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Role of Histidine-50, Glutamic Acid-96, and Histidine-137 in the Ribonucleolytic Mechanism of the Ribotoxin α-Sarcin

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Abstract **α-Sarcin is a ribotoxin secreted by the mold *Aspergillus giganteus* that degrades the ribosomal RNA by acting as a cyclizing ribonuclease. Three residues potentially involved in the mechanism of catalysis—histidine-50, glutamic acid-96, and histidine-137—were changed to glutamine. Three different single mutation variants (H50Q, E96Q, H137Q) as well as a double variant (H50/137Q) and a triple variant (H50/137Q/E96Q) were prepared and isolated to homogeneity. These variants were spectroscopically (circular dichroism, fluorescence emission, and proton nuclear magnetic resonance) characterized. According to these results, the three-dimensional structure of these variants of α-sarcin was preserved; only very minor local changes were detected. All the variants were inactive when assayed against either intact ribosomes or poly(A). The effect of pH on the ribonucleolytic activity of α-sarcin was evaluated against the ApA dinucleotide. This assay revealed that only the H50Q variant still retained its ability to cleave a phosphodiester bond, but it did so to a lesser extent than did wild-type α-sarcin. The results obtained are interpreted in terms of His137 and Glu96 as essential residues for the catalytic activity of α-sarcin (His137 as the general acid and Glu96 as the general base) and His50 stabilizing the transition state of the reaction catalyzed by α-sarcin. Proteins 1999;37: 474–484.** © **1999 Wiley-Liss, Inc.**

Key words: ***Aspergillus*** **ribotoxin; enzyme mechanism; protein spectroscopy; ribonuclease; ribosome-inactivating-protein**

INTRODUCTION

α-Sarcin is a cytotoxic protein composed of a single polypeptide chain of 150 amino acid residues and is secreted by the mold *Aspergillus giganteus* MDH18894.[1,2] It is the most representative member of the family of proteins named *Aspergillus* ribotoxins,[3,4] which also includes restrictocin and mitogillin,[5,6] among others. The cytotoxicity of proteins in this family is related to the inactivation of ribosomes by a specific ribonucleolytic action[7,8] against a single phosphodiester bond located at a highly conserved sequence of the 28S ribosomal RNA, the so-called sarcin/ricin loop,[9–11] whose well-known three-dimensional structure seems to be crucial for the specific protein-RNA recognition.[12] The three-dimensional structures of α-sarcin[13,14] and restrictocin[15] have also been elucidated. However, knowledge about the structural elements responsible for the specific ribonucleolytic action of these cytotoxins is scarce.

In addition to the primary structure conservativeness (more than 85% amino acid sequence identity) among the *Aspergillus* ribotoxins,[4] α-sarcin also displays some sequence and three-dimensional structure (Fig. 1) similarities with other nontoxic fungal RNases, RNases T1 and U2 being the most representative, although they contain about 40 amino acid residues less than does α-sarcin.[16–20] The enzymatic mechanism of RNase T1 has been clearly established (for a review, see Steyaert[21]), and the catalytic residues identified by different experimental approaches, including site-directed mutagenesis.[21–26] This enzyme behaves as a cyclizing RNase, displaying a mechanism similar to that of RNase A. Two histidine residues, His12 and His119, are unequivocally responsible for the general acid-base catalysis by RNase A.[27] However, in RNase T1, the overall reaction—cyclization followed by hydrolysis of the cyclic nucleotide (Fig. 2)—is performed by His92 and Glu58. During the first reaction, the formation of the 2′,3′-cyclic product, Glu58 acts as a general base (B in Fig. 2) and His92 as a general acid (A in Fig. 2) (the Glu/His pair is the most common pair of catalytic residues in microbial ribonucleases[28]). The hydrolysis of this cyclic derivative is performed by the same groups, but their roles are reversed. In addition, there is another His residue at position 40 (C in Fig. 2), which somehow assists the catalysis exerted by Glu58.[25,29] In fact, for a long time it was controversial whether the catalytic residue was Glu58 or His40,[25] but it is now clear that His 40 is required under

Abbreviations: 2′-AMP, adenosine 2′-monophosphate; 3′-AMP, adenosine 3′-monophosphate; ApA, adenylyl(3′,5′)adenosine; 2′,3′-cAMP, adenosine 2′,3′-cyclic monophosphate; CD, circular dichroism; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; poly(A), polyadenylic acid; RNase, ribonuclease; SDS, sodium dodecylsulfate; UV, ultraviolet.

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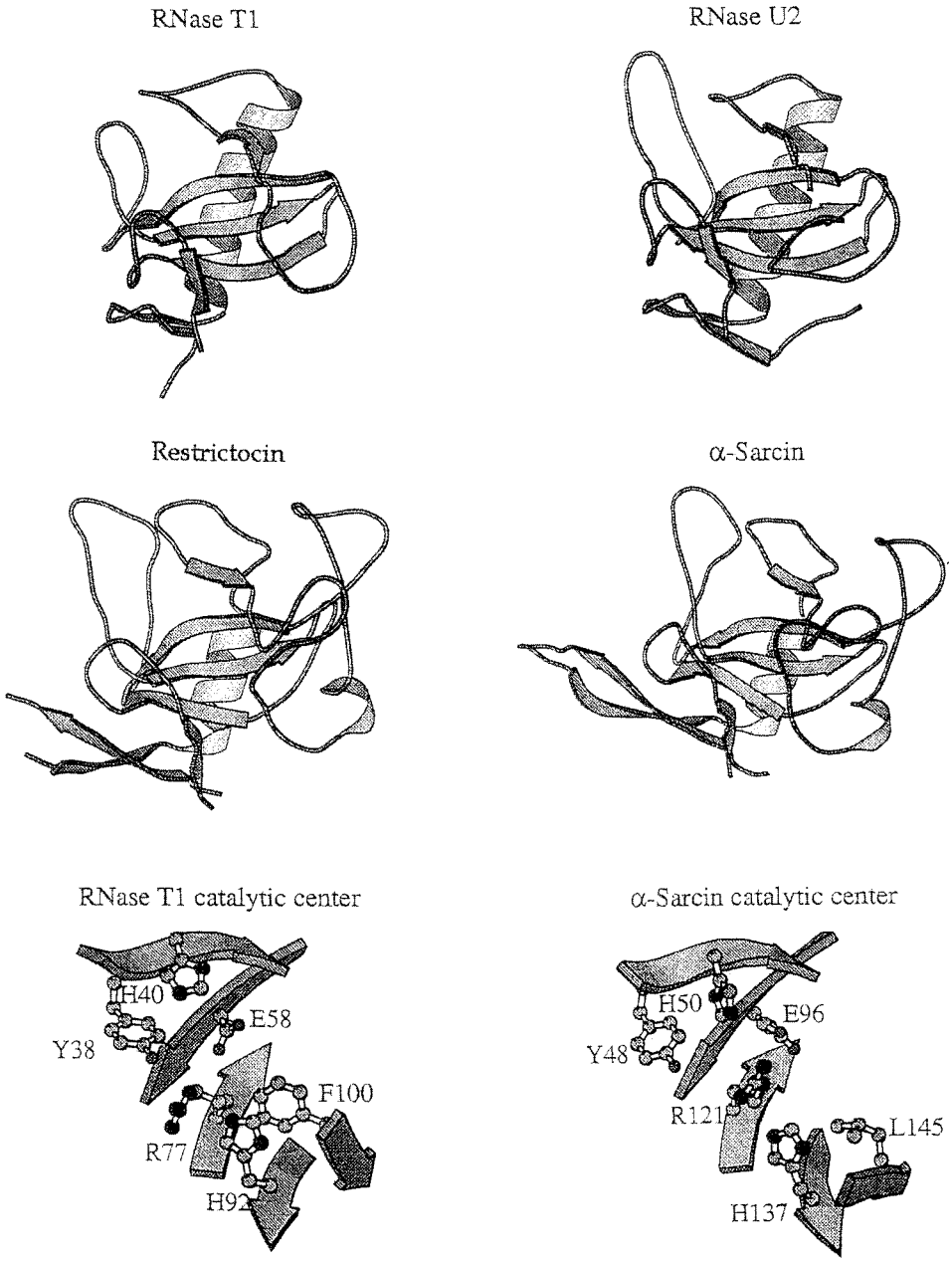

