Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin

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The α-sarcin cytotoxin is an extracellular fungal protein that inhibits protein biosynthesis by specifically cleaving one phosphodiester bond of the 28 S rRNA. The His^{137} residue of α-sarcin is suggested to be involved in the catalytic activity of this protein, based on the observed sequence similarity with some fungal ribonucleases. Replacement of this residue by Gln (H137Q mutant variant of α-sarcin) abolishes the ribonuclease activity of the protein. This has been demonstrated for an homogeneous preparation of the H137Q α-sarcin by measuring its effect against both intact rabbit ribosomes and the homopolymer poly(A). The conformation of H137Q α-sarcin is highly similar to that of the wild-type protein, which has been analysed by CD and fluorescence spectroscopy. Both H137Q and wild-type α-sarcin exhibit identical CD spectra in the peptide-bond region, indicating that no changes at the level of the secondary structure are produced upon mutation. Only minor differences are observed in both near-UV CD and fluorescence emission spectra in comparison to those of the wild-type protein. Moreover, H137Q α-sarcin interacts with phospholipid vesicles, promoting the same effects as the native cytotoxin. Therefore, we propose that His^{137} is part of the ribonucleolytic active site of the cytotoxin α-sarcin.

INTRODUCTION

α-Sarcin is a type I ribosome-inactivating protein (RIP) secreted by the mould *Aspergillus giganteus* MDH 18894 (see [1] for a review). It cleaves only one phosphodiester bond of the 28 S rRNA when ribosomes are used as substrate, producing the α-fragment (about 400 nt) and consequently inhibiting protein biosynthesis. The nucleotide sequence at the cleavage site is evolutionarily conserved in all eukaryotes and prokaryotes so far tested [1]. This region has an essential role in ribosome function because it is involved in both elongation factor-1 (EF-1)-dependent binding of aminoacyl-tRNA and EF-2-catalysed GTP hydrolysis and translocation [1].

The elements of the nucleic acid structure that are recognized by α-sarcin have been studied and determined in considerable detail [1–3] but very little is known about the amino acid residues that form the active site of this RIP. A significant degree of sequence similarity between α-sarcin and other fungal ribonucleases from the RNase T1 subfamily has been reported [4,5], although these RNases contain about 40 residues less than α-sarcin. According to these observations, the residues His^{50}, Glu^{96}-Phe^{97}-Pro^{98}, Arg^{121} and His^{137} would be involved in the ribonucleolytic activity of α-sarcin since the corresponding equivalent amino acids form the active sites of these fungal RNases [4–6]. Restrictocin, another RIP, produced by *Aspergillus restrictus*, exhibits about 85% sequence similarity with α-sarcin [7,8]. Yang and Kennealy [9] studied the cytotoxic activities of restrictocin with N-terminal extensions and specific mutations using systems *in vitro* and *in vivo*. According to their results, only the *Saccharomyces cerevisiae* transformant expressing a H136L mutant (His^{136} replaced by Leu) was able to grow. Indeed, translation *in vitro* of H136L did not result in significant self-inhibition of protein biosynthesis. Therefore, they proposed that His^{136} is putatively in the active site of restrictocin [9]. These authors have recently reported [10] the expression of H136L in *Aspergillus niger* and *Aspergillus nidulans*. Both native and mutant proteins reacted identically to anti-restrictocin serum and eluted at the same time from a reverse-phase HPLC column, which suggests that the surface amino acids of the mutant protein were not significantly altered [10]. However, the altered activity of the H136L mutant protein may be attributed to either conformation or active site changes, as these authors pointed out [9,10], since the structures of restrictocin and its mutants are not known. His^{136} in restrictocin is the homologous residue to His^{137} in α-sarcin since the former protein contains 149 amino acids instead of the 150 of α-sarcin [8]. In the present study we show that a purified mutant variant of α-sarcin, where His^{137} has been replaced by Gln, is catalytically inactive. Indeed, this mutant protein promotes in phospholipid vesicles the same effects as the wild-type protein [11–15] and no significant conformational changes are deduced from its spectroscopic characterization.

