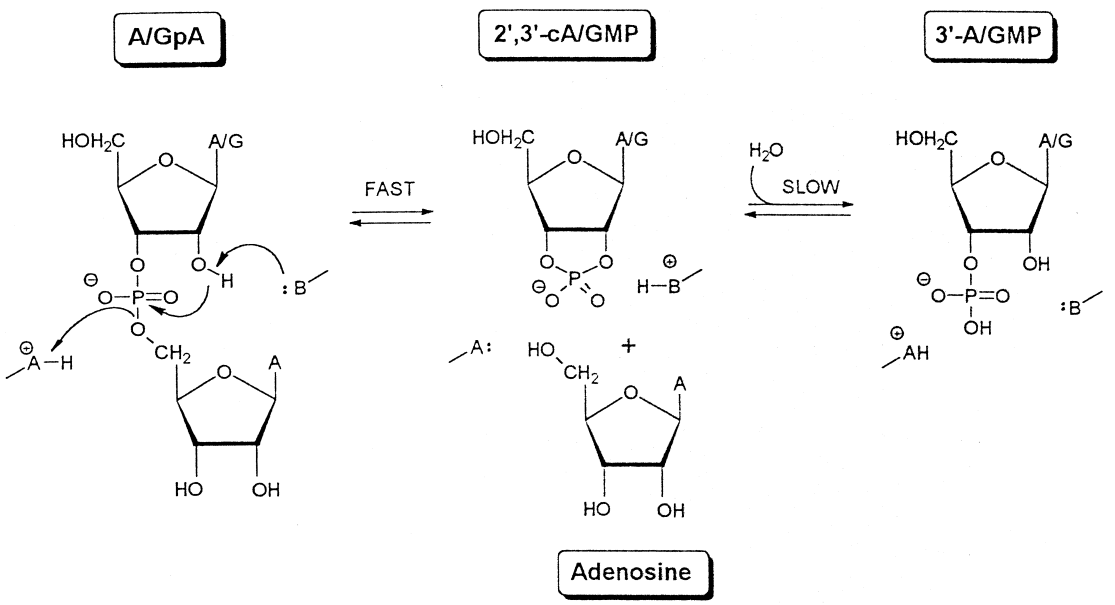


Fig. 2. Percentages of the different reaction products resulting from the incubation of different α-sarcin concentrations with ApA (100%) for 18 h at room temperature. (●) ApA substrate, (▲) adenosine, (○) 2′,3′-cAMP and (△) 3′-AMP.

identified from the chromatographic analysis of the corresponding standards. It is important to note that the sum of the amounts of 2′,3′-cAMP and 3′-AMP produced is identical to the total adenosine production (Fig. 2). When GpA was used as substrate, similar results were obtained (Fig. 1B), and guanosine was not detected in any case either.

These results suggest that the enzymatic mechanism of α-sarcin would be first the transphosphorylation of ApA (or GpA), rendering adenosine and 2′,3′-cAMP (or 2′,3′-cGMP), followed by the hydrolysis of the cyclic nucleotide to the 3′-monophosphorylated derivatives (Scheme 1). Actually, when α-sarcin was incubated with 2′,3′-cAMP as the only substrate, the hydrolysis of this compound was observed with the concomitant production of 3′-AMP, the yield of the process being dependent on the α-sarcin concentration (data not shown). This reaction was not observed in the absence of the protein, eliminating the possibility of a non-enzymatic



Scheme 1. Proposed mechanism for the catalytic action of α-sarcin, based on the two-step model admitted for RNases A and T1. During the transphosphorylation step (fast), A would behave as a general acid and B as a general base. Then, in the hydrolysis step (slow), B would be the general acid and A the general base. A and B are groups of the enzyme.

Table 1
Kinetic constants for α-sarcin against the two nucleotides employed as substrates

Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
ApA	40.0	1×10^{-4}	2.5
GpA	3.6	9×10^{-6}	2.5

α-Sarcin (3 μM) was incubated at room temperature for 14.5 h in the presence of increasing concentrations of ApA or GpA. Adenosine production and the initial concentration of the corresponding dinucleotide were used to calculate K_M and k_{cat} by means of double reciprocal plots.

hydrolysis of the cyclic compound. Moreover, HPLC analyses reveal a significant production of 2′,3′-cAMP after incubation of 3 μM α-sarcin with 2′(3′)-AMP for 18 h which does not occur in the absence of the protein (data not shown). This suggests the reversibility of the second step in Scheme 1. The experiments summarized in Fig. 1 were also performed for RNases A and T1 and the nature of the products obtained was identical to that of α-sarcin. The well known reaction mechanism of these two RNases is also given in Scheme 1.

Double recipocal plots were used to calculate K_M and k_{cat} from experiments where a fixed amount of α-sarcin was incubated with a range of ApA or GpA concentrations. The same results were obtained for two different batches of α-sarcin and in either Tris or phosphate buffer. Based on the values obtained (Table 1), α-sarcin would display a higher relative affinity for GpA than for ApA which might be related to the fact that the protein cleaves a guanosine/adenosine phosphodiester bond in ribosomes [3]. In fact, the calculated apparent K_M for intact ribosomes is 5.3 μM [3]. Nevertheless, it is worth indicating that these values are only meaningful for comparison of both substrate compounds because the experiments have been performed under suboptimal conditions and the dinucleotides do not accomplish the structural restrictions for the specific α-sarcin cleavage in ribosomal RNA.

On the other hand, the k_{cat} and the catalytic efficiency (k_{cat}/K_M) of α-sarcin against these two dinucleotides is extremely low (Table 1). This must not be interpreted in terms of a non-enzymatic hydrolysis, since adequate controls without α-sarcin were perfectly stable and cleavage was not observed at all. Indeed, it has been described how replacement of His-137 by a Gln renders a catalytically inactive variant of α-sarcin [19]. When this H137Q mutant was assayed against ApA no cleavage was observed, the production of adenosine being less than 3% of the amount produced by α-sarcin hydrolysis. Altogether, these results suggest that some other determinants are needed in order for the substrate to be efficiently cleaved. Actually, experiments with a 25-mer substrate analog have already shown that the optimum recognition of the sarcin/ricin loop oligonucleotide requires a guanosine at position 10 of this substrate analog, even though the hydrolyzed bond is six residues away [9,20].

Consequently, we propose that hydrolysis of the RNA by α-sarcin would fit into a two step mechanism, with the appearance of a cyclic intermediate. The specificity of α-sarcin in ribosomes [9,20] would arise from other structural elements forcing the substrate phosphodiester bond into a restricted configuration, such a restriction not being present in a dinucleotide with several orientations under equilibrium.

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