Fig. 1. Ribbon representation of the three-dimensional structures of various ribonucleases sharing structural elements with α-sarcin, including RNase T1 from *A. oryzae*,[30] RNase U2 from *Ustilago sphaerogena*,[31] restrictocin from *A. restrictus*,[15] and α-sarcin from *A. giganteus*.[13,14] The active site surrounding residues of RNase T1 and α-sarcin are also shown. All figures were generated by the MOLSCRIPT program.[57]

its protonated form for optimal activity because it participates in the electrostatic stabilization of the transition state.[21,25] However, the ability of His40 to adopt the function of general base must account for the significant remaining activity found in Glu58–mutated RNase T1.[21,25,29]

Comparison of the three-dimensional structures of α-sarcin[13,14] and restrictocin[15] with those of RNase T1[30] and RNase U2[31] reveals that all the four proteins share identical secondary structure elements in spite of their different amino acid sequence lengths (Fig. 1). After superimposition of these three-dimensional structures, it is clearly observed that the counterparts of the His40, Glu58, and His92 catalytic residues in RNase T1 are His50, Glu96, and His137 in α-sarcin (Fig. 1). We have previously reported that α-sarcin behaves as a cyclizing ribonuclease, such as RNases T1, U2, and A,[32] a long-time held hypothesis. This evidence strongly supports the concept that His50, Glu96, and His137 of α-sarcin were the candidates to play catalytic roles in this toxic ribonuclease. Within this idea, substitution of His137 by Gln abolishes the activity of α-sarcin on ribosomes.[32,33] In addition, we have recently reported the electrostatic behavior of titrating

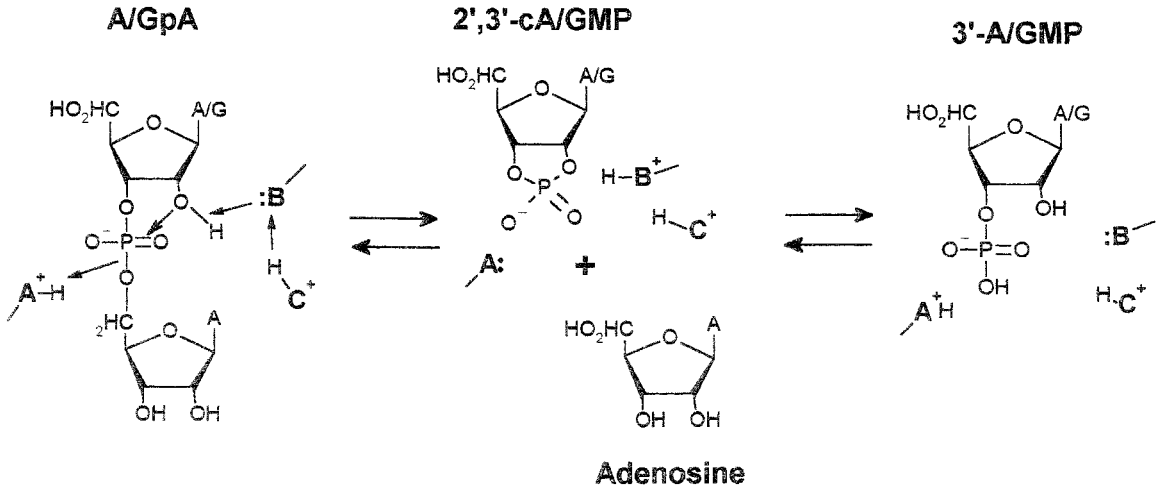

Fig. 2. Mechanism for the catalytic action of RNase T1 against a dinucleotide, ApA or GpA, substrate. A transphosphorylation process (in which the corresponding 2′,3′ cyclic mononucleotide and adenosine are produced) is followed by hydrolysis of the cyclic nucleotide to produce the corresponding 3′-mononucleotide. This mechanism is also proposed for α-sarcin. A, B, and C are His92, Glu58, and His40 in RNase T1, and His137, Glu96 and His50 in α-sarcin, respectively.

groups in α-sarcin by using ^{1}H NMR spectroscopy.[34] His50, Glu96, and His137 display pK_a values of 7.7, 5.2, and 5.8, respectively, far from their intrinsic values. These values are respectively shifted to 7.6, 4.8, and 6.8 in α-sarcin-2′-GMP complex. Based on these results, the previously mentioned three residues were proposed to be involved in the catalysis by α-sarcin. In the work presented in this article, we have engineered and purified variants of α-sarcin, replacing His50, Glu96, and His137 with Gln. These protein forms have been characterized in order to analyze the roles of those residues in the ribonucleolytic mechanism of α-sarcin.

MATERIALS AND METHODS

DNA Manipulations

All materials and reagents used were molecular biology grade. Cloning procedures and bacteria manipulations were carried out according to standard methods[35] as described previously.[33,36] Oligonucleotide site-directed mutagenesis was used to replace the selected residues by Gln. The method is based on that designed by Kunkel et al.,[37] and it was done as previously described.[33,36] The plasmids used for the production of the different variants of α-sarcin were based on pET11b[38] or pINIIIOmpA1.[33,36,39] Starting with the plasmid pINPGαS, where the α-sarcin cDNA was originally cloned,[36] a digestion with *Xba*I and *Bam*HI was used to obtain a fragment containing the complete sequence encoding this protein, fused to a mutated OmpA signal peptide.[36] This fragment was cloned in pEMBL18(+)[40] to obtain the uridine-rich single-stranded DNA needed as a template for the mutagenesis experiments (see below). Once the mutant DNA was obtained, it was completely sequenced to confirm the uniqueness and fidelity of the mutation and cloned again in the corresponding sites of either pET11b or pINPG.[36]

The following mutagenic primers (site of mutation underlined) were used: H50Q: 5'-C TAT CCT CAA TGG TTC ACC-3'; H137Q: 5'-C ATT GCT CAA ACT AAG GAG-3'; E96Q: 5'-CAC TAC CTG CTG CAG TTC CCA ACC-3'. To obtain the double (H50/137Q) and triple (H50/137QE96Q) mutant variants, successive mutagenic rounds were made, using the corresponding single or double mutants as templates and the mutagenic primer for each mutation. The *Escherichia coli* strains used were BW313 (*HfrKL*16 *po*/45 [*lysA* (61–62)] *dut*1 *ung*1 *thi*1 *relA*1), to obtain the uridine-rich ssDNA, DH5αF′({[F′] *endA*1 *hsdR*17 (r^-_k m^-_k) *supE*44 *thi*-1 *recA*1 *gyrA* (NaI^R) *relA*1 Δ(*lacZYA-argF*) U169 *deoR* [Θ80 *dlac*Δ (*lacZ*) M15]}) or TG1 (F′ *traD*36 $lacI^q$ Δ(*lacZ*) *M*15 *proA*$^+$*B*+/*supE* Δ(*hsdM-mcrB*)5 (r_k^- m_k^+ *McrB*−) *thi* Δ(*lac-proAB*)), to obtain the expression constructs, and RB791 (W3110 $lacI^q$ L8) and BL21(DE3) (F′ *ompT* [*lon*] hsd_B (r^-_B m^-_B)) to produce the proteins.