EXPERIMENTAL

DNA manipulations

All materials and reagents used were of molecular biology grade. Cloning procedures and bacteria manipulations were carried out according to standard methods [16] as described previously [16,18,19]. Oligonucleotide site-directed mutagenesis was used to replace His^{137} with Gln. The method is based on that designed by Kunkel et al. [17] and it was performed as described before [18,19]. Recombinant α-sarcin has been recently produced in *Escherichia coli* [19]. The plasmid then used (pINPGαS) was the starting material to perform the mutagenesis. It was cleaved with *Xba*I and the small fragment thus obtained, containing the α-sarcin coding DNA, was subcloned in pEMBL18(+) [20]. This allowed the single-stranded DNA necessary to carry out the mutagenesis to be obtained, after hybridization with the following mutagenic primer: 5′-CATTGCTCAAACTAAGGAG-3′ (the

Abbreviations used: DMPG, dimyristoylphosphatidylglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; EF, elongation factor; ET, resonance energy transfer; H136L, a mutant variant of restrictocin where His^{136} has been replaced by Leu; H137Q, a mutant variant of α-sarcin where His^{137} has been replaced by Gln; NBD-PE, *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine; Rh-PE, N-(lissamine rhodamine B sulphonyl)-diacylphosphatidylethanolamine; RIP, ribosome-inactivating protein.

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position changed is underlined). The mutated H137Q (a mutant variant of α-sarcin where His^{137} has been replaced by Gln) α-sarcin-coding DNA was cloned again in the original construct [19], rendering a new plasmid, designated as pINPGαSH137Q.

Proteins production and purification

The plasmid pINPGαSH137Q was used to overproduce the α-sarcin mutant variant under very similar conditions to those previously described [19]. Instead of Luria–Bertani medium, minimal M9 medium was used [16] because this change results in a significant increase in the amount of soluble α-sarcin. The isopropyl-β-D-thiogalactoside-induced cells were grown overnight at 37 °C. The extracellular medium and cellular pellet were treated as described [19]. The fractions containing soluble H137Q were used to purify the protein according to the procedure already described [19]. Fungal wild-type α-sarcin was purified essentially according to [21], with the modifications described in [22]. Polyacrylamide electrophoresis of proteins [23], HPLC fractionations, protein hydrolyses and amino acid analyses were performed as described [19].

Spectroscopic characterization

Absorbance measurements were carried out on a Uvikon 930 spectrophotometer at 100 nm/min scanning speed, at room temperature and in 1 cm optical-path cells. CD spectra were obtained on a Jobin-Yvon Mark III dichrograph fitted with a 250 W xenon lamp, at 0.2 nm/s scanning speed; 0.01 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·cm^2·$dmol^{-1}$. Fluorescence emission spectra were obtained on a Perkin-Elmer MPF 44E spectrofluorimeter at 25 °C and 1 nm/min scanning speed in 0.2 cm optical-path cells.

Interaction with phospholipids

Large dimyristoylphosphatidylglycerol (DMPG) (Avanti Polar Lipids) unilamellar vesicles were prepared as previously described [11–15]. A dry lipid film of DMPG was hydrated in Mops buffer (50 mM Mops, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA) for 60 min at 37 °C. The lipid suspension was further subjected to five cycles of extrusion through polycarbonate filters (0.1 μm pore diameter) (Nuclepore Corporation) [24]. Lipid concentration was determined as described [25]. The aggregation of phospholipid vesicles was monitored by measuring the increase in the absorbance at 360 nm of a suspension of large unilamellar vesicles of DMPG in Mops buffer (30 μM final lipid concentration) after addition of a small aliquot of a freshly prepared solution of protein [11]. The measurements were performed on a Beckman DU-8 thermostated spectrophotometer at 37 °C with 1 cm optical-path cuvettes (1 ml total volume). Controls without protein were also considered. To analyse the effect of the protein on the thermotropic behaviour of the DMPG vesicles, these were labelled with 1,6-diphenyl-1,3,5-hexatriene (DPH) (1000:1 lipid:DPH weight ratio) as previously described [11]. After equilibration of either vesicles or protein–vesicles mixtures at the corresponding temperature, the fluorescence emission of DPH at 425 nm, for excitation at 365 nm, was measured in thermostated cells. Lipid-mixing of the vesicles was assayed as described [12]. Freshly prepared α-sarcin (wild-type or mutant) in Mops buffer was added to a vesicle suspension composed of a 1:9 mixture of fluorescence-labelled and unlabelled DMPG vesicles (145 μM final lipid concentration; 1 ml total volume). Labelled vesicles contained 1% *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine (NBD-PE) and 0.6% *N*-(lissamine rhodamine B sulphonyl)-diacylphosphatidylethanolamine (Rh-PE) (Avanti Polar Lipids). The percentage of energy transfer (%ET) was calculated according to the equation $(\%ET) = (1 - F/F_0) \times 100$, where F is the fluorescence intensity at 530 nm of the sample at equilibrium, after addition of the corresponding amount of protein, and F_0 is the fluorescence intensity at 530 nm of a DMPG vesicle suspension labelled with 0.1% NBD-PE (145 μM total lipid concentration). All these fluorescence measurements were carried out on an SLM Aminco 8000 spectrofluorimeter, as described [11,12], in cells of 0.2 or 0.4 cm optical-path, for excitation at 450 nm. Glan–Thompson polarizers (90°/0°) were used to avoid the potential contribution of the sample light scattering to the fluorescence signal.

Ribonucleolytic activity

The specific ribonucleolytic activity of α-sarcin was followed by detecting the release of the 400 nt α-fragment [26,27] from the 28 S rRNA of eukaryotic ribosomes. This activity was measured by using a cell-free rabbit reticulocyte lysate (Promega) as described [19,28]. This lysate was treated with wild-type or H137Q α-sarcin (40 ng). The resulting RNA was analysed on 2.4% agarose gel and visualized after ethidium bromide staining. The ribonucleolytic activity of the purified proteins against poly(A) (Sigma) [29] was studied as described [30], by using 0.1% SDS/15% PAGE [23] containing 0.3 mg/ml of poly(A). Once the electrophoresis was performed, the gel was treated as described in [30] and incubated in 0.1 M Tris-HCl, pH 7.5, at 37 °C for 3 h. Then, the gel was stained with 0.2% Toluidine Blue and washed with water to obtain the desired contrast.

RESULTS

Protein purification

The α-sarcin coding DNA region of pINPGαSH137Q was completely sequenced, confirming that the only change produced was the substitution of T^{492} [31] by A, rendering a plasmid

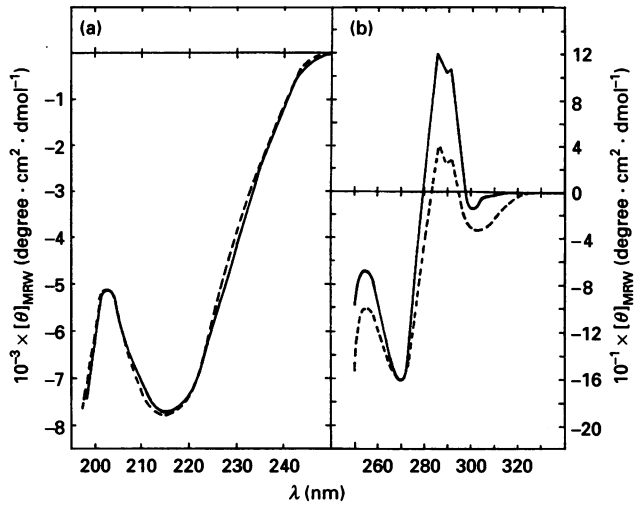

Figure 1 CD spectra of α-sarcin and its H137Q variant

(**a**) Far-UV and (**b**) near-UV CD spectra of wild-type α-sarcin (——) and its mutant variant H137Q (– – – –). The proteins were dissolved in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl. Mean residue weight (MRW) ellipticity values are expressed in units of degree·cm^2·$dmol^{-1}$, 113 being the mean residue weight for the amino acids of these proteins.