Protein Production and Purification

E. coli RB791 cells harboring a plasmid based on pINIIIOmpA1 (pINPGαSH137Q) were used to produce the α-sarcin mutant variant H137Q, as described previously.[33] However, when the same procedure was used for the production of H50Q, most of the protein remained as the nonproteolytically processed precursor form and could not be purified. Therefore, the system based on pET11b and *E. coli* cells BL21(DE3) was used to produce the other four variants of α-sarcin, H50Q, H50/137Q, E96Q, and H50/137Q/E96Q. Thus, cells were grown to an optical density at 600 nm of 1.0 and induced with 0.4 mM IPTG. Then they were cultured at 37°C for 4 hours longer. The different cellular fractions containing soluble protein were used to purify each mutant according to the procedure described previously[33,36]. Fungal wild-type α-sarcin was produced and purified to homogeneity essentially according to meth-

ods previously reported.[1,2] Polyacrylamide gel electrophoresis of proteins, protein hydrolysis, and amino acid analyses were also performed according to standard procedures as described previously.[36]

Spectroscopic Characterization

Absorbance measurements were carried out on a Uvikon 930 spectrophotometer (Kontron Instruments, Milan, Italy) at 100 nm/minute scanning speed, at room temperature, and in 1-cm optical-path cells. CD spectra were obtained on a Jasco 715 spectropolarimeter (Easton, MD) at 0.2 nm/second 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 degree $\times$ cm^2 $\times$ dmol^{-1}. Extinction coefficients E(0.1%, 1 cm, 280 nm) were calculated from their absorbance spectra and amino acid analyses. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 220 nm in the range of 25°–65°C; the temperature was continuously changed at a rate of 0.5°C/minute. Fluorescence emission spectra were obtained on an SLM Aminco 8000 spectrofluorimeter (Urbana, IL) at 25°C and in 0.2-cm optical-path cells. Protein samples for NMR experiments were dissolved in (9:1 by volume) H_2O/D_2O at 1.5 mM concentration. The pH (not corrected for isotope effects) was adjusted to 4.0. The experiments were performed on a Brucker AMX600 spectrometer (Karlsruhe, Germany) at 25°C by using conventional pulse sequences.[13]

Ribonucleolytic Activity

The specific ribonucleolytic activity of α-sarcin was followed by detecting the release of the 400 nt α-fragment[9,41,42] from a cell-free reticulocyte lysate (Promega, Madison, WI) as described previously.[33,36] This qualitative assay is required to adscribe a protein to the *Aspergillus* ribotoxin family. Briefly, the different proteins were incubated with the lysate for 15 minutes at room temperature. The reaction was stopped by addition of 0.5% SDS. The production of the 400 nt α-fragment was visualized by ethidium bromide staining after electrophoresis on 2.4% agarose gels of the RNA extracted from the reaction mixture.

The activity of the purified proteins against poly(A)[42] was assayed after subjecting the samples to an electrophoretic procedure in 15% polyacrylamide gels containing 0.1% SDS and 0.3 mg/mL poly(A). This method, designated as zymogram, was based on one peviously described.[33,43] After electrophoresis, the gel was incubated at 37°C for 3 hours at either of two different pH values, 4.5 or 7.5 (acetate buffer instead of phosphate buffer was used for the reaction at pH 4.5). It was then stained with 0.2% toluidine blue. Proteins exhibiting ribonuclease activity appear as colorless bands after proper destaining treatment.

Hydrolysis of ApA by α-sarcin and the mutant variants herein described was performed as described elsewhere[32] in acetate or phosphate buffer, at different pH values, containing 5 mM EDTA. The reaction products were resolved by HPLC according to the method of Shapiro et al.,[44] with a phosphate-methanol gradient as previously described.[32] Under these conditions, the amounts of 2′,3′-cAMP, adenosine, and 3′-AMP are proportional to the reaction time up to at least 24 hours of incubation. Therefore, the amounts of such reaction products at a fixed incubation time, up to 24 hours, represent the rate of the corresponding reaction. It is important to mention that long incubation times are required because of the low ribonuclease activity of α-sarcin out of the specific degradation of the ribosomal RNA.

All of these assays were performed with the corresponding controls to test potential nonspecific degradation of the substrates, which does not occur under the conditions used.

TABLE I. Extinction Coefficient ($E^{0.1\%}$) at 280 nm in 1-cm Optical Path Cells and Temperature at the Midpoint (Tm) of the Thermally Induced Denaturation†

	$E^{0.1\%}$	Tm (°C)
Wild-type α-sarcin	1.34	51.8
H50Q	1.27	52.0
H137Q	1.34	48.0
E96Q	1.34	52.3
H50/137Q	1.27	48.0
H50/137Q/E96Q	1.27	50.9

†Determined from the profile obtained by continuous recording of the ellipticity at 220 nm of wild-type and variants of α-sarcin.

RESULTS

Structural Characterization of the Mutant Variants of α-Sarcin

We prepared five variants of α-sarcin, H50Q, H137Q, E96Q, H50/137Q, and H50/137Q/E96Q. All were purified to homogeneity, according to their behavior on PAGE-SDS, with yields ranging from 1.0 to 2.5 mg of protein per liter of original culture. The amino acid composition of each purified protein was consistent with the corresponding mutation planned. All of these protein forms were characterized spectroscopically to evaluate the influence of the mutation on the three-dimensional structure, by considering wild-type fungal α-sarcin as control. Only very minor changes were observed when the UV-absorption spectra were recorded (data not shown). In fact, the calculated extinction coefficient ($E^{0.1\%}$) at 280 nm changed only when His50 was the residue replaced (Table I). Thus, the value obtained was 1.27 for H50Q, H50/137Q, and H50/137Q/E96Q; it was 1.34 for the other two variants (H137Q and E96Q) and the wild-type protein.[45] This slight difference, within the range of experimental error, may be related to the spatial proximity of His50 to the aromatic ring of Tyr48.[13,14]

The far-UV circular dichroism (CD) spectra of the five variants (Fig. 3A) were also highly similar to that of the fungal protein. Deconvolution of these spectra by convex constraint analysis[46] does not show significant differences in terms of secondary structure contents (data not shown). Indeed, only H50/137Q/E96Q showed a slight significant

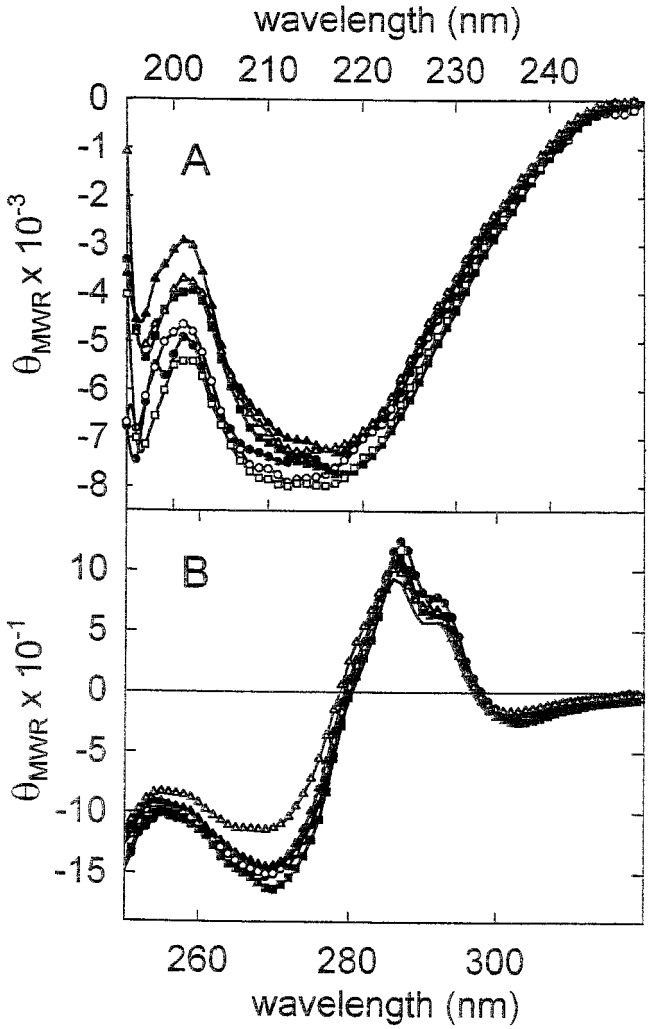

Fig. 3. Circular dichroism spectra of α-sarcin and its variants. Far-UV (**A**) and near-UV CD (**B**) spectra of wild-type α-sarcin (●) and its variants H50Q (○), H137Q (■), E96Q (□), H50/137Q (▲), and H50/137Q/E96Q (△). The proteins were dissolved in 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. Mean residue weight ellipticity values (θ_{MRW}) are expressed in units of degree × cm^2 × $dmol^{-1}$, 113 being the mean residue mass for the amino acids of these proteins.