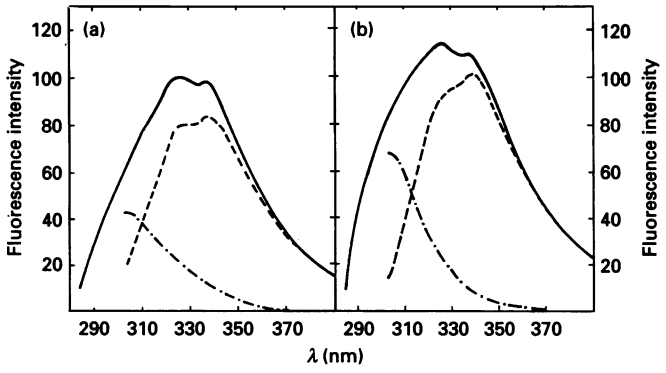

Figure 2 Fluorescence emission spectra of α-sarcin and its H137Q variant

Fluorescence spectra of wild-type α-sarcin (**a**) and its H137Q variant (**b**). These spectra have been recorded for excitation at 275 (——) and 295 (– – – –) nm after normalization. The Tyr contribution calculated as described in [46] is also indicated (–·–·–). The proteins were dissolved in 50 mM Mops 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 an excitation wavelength of 275 nm, as 100%.

that produces the desired mutant. This H137Q variant of α-sarcin was produced in *E.coli* RB791 (W3110 *lacI*^{q}L8) cells, and purified to homogeneity, according to its electrophoretic and HPLC patterns and amino acid composition. Indeed, the fungal wild-type α-sarcin and this H137Q variant behaved identically when analysed by HPLC and 0.1% SDS/17% PAGE. The yield of this purification was about 1.5–2.0 mg per litre of original cell culture, which is within the same range as that obtained for the wild-type α-sarcin [19].

Spectroscopic characterization

The purified H137Q α-sarcin variant was analysed spectroscopically. An extinction coefficient ϵ (0.1%, 1 cm, 280 nm) of 1.34 was calculated from its UV absorbance spectrum. This value is identical with that previously reported for the wild-type fungal protein [7]. The far-UV CD spectrum of H137Q is indistinguishible from that of the fungal protein (Figure 1a). This indicates that the secondary structure of the protein is not altered by the mutation. However, the near-UV CD spectrum, although similar in shape to that of the wild-type protein, exhibited some differences. The ellipticity values at the different significant wavelengths of the spectrum are modified, although the position of the observed peaks and minima remain essentially unaltered (Figure 1b). This indicates that the micro-environment of some of the aromatic residues of the protein is modified when His^{137} is replaced by Gln, which is confirmed after inspection of the fluorescence emission spectrum of the H137Q variant (Figure 2). In fact, the fluorescence quantum yields of the Tyr and Trp residues are increased about 1.5-fold (1.5 and 1.3 for the Tyr and Trp contributions respectively) in comparison to those in the wild-type α-sarcin.

Interaction with phospholipid vesicles

α-Sarcin is able to interact with negatively charged phospholipid bilayers [11–15,22], promoting vesicle aggregation, lipid-mixing and modification of the thermotropic behaviour of the phospholipids. Thus, we have studied the ability of the α-sarcin H137Q variant to induce these alterations. Part of the results obtained are summarized in Figure 3. The H137Q variant of α-sarcin promotes the same effects as the wild-type protein. The kinetics of the promoted vesicle aggregation, the extent of the lipid-mixing between the vesicles produced, and the induced modi-