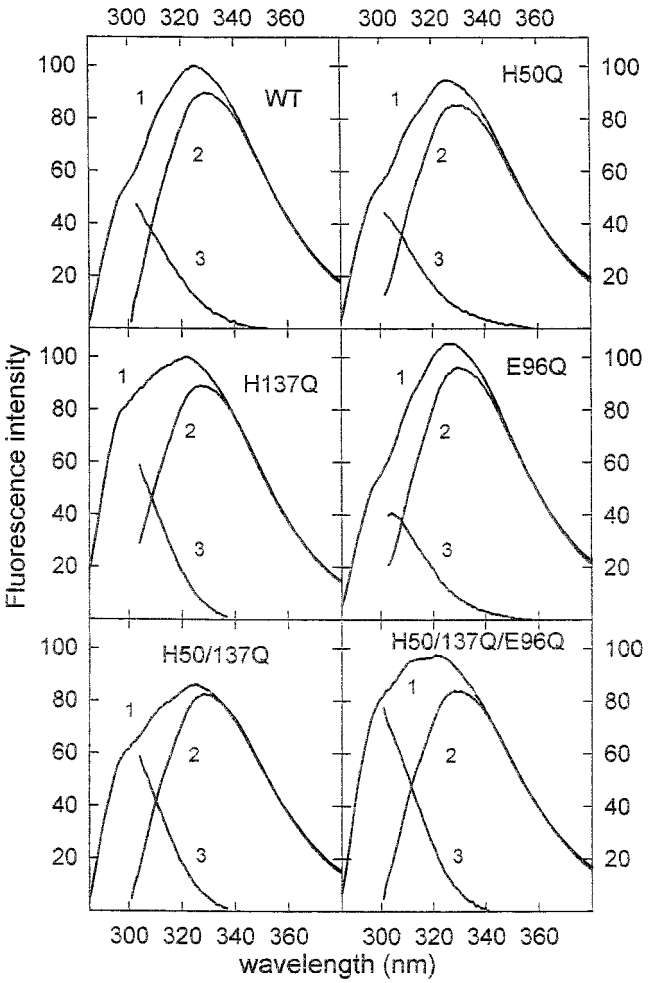

Fig. 4. Fluorescence emission spectra of wild-type (WT) α-sarcin and five variants of α-sarcin. The spectra have been recorded for excitation at 275 nm (*1*) and 295 nm. These two spectra have been normalized by considering that the tyrosine contribution above 380 nm is negligible. The normalized emission spectra for excitation at 295 nm (*2*), corresponding to the Trp contribution, are also shown. The tyrosine contribution, calculated as the difference between the two above-mentioned spectra, is also given (*3*). The proteins were dissolved in 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. Fluorescence emission is expressed in arbitrary units, considering the intensity of the wild-type protein at 325 nm for excitation at 275 nm as 100%.

difference when the near-UV CD spectra were recorded (Fig. 3B).

Regarding the fluorescence emission spectra of the protein variants, we also observed only minor differences when we compared them with those of wild-type α-sarcin (Fig. 4). The Trp contribution, emission spectrum for excitation at 295 nm, shows an emission maximum centered around 330 nm, and the fluorescence intensity at this wavelength is within the range ± 10% for all the proteins. Larger differences are observed for the Tyr contribution, in terms of increased fluorescence emission, in the three variants involving His137.

The thermal denaturation profiles of the purified proteins were analyzed by measuring the temperature dependence of the ellipticity at 220 nm. A single transition trace was obtained for all the protein forms studied. The temperature value at the midpoint of the thermal transitions (Tm) is given in Table I. The Tm of the variants involving His137 is decreased up to 4°C in comparison to the corresponding value of the wild-type protein.

An NMR study of the H50/137Q variant was done using 1D and 2D methods. The assignment of the spectrum was assisted by comparison with those of wild-type α-sarcin[13] following the standard NOE-based methodology.[47] The double mutation does not modify the characteristic chemical shift dispersion of the native protein. Significant changes were observed only for the chemical shifts of the nucleus belonging to atoms of the mutated residues and their surrounding residues. The NOE pattern of the protein cross-peaks displayed no substantial differences between wild-type α-sarcin and H50/137Q α-sarcin (Fig. 5).

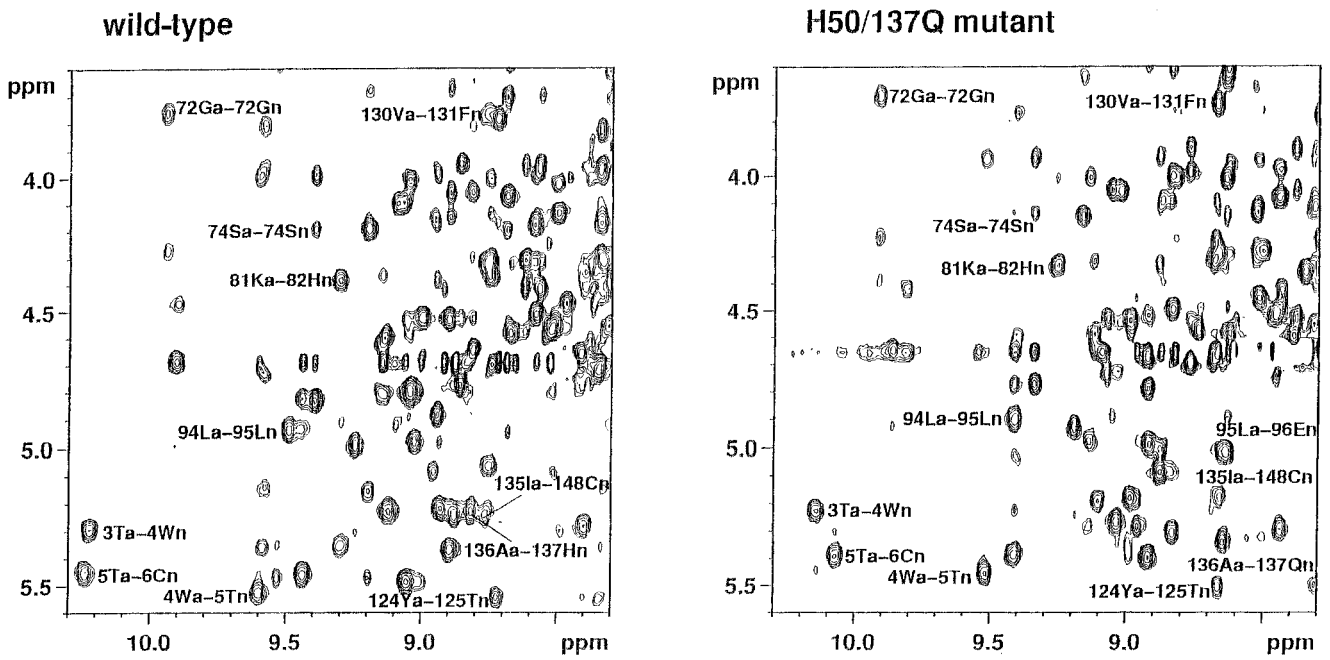

Fig. 5. Selected region of the two-dimensional NOESY spectra of wild-type α-sarcin and H50/137Q variant α-sarcin showing intraresidual, sequential, and long-range Hα-HN NOEs. Significant changes are observed for the NOEs corresponding to protons from the mutated residues, such as 136Hα-137HN, and from those of other closely located residues, such as 95La-96En, which is outside the contour map in the spectrum of the wild-type protein.

Altogether, the results presented above demonstrate that the three-dimensional structure of all the variants is largely preserved.

Enzymatic Characterization of the Mutant Variants of α-Sarcin

The enzyme activity of the five variants was first studied against a cell-free rabbit reticulocyte lysate at neutral pH. Under these conditions, wild-type α-sarcin produced the characteristic α-fragment resulting from the hydrolysis of a single phosphodiester bond in the 28S rRNA (Fig. 6A). This assay is able to detect about 0.6 pmol of wild-type α-sarcin, and this is the only activity that a protein must perform to be considered a member of the *Aspergillus* ribotoxins family. Accordingly, all the variants prepared lack the ribonucleolytic activity involved in the specific cytotoxic action of α-sarcin (at most, their activity would be less than 10% that of the wild-type protein based on the detection limit of this assay). Also, wild-type α-sarcin produced a nonstained band resulting from the absence of poly(A) at the region occupied by the ribonucleolytic activity because of the nonspecific degradation of the polynucleotide absorbed into the polyacrylamide gel (Fig. 6B). These variants were not active against this polynucleotide at either pH 4.5 or 7.5 (less than 1% that of the wild-type protein, according to the detection limit of this assay).

These are the two qualitative assays widely used to analyze the activity of ribotoxins from the α-sarcin family. To evaluate the enzyme activity, we have adapted a previously reported assay.[44] Using dinucleotides as substrates,[32] we have shown that α-sarcin behaves as a cyclizing ribonuclease by cleaving either ApA or GpA, the reaction products being adenosine, the 2′,3′-cyclic intermediate, and the corresponding 3′-monophosphate derivative (Fig. 2). This assay has shown that α-sarcin is an acid ribonuclease with an optimum pH value of 5.0.[34] Thus, wild-type α-sarcin and the five variants were assayed against ApA at both pH 7.0 and pH 5.0. This assay was performed under conditions in which the amount of the reaction products is proportional to the incubation time. Thus, such amounts are proportional to the corresponding reaction rates (10 to 20 pmol of reaction product can be detected by this assay[32]). Among the mutant forms, only the H50Q variant displayed detectable activity (about 15% of the activity corresponding to the wild-type enzyme at pH 7.0 and 5% at pH 5.0), whereas the other four variant forms were inactive. Table II summarizes the activities of wild-type and H50Q α-sarcin at both pH values. The mutation reduces k_{cat} by about one order of magnitude at both pH values, whereas no significative differences are observed in terms of the K_M values. This indicates that His50 is involved in catalysis rather than in substrate binding.

The ionization state of the groups involved in a cyclizing ribonucleolytic catalysis is crucial. Thus, we studied in further detail the effect of pH on the activity of this protein variant. As indicated above, the optimum pH for the degradation of ApA by α-sarcin is 5.0, but it is extended from 5.0 to 7.0 for the H50Q variant, although displaying a lower activity than does the wild-type protein throughout the whole pH range studied (Fig. 7). The absolute values of both ascending and descending slopes of the plots of log

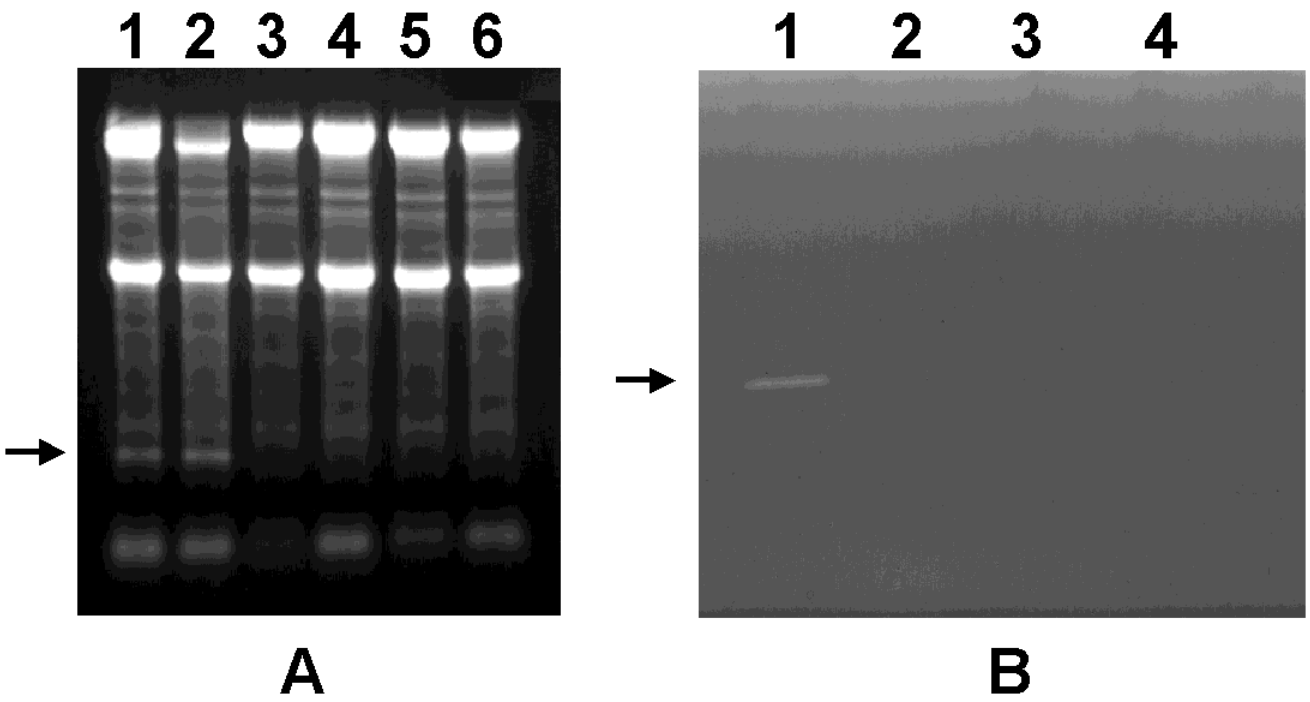

Fig. 6. **A**: Ribosome-inactivating activity assay of α-sarcin. The highly specific RNase activity of this protein is shown by the release of the 400 nt α-fragment (*arrow*) from the 28S rRNA of eukaryotic ribosomes. Cell-free reticulocyte lysates were incubated in the presence of 40 ng (lane 1) and 100 ng (lane 2) of wild-type α-sarcin, and 100 ng (5.8 pmol) of H50Q (lane 3), H137Q (lane 4), E96Q (lane 5), and H50Q/H137Q (lane 6). The result obtained for the triple mutation variant was identical to those of the other mutation variants. The lowest detection limit of this assay is about 10 ng (0.6 pmol) of wild-type α-sarcin. The reaction mixture was analyzed on 2.4% agarose gel and stained with ethidium bromide. **B**: Zymogram corresponding to the ribonucleolytic activity of wild-type α-sarcin (lane 1) and H50Q (lane 2), H137Q (lane 3), and E96Q (lane 4) variants against poly(A) at pH 4.5. The same results are obtained when the experiment is performed at pH 7.5. The amount of protein analyzed at each lane was 100 ng (5.8 pmol). The poly(A)-degrading activity of wild-type α-sarcin produces a nonstained region (*arrow*) in this negative-staining analysis. No activity was detected when amounts of mutant proteins as large as 5 mg (290 pmol) were assayed. The lowest detection limit of this assay is 50 ng of wild-type α-sarcin.

TABLE II. Activities of Wild-Type and H50Q α-Sarcin[†]

	pH 7.0		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Wild-type	40 ± 4	$(1.0 \pm 0.1) \times 10^{-4}$	2.5 ± 0.4
H50Q	38 ± 4	$(1.4 \pm 0.2) \times 10^{-5}$	0.3 ± 0.1
	pH 5.0		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Wild-type	40 ± 4	$(2.0 \pm 0.1) \times 10^{-4}$	5.0 ± 0.7
H50Q	45 ± 4	$(1.6 \pm 0.2) \times 10^{-5}$	0.3 ± 0.1

[†]The kinetic parameters (±standard errors) were determined from the transesterification of ApA by linear regression analysis of double reciprocal plots.

(k_{cat}) versus pH are ≤ 1 (Fig. 7), suggesting the involvement of one base and one acid group in the transesterification reaction. The pH dependence of the k_{cat} parameter usually resembles the critical ionizations of the enzyme-substrate complex whose decomposition is rate limiting. The experimental data are fairly adjusted to a curve generated by considering two groups with macroscopic pK values estimated at 3.8 and 6.7 for the wild-type protein and at 4.4 and 8.0 for the H50Q variant (Fig. 7).