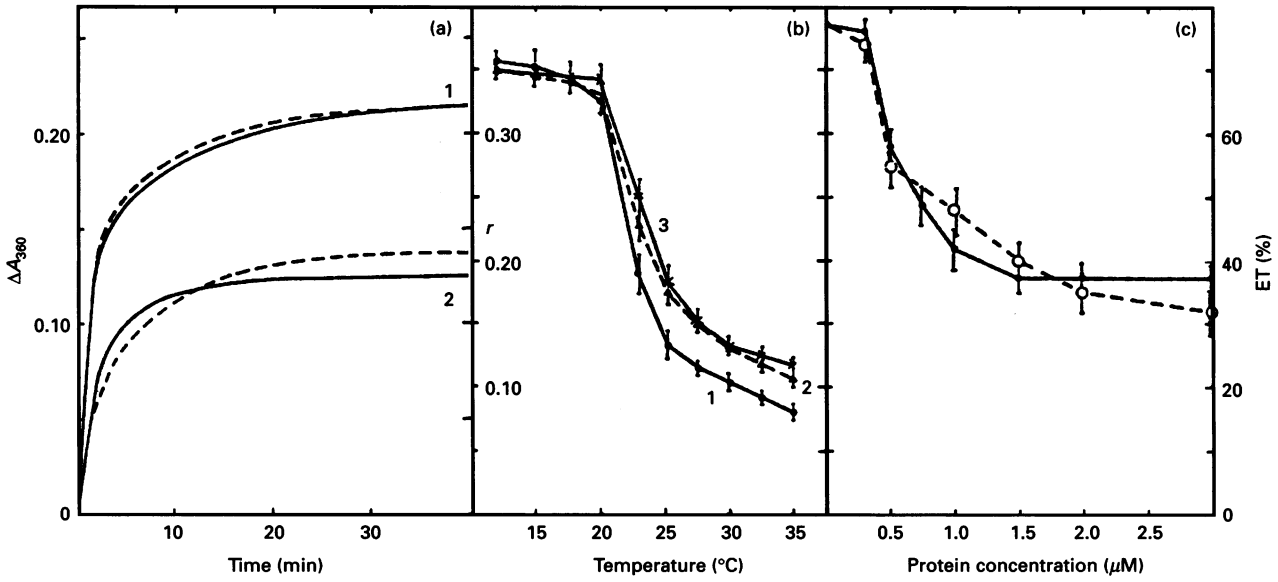

Figure 3 Interaction of H137Q α-sarcin with DMPG vesicles

All the experiments were performed in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, as described [11,12]. (**a**) Typical time-course of the absorbance increase at 360 nm produced by interaction of wild-type (——) and H137Q (– – – –) α-sarcin with DMPG vesicles (30 μM). Two different protein concentrations were used: (1) 1.0 μM and (2) 0.3 μM. (**b**) Phase-transition temperature profiles of DPH-labelled DMPG vesicles. Anisotropy values (r) were recorded as a function of temperature. DMPG vesicles in the absence of protein (1), and in the presence of wild-type α-sarcin (2) and its mutant variant H137Q (3), at a 1:50 protein/lipid molar ratio. The DMPG concentration was 116 μM. Values are expressed as average ± SD of three determinations. (**c**) Effect of α-sarcin (●) and its mutant variant H137Q (○) on the energy transfer between NBD-PE and Rh-PE incorporated into the same DMPG vesicles. The phospholipid concentration was 145 μM. Values are expressed as the average ± SD of three different determinations.

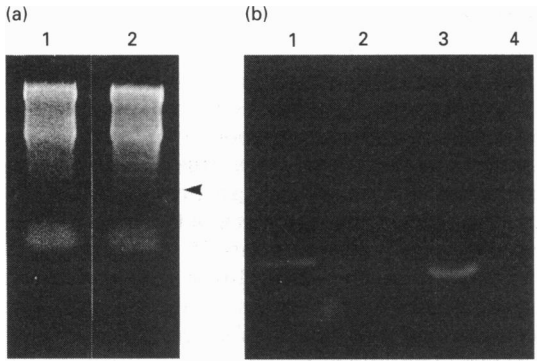

Figure 4 Enzyme characterization of H137Q α-sarcin

(**a**) Specific RNase activity of α-sarcin shown by release of the 400 nt α-fragment (indicated by an arrowhead) from the 28 S rRNA of eukaryotic ribosomes. These ribosomes were incubated with 40 ng of the mutant variant H137Q (lane 1), and with 40 ng of wild-type α-sarcin (lane 2). The limit of detection of this assay is about 10 ng of native α-sarcin. The RNA was separated on 2.4% agarose gel as described previously [19,28]. (**b**) 0.1% SDS/15% PAGE of different amounts of wild-type (lanes 1 and 3) and purified H137Q variant (lanes 2 and 4) α-sarcin. The samples were boiled in loading buffer containing 0.1% SDS, but they were not reduced with 2-mercaptoethanol. In addition, the running-gel contained 0.3 mg/ml of poly(A). Once the electrophoresis was performed, the gel was incubated and stained according to [30] to develop the presence of the RNase activities. Two different amounts of protein were assayed: 0.5 μg (lanes 1 and 2) and 1.0 μg (lanes 3 and 4). This assay allows clear detection of 50 ng of native α-sarcin.

fication of the thermotropic behaviour of DMPG vesicles are identical, within the range of experimental error, when both protein forms are analysed. These results indicate that His^{137} is not a crucial residue for the interaction of the protein with phospholipid bilayers, thus the membrane-destabilizing ability of the protein is fully conserved after the mutation.