This quantitative assay also allows for an independent analysis of the two reactions catalyzed by a cyclizing ribonuclease. The amounts of adenosine and 3′-AMP represent the extent of the first and the second reactions, respectively, and both can be evaluated from the corresponding chromatogram (Fig. 8). The ratio of adenosine produced to initial ApA is an index of the transphosphorylation reaction (first reaction, Fig. 2) as well as of the overall ribonucleolytic process because the substrate is degraded at this stage. The 3′-AMP/adenosine ratio represents the extent of the hydrolysis reaction (second reaction, Fig. 2). We have studied the variation of this ratio along the pH range considered for both wild-type and H50Q α-sarcin forms (Fig. 8). The H50Q variant showed a preponderance of the second reaction over the cyclizing reaction at pH 7.0, which is not observed for the wild-type protein (Fig. 8). This is also clear from the elution profiles (Fig. 8) corresponding to the reaction mixtures produced by H50Q and wild-type α-sarcin at pH 7.0, at which a higher 3′-AMP to adenosine ratio is evident for the H50Q variant.

DISCUSSION

Three residues in α-sarcin—His50, Glu96, and His137—have been proposed to be involved in catalysis and/or substrate binding based on the similarities in terms of three-dimensional structure between this protein and

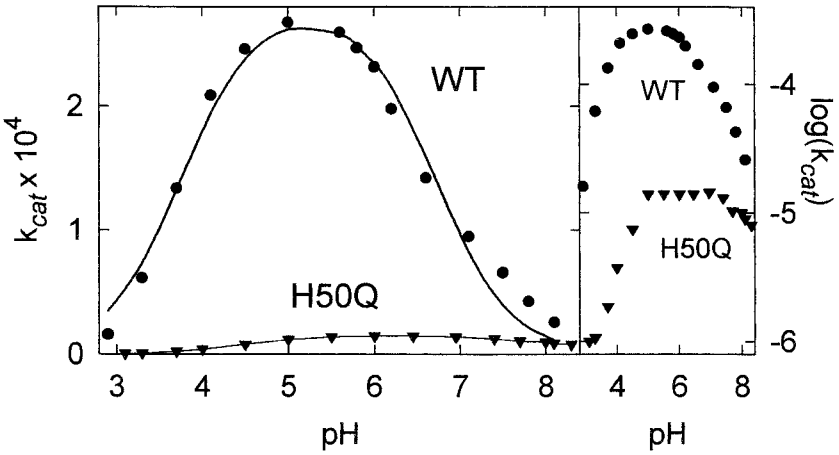

Fig. 7. pH Dependence of the activity (k_{cat}) of α-sarcin and H50Q variant for the transesterification of ApA; wild-type α-sarcin (●) and its H50Q variant (▼). The lines were generated by considering the involvement of two ionization equilibria (see Table III). The hydrolysis reaction was performed in 50 mM acetate or phosphate buffer containing 0.1 M NaCl and 5 mM EDTA.

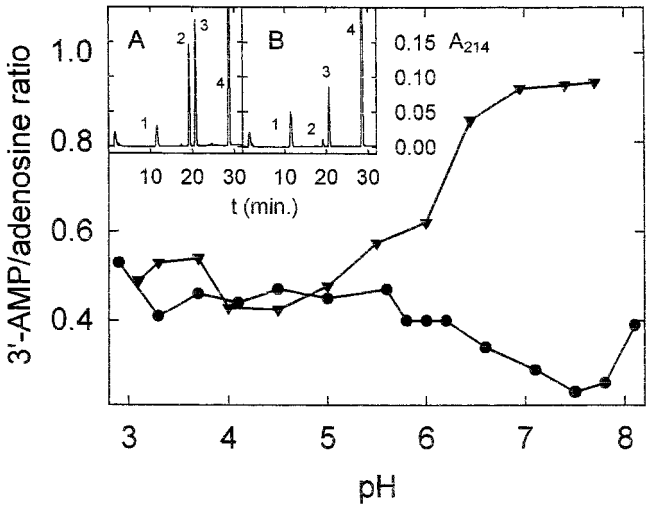

Fig. 8. Dependence on pH of the production of 3′-AMP (reaction 2; Fig. 2), expressed in terms of the ratio of 3′-AMP concentration to adenosine concentration for wild-type (●) and H50Q (▼) α-sarcin. **Inset**: HPLC separation of the reaction products resulting from the hydrolysis of 180 μM ApA when incubated with 3.8 μM wild-type α-sarcin (**A**) or H50Q variant (**B**) for 14 hours at room temperature in 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 5 mM EDTA. Peaks 1 to 4 correspond to 3′-AMP, 2′,3′-cAMP, adenosine, and ApA, respectively.

RNase T1. The results obtained from NMR measurements revealed microscopic pK_a values of 5.2, 5.8, and 7.7 for Glu96, His137, and His50, respectively.[34] Because α-sarcin behaves as a cyclizing acid RNase against dinucleotide substrates with an optimum pH value at 5.0, Glu96 and His137 were proposed as the general base and general acid residues involved in catalysis.[34] Five variant proteins involving mutations at these three residues have been produced in *E. coli*. The spectroscopic characterization of these protein forms has revealed that all of them essentially preserve the three-dimensional structure of the wild-type protein. However, variants involving His137 residue display significantly decreased Tm values and some differences in their fluorescence emission spectra compared with the wild-type protein. These variations may be a consequence of the internal location of the His137 residue. Its substitution by glutamine probably modifies the electrostatic microenvironment of the nearby residues, and it is well known that these changes at places where the dielectric constant value is low, such as in the protein interior where most of the hydrophobic side-chains are located, may result in small perturbations in the hydrogen bonding, and in hydrophobic and electrostatic interactions.

The characterization of the activity of the studied protein forms has shown that His50, Glu96, and His137 are three residues required for the inactivation of the ribosome by α-sarcin because each individual variant lacks this activity, as do double and triple mutation variants. The same conclusion can be drawn from a polynucleotide substrate. The involvement of His137 in the catalysis by α-sarcin is clear from the results herein presented and those previously reported for the H137Q variant.[32,33] Moreover, the recently determined pK_a value of this residue[34] confirms that His137 residue might act as general acid in the transphosphorylation reaction. The results obtained for mitogillin H136Y[3,48] and restrictocin H136L[49,50] are in agreement. Restrictocin and mitogillin are also ribotoxins produced by *Aspergillus* strains and share 86% sequence identity with α-sarcin. They show a gap at the second residue at the NH_2-terminal end of the amino acid sequence in comparison with that of α-sarcin.[51] Thus, His49, Glu95, and His136 are the residues corresponding to His50, Glu96, and His137 in α-sarcin and His40, Glu58, and His92 in RNase T1.[19,20]

Some mutagenesis experiments regarding the substitution of the glutamic acid residue at the active site of these ribotoxins have been reported. Thus, restrictocin E95G[49] and α-sarcin E96A[52] mutant variants still retain toxic activity. The former is partially active because it could kill yeast cells when produced intracellularly in vivo,[49,50] and α-sarcin E96A is still cytotoxic, although about 80-fold less than the wild-type protein.[52] This was interpreted as if the glutamic acid residue might not be directly involved in the catalysis of the phosphodiester bond cleavage or, if it does act as a general base in catalysis, some other residue in the protein may fill its role as a general base but with a lower ribonuclease activity. In RNase T1 it is now clear that

Glu58 serves as the catalytic base in the transphosphorylation reaction,[21] although the RNase T1 Glu58-mutated enzymes still show significant ribonucleolytic activity.[24,25,53] When Glu58 in Rnase T1 is substituted by either Ala or Gln, the variant displays 10% and 1% activity, respectively.[23,24] These results were explained in terms of the ability of His40 in RNase T1 and His49 in restrictocin to adopt the function of general base.[21,25,29,54] In α-sarcin, Glu96 plays the role of the catalytic base, but its function cannot be replaced by His50. This agrees with the large difference in terms of intrinsic pK_a values of these two residues, 5.2 and 7.7 for Glu96 and His50, respectively.[34] Accordingly, α-sarcin could not in principle operate as RNase T1 with two potential catalytic base residues. In fact, the E96Q mutant form does not show the essential features of a ribonuclease because it does not cleave ribosomal RNA, poly(A), or ApA. This disagrees with the above-mentioned results reported for the E96A α-sarcin variant. This apparent discrepancy may be explained by considering the different nature of the residue replacing Glu96. In the three-dimensional structure of α-sarcin, Glu96 is involved in a network of interactions, most of them hydrogen bonds but also some ion pair bonds, with some other neighbor residues. The neutral nature of the alanine side-chain cannot replace these interactions, and the ionization properties of the surrounding residues may be modified. NMR studies performed on RNase T1 E58A mutant have revealed that the absence of the glutamic acid residue decreased by 1.0 unit and 0.5 unit the pK_a values of His40 and His92, respectively.[25] If this occurred in the α-sarcin E96A variant, the His50 residue might play the role of general base, replacing Glu96, although with a lower efficiency, thus explaining the observed 10% activity of this mutant form.[52]