Enzymic characterization

We have studied the enzymic activity of the H137Q variant of α-sarcin by considering two different substrates: intact ribosomes and poly(A). From the results presented in Figure 4(a), we conclude that the α-fragment, the characteristic product of the α-sarcin activity in ribosomes, is not produced by the H137Q protein. In addition, the mutant protein does not hydrolyse the poly(A) substrate. We have also considered this homopolymer as substrate because it has been reported that α-sarcin is specific for purines when naked RNA is used to evaluate its ribonucleolytic activity [29]. In Figure 4(b), it can be seen that the mutant is devoid of catalytic activity whereas the wild-type α-sarcin activity is clearly visible in the poly(A)-containing gels.

DISCUSSION

A mutant variant of α-sarcin where His^{137} has been changed to Gln (H137Q) has been produced and purified to homogeneity. The H137Q variant is indistinguishable from the wild-type α-sarcin when analysed by HPLC. Improper folding or partial denaturation would probably have changed the retention times of the H137Q protein. Indeed, the CD spectra in the peptide-bond region of both the wild-type and the H137Q α-sarcins are identical (Figure 1a). This indicates that the secondary structure of the protein is not significantly affected by the mutation. As indicated above, α-sarcin exhibits a significant degree of sequence similarity with several fungal ribonucleases belonging to the RNase T1 subfamily [5]. The three-dimensional structure of three of these proteins, RNases T1, Ms and F1, has been resolved at high resolution by X-ray diffraction studies [32–34]. Chemical and crystallographic studies have shown that the hydrolysis of RNA catalysed by RNase T1 occurs in two steps: first, transesterification of RNA to yield oligonucleotides with terminal guanosine 2′,3′-cyclic bisphosphate, and secondly, hydrolysis of this bisphosphate to produce terminal guanosine 3-monophosphate. The amino acid residues involved in this reaction are: (1) the pairs His^{40}/Glu^{58}, which take the proton from O_2,H (His^{40} is probably involved in the positioning or activation of O_2,H) in the first step; (2) His^{92}, which donates a proton to the leaving $O_{5'}$ group and activates the water molecule for hydrolysis; and (3) Tyr^{38} and Arg^{77}, which are involved in positioning, and the latter in addition neutralizes the incoming negatively charged phosphate [35–39]. Indeed, these crystallographic studies have also shown that the imidazole side-chain of the active-site His^{92} fulfils two purposes: it serves as a subsite and forms a stack with base N of an enzyme-bound substrate GpN, thereby orientating the scissile phosphodiester group in the active site for proper attack [40]; and it serves as a general acid in the transesterification step of the reaction, as described above, and as a general base that activates a water molecule for the hydrolysis step [37]. A sequence alignment that considers the three-dimensional structures of the mentioned fungal RNases revealed that His^{137} in α-sarcin would be equivalent to His^{92} in RNases T1 and Ms, and to His^{91} in RNase F1 [5]. This histidine (H^{91} or H^{92}) residue is located in the three RNases in a β-bend. His and Gln have very similar β-turn propensities and therefore a change in the secondary structure of α-sarcin should not be expected upon H137Q mutation, in agreement with the observation that the far-UV CD spectrum is unaltered.

The near-UV CD spectrum of H137Q α-sarcin (Figure 1b) shows a similar shape to that of the wild-type α-sarcin. An ellipticity maximum can be seen at 286 nm and a shoulder at 292 nm is also visible. Extreme ellipticity values at 302, 270 and 255 nm are also observed in this spectral analysis. However, significant quantitative differences are detected when the spectra of H137Q and wild-type α-sarcin are compared. The ellipticity values at 286 nm are 120 and 40 degree·cm^2·$dmol^{-1}$ for the wild-type and H137Q proteins respectively. The CD bands at 286 and 292 nm should arise from tryptophan contributions, based on the known spectral properties of this protein chromophore. Consequently, the residue at position 137 would affect the micro-environment of Trp^4 and/or Trp^{51} of α-sarcin, the two only tryptophans of this protein. Actually, these two Trp residues are predicted to be in β-strands [5] and the β-strand involving Trp^4 would be close enough to the residue in position 137 if the folding of α-sarcin were similar to that of RNase T1, as has been proposed [5]. From this, we can speculate that the modification in the near-UV CD spectrum of H137Q is due to changes in the micro-environment of Trp^4. It is also remarkable that the near-UV CD spectrum of H137Q is almost identical with that of both restrictocin and mitogillin [7], two proteins that are similar to α-sarcin. Mitogillin and restrictocin both contain 149 amino acids and only one substitution is observed between them (Asn^{26} in mitogillin is substituted by Ser^{26} in restrictocin) [8,41]. When the amino acid sequences of mitogillin and restrictocin are compared with that of α-sarcin only 19 substitutions are observed. Three of them are located in the stretch 137–139 (Gln-Arg-Gly in mitogillin and restrictocin), which is equivalent to residues 138–140 in α-sarcin (Thr-Lys-Glu) (α-sarcin contains 150 amino acids) [4,8,41]. As mentioned above, the H137Q mutation performed in α-sarcin renders a near-UV CD spectrum which is very similar to those of the other two proteins. Therefore, it seems that the amide group of Gln^{137} in H137Q α-sarcin could be equivalent to Gln^{137} in mitogillin and restrictocin.

Figure 2 shows the fluorescence emission spectra of wild-type and H137Q α-sarcin (Figures 2a and 2b respectively). The fluorescence emission of α-sarcin is dominated by the tryptophan contribution. Thus, some change in the fluorescence emission properties of the protein could be expected, based on the results obtained from the near-UV CD measurements. In fact, the H137Q α-sarcin fluorescence emission is increased when compared with that of the wild-type protein. The emission band at 300–305 nm, due to the Tyr contribution, is increased 1.5-fold. The spliced band around 330 nm, the Trp contribution, is increased by a factor of about 1.3. Furthermore, when using the BIOSYM program to calculate the distances existing between the RNase T1 His^{92} imidazole $N_{\epsilon 2}$ and the C_ζ of every Tyr residue contained in this protein, it was observed that this distance is less than 10 Å (1 Å = 0.1 nm) for the residues in sequence positions 38, 42 and 45. These measurements were 6.71, 9.20 and 8.88 Å for Tyr^{38}, Tyr^{42} and Tyr^{45} respectively. Sequence alignment revealed that these RNase T1 residues would be equivalent to Tyr^{48}, Phe^{52} and Tyr^{54} in α-sarcin [5]. Assuming that the α-sarcin folding is similar to that of RNase T1, as has been proposed [5], this spatial proximity would be short enough to allow the influence of α-sarcin His^{137} on the spectroscopic properties of the aromatic residues mentioned. It follows from this that deprotonation of this His, or its replacement by Gln, could modify the micro-environment of these Tyr residues in such a way that it could also explain the spectroscopic changes observed. Indeed, we have also reported that α-sarcin binds one Zn(II) atom, which results in the inhibition of its ribonucleolytic activity [6]. Binding of Zn(II) promoted an increase in the fluorescence emission of α-sarcin as well as a decrease in the ellipticity around 286 nm [6]. Interestingly, this binding did not promote any change in the far-UV CD spectrum. Considering that this Zn(II)-induced inhibition of the α-sarcin ribonucleolytic activity was similar to that produced in RNase A by the binding of Zn(II) or Cu(II) to some His residues [42–44], it was proposed that this process would also take place in α-sarcin via Zn(II) binding to His^{50} and His^{137}[6]. All these spectral changes were similar to those now described for the H137Q α-sarcin, which further supports this possibility.