Substitution of His50 by Gln in α-sarcin does not completely inactivate the enzyme but decreases the k_{cat} value, indicating this residue would be involved in catalysis. It has been reported that H49A restrictocin mutant does not produce the α-fragment when assayed against ribosomes at low concentrations (60–300 nM), but extensive degradation of ribosomal RNA was observed with increasing amounts of protein (1.5–9.0 μM).[54] A recent article notes that H49Y mitogillin is not cytotoxic for yeast and shows only residual activity when assayed against ribosomes.[48] α-Sarcin is a highly specific ribonuclease when assayed against cell-free intact ribosomes. However, it can cause extensive progressive digestion of total rRNA or 28S rRNA with no formation of the α-fragment when used at higher concentrations.[10,42] Even DNA can be digested by α-sarcin when large enzyme:substrate ratios (more than 100) are assayed.[42] The H50Q mutant is not able to hydrolyze the rRNA with subsequent production of the α-fragment when used against ribosomes at very low concentration (15–40 nM), and it is also inactive even when 5 μg are assayed against poly(A) in a zymogram experiment at either neutral or acid pH. However, it is still active against ApA substrate at *micromolar* concentrations, indicating that it is not devoid of its ability to hydrolyze a phosphodiester bond. His40 in RNase T1 has been proposed to participate under its protonated form in electrostatic stabilization of the transition state.[25] The protonated His40 side-chain accounts for a factor of approximately 2,000 in the overall rate acceleration of dinucleotide transesterification by wild-type RNase T1.[25] This may be the case for His50 in α-sarcin, and its mutation would render a variant protein with lower activity.

TABLE III. Best-fit Parameters for the pH Dependence of k_{cat} for ApA Transesterification Compared with the pK_a Values Derived from NMR

	Kinetics[a]		NMR[b]
	Wild-type	H50Q	Wild-type-2′GMP complex
pK_B	3.8 ± 0.1	4.4 ± 0.1	
pK_A	6.7 ± 0.1	8.0 ± 0.2	
pK_{His50}			7.6
pK_{Glu96}			~4.8
pK_{His137}			6.8

[a]Determined by nonlinear regression analysis using the equation $k_{cat} = (k_{cat})^*/(1 + H/K_B + K_A/H)$, where $(k_{cat})^*$ is the pH-independent value of k_{cat}, H is the proton concentration, and K_A and K_B are macroscopic acid dissociation constants. The pH dependence of k_{cat} has been also analyzed by considering the involvement of the ionization equilibria of three and four residues. Standard errors are also given.
[b]Microscopic acid dissociation constants determined from NMR measurements.[34]

The profile obtained for the pH-dependence of the α-sarcin activity is typical for an acid-base catalyst, involving a proton-donor group (general acid), His137, and a proton-accepting group (general base), Glu96. The absence of a charged residue as His50 in the proximity of the Glu96 side-chain obviously destabilizes the carboxylate form and would consequently raise its pK_a value, allowing Glu96 to act as general base at higher pH than in wild-type α-sarcin. This fact could explain the differences observed between wild-type and H50Q α-sarcin in terms of their activity dependence on pH (Fig. 7). In fact, the pK_a of the proton-accepting group is increased in the H50Q variant (Table III).

Finally, it is remarkable how the relative amount of the products resulting from the ApA hydrolysis is different when either α-sarcin or H50Q is used (Fig. 8). α-Sarcin, as most cyclizing ribonucleases do,[55] displays a low efficiency in catalyzing the conversion of the cyclic to the 3′-mononucleotide; however, this behavior is altered in the H50Q variant, in which the production of 3′-AMP is clearly increased at neutral pH (Fig. 8). It has been recently reported that the second step of the reaction catalyzed by RNases A and T1 is readily reversible.[56]. Therefore, a potential explanation for the result obtained with the H50Q variant would be related to a change in the rate constant of such a step upon the mutation.

In summary, the results presented here are consistent with glutamic acid 96 and histidine 137 acting as general base and general acid, respectively, in the catalysis by α-sarcin. The role of histidine 50 would be related to the stabilization of the transition state of the transesterification process.

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REFERENCES

1. Olson BH, Goerner GL. α-Sarcin, a new antitumor agent. I. Isolation, purification, chemical composition and the identity of a new amino acid. Appl Microbiol 1965;13:314–321.
2. Olson BH, Jennings JC, Roga V, Junek AJ, Schuurmans DM. α-Sarcin, a new antitumor agent. II. Fermentation and antitumor spectrum. Appl Microbiol 1965;13:322–326.
3. Kao R, Davies J. Fungal ribotoxins: a family of naturally engineered targeted toxins? Biochem Cell Biol 1995;73:1151–1159.
4. Wirth J, Martínez del Pozo A, Mancheño JM, et al. Sequence determination and molecular characterization of gigantin, a cytotoxic protein produced by the mould *Aspergillus giganteus* IFO 5818. Arch Biochem Biophys 1997;343:188–193.
5. Fando JL, Alaba I, Escarmis C, Fernández-Luna JL, Méndez E, Salinas M. The mode of action of restrictocin and mitogillin on eukaryotic ribosomes. Inhibition of brain protein synthesis, cleavage and sequence of the ribosomal RNA fragment. Eur J Biochem 1985;149:29–34.
6. Lamy B, Davies J, Schindler D. The *Aspergillus* ribonucleolytic toxins (ribotoxins). In: Frankel AE, editor. Genetically engineered toxins. New York: Marcel Dekker, Inc.; 1992. p 237–258.
7. Wool IG, Glück A, Endo Y. Ribotoxin recognition of ribosomal RNA and a proposal for the mechanism of translocation. Trends Biochem Sci 1992;17:266–269.
8. Gasset M, Mancheño JM, Lacadena J, et al. α-Sarcin, a ribosome-inactivating protein that translocates across the membrane of phospholipid vesicles. Curr Top Pept Protein Res 1994;1:99–104.
9. Endo Y, Wool IG. The site of action of α-sarcin on eukaryotic ribosomes: the sequence at the α-sarcin cleavage site in 28S ribosomal ribonucleic acid. J Biol Chem 1982;257:9054–9060.
10. Wool IG. The mechanism of action of the cytotoxic nuclease α-sarcin and its use to analyze ribosome structure. Trends Biochem Sci 1984;9:14–17.
11. Glück A, Wool IG. Determination of the 28S ribosomal RNA identity element (G4319) for α-sarcin and the relationship of recognition to the selection of the catalytic site. J Mol Biol 1996;256:838–840.
12. Szewczak AA, Moore PB. The sarcin/ricin loop, a modular RNA. J Mol Biol 1995;247:81–98.
13. Campos-Olivas R, Bruix M, Santoro J, et al. ^{1}H and ^{15}N nuclear magnetic resonance assignment and secondary structure of the cytotoxic ribonuclease α-sarcin. Protein Sci 1996;5:969– 972.
14. Campos-Olivas R, Bruix M, Santoro J, et al. Structural basis for the catalytic mechanism and substrate specificity of the ribonuclease α-sarcin. FEBS Lett 1996;399:163–165.
15. Yang X, Moffat K. Insights into specificity of cleavage and mechanism of cell entry from the crystal structure of the highly specific *Aspergillus* ribotoxin, restrictocin. Structure 1996;4:837–852.
16. Sacco G, Drickamer K, Wool IG. The primary structure of the cytotoxin α-sarcin. J Biol Chem 1983;258:5811–5818.
17. López-Otín C, Barber D, Fernández-Luna JL, Soriano F, Méndez E. The primary structure of the cytotoxin restrictocin. Eur J Biochem 1984;143:621–634.
18. Fernández-Luna JL, López-Otín C, Soriano F, Méndez E. Complete amino acid sequence of the *Aspergillus* cytotoxin mitogillin. Biochemistry 1985;24:861–869.
19. Martínez del Pozo A, Gasset M, Oñaderra M, Gavilanes JG. Conformational study of the antitumor protein α-sarcin. Biochim Biophys Acta 1988;953:280–288.
20. Mancheño JM, Gasset M, Lacadena J, Martínez del Pozo A, Oñaderra M, Gavilanes JG. Predictive study of the conformation of the cytotoxic protein α-sarcin: a structural model to explain α-sarcin-membrane interaction. J Theor Biol 1995;172:259–267.
21. Steyaert J. A decade of protein engineering on ribonuclease T1. Atomic dissection of the enzyme-substrate interactions. Eur J Biochem 1997;247:1–11.
22. Heinemann U, Saenger W. Specific protein-nucleic acid recognition in ribonuclease T1–2′-guanylic acid complex. An X-ray study. Nature 1982;299:27–31.
23. Nishikawa S, Morioka H, Fuchimura K, et al. Modification of Glu58, an amino acid of the active center of ribonuclease T1, to Gln and Asp. Biochem Biophys Res Commun 1986;138:789–794.
24. Nishikawa S, Morioka H, Kim HJ, et al. Two histidine residues are essential for ribonuclease T1 activity as is the case for ribonuclease A. Biochemistry 1987;26:8620–8624.
25. Steyaert J, Hallegh K, Wyns L, Stansens P. Histidine-40 of ribonuclease T1 acts as base catalyst when the true catalytic base, glutamic acid-58, is replaced by alanine. Biochemistry 1990;29: 9064–9072.
26. Georgalis Y, Zouni A, Hahn U, Saenger W. Synthesis and kinetic study of transition state analogs for ribonuclease T1. Biochim Biophys Acta 1991;1118:1–5.
27. del Cardayré SB, Ribó M, Yokel EM, Quirk DJ, Rutter WJ, Raines RT. Engineering ribonuclease A: production, purification and characterization of wild-type enzyme and mutants at Gln 11. Protein Eng 1995;8:261–273.
28. Nonaka T, Nakamura KT, Uesugi S, Ikehara M, Irie M, Mitsui Y. Crystal structure of ribonuclease Ms (as a ribonuclease T1 homologue) complexed with a guanylyl-3′,5′-cytidine analogue. Biochemistry 1993;32:11825–11837.
29. Egami F, Oshima T, Uchida T. Specific interactions of base-specific nucleases with nucleosides and nucleotides. Mol Biol Biochem Biophys 1980;32:250–277.
30. Pfeiffer S, Karini-Nejad Y, Rüterjans H. Limits of NMR structure determination using variable target function calculations: ribonuclease T1 a case study. J Mol Biol 1997;266:400–426.
31. Noguchi S, Yoshinori S, Uchida T, Sasaki C, Natsuzaki T. Crystal structure of *Ustilago sphaerogena* ribonuclease U2 at 1.8 Å resolution. Biochemistry 1995;34:15583–15591.
32. Lacadena J, Martínez del Pozo A, Lacadena V et al. The cytotoxin α-sarcin behaves as a cyclizing ribonuclease. FEBS Lett 1998;424: 46–48.
33. Lacadena J, Mancheño JM, Martínez-Ruiz A, et al. Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin. Biochem J 1995;309:581–586.
34. Pérez-Cañadillas JM, Campos-Olivas R, Lacadena J, et al. Characterization of pKa values and titration shifts in the cytotoxic ribonuclease α-sarcin by NMR. Relationship between electrostatic interactions, structure and catalytic function. Biochemistry 1998; 37:15865–15876.
35. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.
36. Lacadena J, Martínez del Pozo A, Barbero JL, et al. Overproduction and purification of biologically active native fungal α-sarcin in *Escherichia coli*. Gene 1994;142:147–151.
37. Kunkel TA, Roberts JD, Zakour RA. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol 1987;154:367–382.
38. Rosenberg AH, Lode BN, Chui D, Liu S, Dunn JJ, Studier FW. Vectors for expression of cloned DNAs by T7 RNA polymerase. Gene 1987;56:125–135.
39. Ghrayeb J, Kimura H, Tanaka M, Hsiung H, Masui Y, Inouye M. Secretion cloning vectors in *Escherichia coli*. EMBO J 1984;3:2437–2442.
40. Dente L, Cortese R. pEMBL: a new family of single standed plasmids for sequencing DNA. Methods Enzymol 1987;155:111–119.
41. Schindler DG, Davies JE. Specific cleavage of ribosomal RNA caused by α-sarcin. Nucleic Acids Res 1977;4:1097–1110.
42. Endo Y, Huber P, Wool IG. The ribonuclease activity of the cytotoxin α-sarcin: the characteristics of the enzymatic activity of α-sarcin with ribosomes and ribonucleic acids as substrates. J Biol Chem 1983;258:2662–2667.
43. Blank A, Sugiyama RH, Dekker C. Activity staining of nucleolytic enzymes after sodium dodecyl-sulfate-polyacrylamide gel electrophoresis: use of aqueous isopropanol to remove detergent from gel. Anal Biochem 1982;120:267–275.
44. Shapiro R, Fett JW, Strydom DJ, Vallee BL. Isolation and characterization of a human colon carcinoma-secreted enzyme with pancreatic-ribonuclease-like activity. Biochemistry 1986;25: 7255–7264.
45. Gavilanes JG, Vázquez D, Soriano F, Méndez E. Chemical and spectroscopic evidence of the homology of three antitumor pro-

teins: α-sarcin, mitogillin and restrictocin. J Protein Chem 1983;2: 251–261.
46. Perczel A, Hollósi M, Tusnády G, Fasman GD. Convex constraint analysis: a natural deconvolution of circular dichroism curves. Protein Eng 1991;4:669–679.
47. Wüthrich K. NMR of proteins and nucleic acids. New York: John Wiley & Sons; 1986.
48. Kao R, Shea JE, Davies JE, Holden DW. Probing the active site of mitogillin, a fungal ribotoxin. Mol Microbiol 1998;29:1019–1027.
49. Yang R, Kenealy WR. Effects of amino terminal extensions and specific mutations on the activity of restrictocin. J Biol Chem 1992;267:16801–16805.
50. Brandhorst T, Yang R, Kenealy WR. Heterologous expression of the cytotoxin restrictocin in *Aspergillus nidulans* and *Aspergillus niger*. Protein Expr Purif 1994;5:486–497.
51. Rodríguez R, López-Otín C, Barber D, Fernández-Luna JL, González G, Méndez E. Amino acid sequence homologies in α-sarcin, restrictocin and mitogillin. Biochem Biophys Res Commun 1982; 108:315–321.
52. Sylvester ID, Roberts LM, Lord JM. Characterization of prokaryotic recombinant *Aspergillus* ribotoxin α-sarcin. Bichim Biophys Acta 1997;1358:53–60.
53. Grunert H, Zouni A, Beineke M, et al. Studies on RNase T1 mutants affecting enzyme catalysis. Eur J Biochem 1991;197:203–207.
54. Nayak SK, Batra JK. A single amino acid substitution in ribonucleolytic toxin restrictocin abolishes its specific substrate recognition activity. Biochemistry 1997;36:13693–13699.
55. Thompson JE, Venegas FD, Raines RT. Energetics of catalysis by ribonucleases: fate of the 2′,3′-cyclic phosphodiester intermediate. Biochemistry 1994;33:7408–7414.
56. Loverix S, Laus G, Martins JC, Steyaert J. Reconsidering the energetics of ribonuclease catalysed RNA hydrolysis. Eur J Biochem 1998;257:286–290.
57. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Crystallogr 1991;24: 946–950.