In addition, three His residues (sequence positions 27, 40 and 92) of RNase T1 have an anomalous high pK_a value [45]. These three histidines correspond to residues 36, 50 and 137 in α-sarcin, based on the above-mentioned sequence alignment [5]. The pK_a of the three mentioned RNase T1 histidines are 7.2, 7.8 and 7.9 respectively, when a pK_a of about 6.4 would be expected for histidine residues in an unfolded conformation [45]. α-Sarcin exhibits a poorly explained conformational transition centered around pH 8.0, which results in increased fluorescence emission and decreased ellipticity at 286 nm [46]. Removal of His^{137} could be considered equivalent to a deprotonation of this residue. Therefore, substitution of this His by Gln could mimic a deprotonated imidazole ring in that sequence position. The spectral changes observed would reinforce this hypothesis. Therefore, the conformational transition observed at pH 8.0 in α-sarcin would be related to these histidine residues.

The α-sarcin passage across cell membranes could be related to its recognition by some membrane protein receptor. However, no results revealing such a possibility have been reported so far. On the other hand, although the molecular mechanism of this internalization may remain elusive, the involvement of membrane phospholipids seems to be clear. Actually, we have described how α-sarcin produces aggregation of phospholipid vesicles and exhibits specific requirements for acid lipids [11]. The protein also promotes the fusion of lipid bilayers [12]. In this regard, the cytotoxicity of α-sarcin can be modelled as occurring in two steps: first, interaction with the membrane lipids and entering cells and secondly, specific ribonucleolytic action on ribosomes. We have also studied the potential interaction of the α-sarcin mutant variant H137Q with lipid vesicles. The observed preservation of its ability to perturb negative phospholipid vesicles is further proof in support of the notion that the three-dimensional structure of the protein is preserved after the mutation. H137Q α-sarcin induces the aggregation of DMPG liposomes to the same extent as its wild-type counterpart. The same result is obtained whether saturating or non-saturating protein/lipid ratios are employed (Figure 3a) [11]. Indeed, it has been described how α-sarcin modifies the thermotropic behaviour of DMPG vesicles [11]. This analysis has also been performed with the H137Q mutant variant, and both proteins, wild-type and mutant, behave undistinguishably (Figure 3b). Finally, the intermixing of membrane lipids resulting from membrane fusion can be monitored by using ET measurements. The concept relies upon the ability of a fluorescence donor to excite an energy acceptor if both are free to diffuse in the same membrane at an appropriate surface density [47]. From this type of experiment it was concluded that α-sarcin is able to promote the fusion of bilayers [12]. The behaviour of the two protein forms is also identical within the range of experimental error (Figure 3c). Therefore, the replacement of α-sarcin His^{137} by Gln does not impair the ability of this protein to interact with negatively charged phospholipid vesicles.

The results presented above indicate that H137Q displays a highly similar three-dimensional structure to that of the native protein. Only small local changes in the environment of some aromatic residues are produced. Indeed, the ability to interact with negative phospholipid bilayers is also preserved. Therefore, this purified mutant variant of α-sarcin was a good candidate to test the potential catalytic role of His^{137}. We therefore tested the ability of the wild-type and H137Q α-sarcin to degrade either intact ribosomes or the naked homopolymer poly(A). In both sets of experiments, no activity was found for the mutant preparation, in contrast to the wild-type protein which was fully active against both types of substrate (Figure 4). Therefore, it can be concluded that α-sarcin His^{137} is an essential residue for its catalytic activity. Whether this residue is strictly catalytic or just contibutes to the accommodation of the substrate cannot be unequivocally discerned. His^{92} seems to play both roles in RNase T1 [37,40,48,49], as mentioned above. However, when this RNase T1 residue is mutated to Gln, the dominant effect of the mutation is on $k_{cat.}$, suggesting its involvement in catalysis rather than in substrate binding [48]. If the mentioned similarity between RNase T1 and α-sarcin were extended to the mechanism of action of both proteins, His^{137} of α-sarcin would be involved in catalysis.

In summary, substitution of His^{137} by Gln in α-sarcin renders a catalytically inactive protein without greatly modifying its structural organization. As far as we know, this is the first time that any amino acid residue of this protein has been directly identified as part of the active site. Indeed, this information could be very valuable in the design of attenuated forms of α-sarcin for use in a protein cytotoxicity context for the production of non-toxic antigenic variants of this cytotoxin.

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