the FEBS Journal

The bactericidal action on *Escherichia coli* of ZF-RNase-3 is triggered by the suicidal action of the bacterium OmpT protease

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Keywords
bactericidal activity; *Danio rerio; Escherichia coli;* RNase; zebrafish

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(Received 17 December 2009, revised 4 February 2010, accepted 8 February 2010)

doi:10.1111/j.1742-4658.2010.07614.x

ZF-RNase-3 is one of the RNases from zebrafish (*Danio rerio*) with special (i.e. noncatalytic) properties. These include angiogenic and bactericidal activities. Given the interest of fish RNases as host-defense effectors, we studied the mechanism of the bactericidal action of ZF-RNase-3 on *Escherichia coli* as a model Gram-negative bacterium. The results obtained indicate that the bactericidal activity of ZF-RNase-3 is not lost when its catalytic RNase activity is obliterated. On the other hand, fully denatured ZF-RNase-3 conserves its bactericidal activity. When ZF-RNase-3 is added to *E. coli* cultures, it is cleaved at a specific Arg-Arg peptide bond, thus engendering two peptide fragments. The larger fragment (residues 31–124), produced by proteolysis and reduction of a disulfide, is recognized as the actual bactericidal agent. The protease responsible for the proteolytic attack has been identified with OmpT, an outer membrane *E. coli* omptin protease. However, the most remarkable result obtained in the present study is the finding that the microbicidal action of ZF-RNase-3 can be achieved only with the suicidal cooperation of the bacterium itself.

Introduction

The vertebrate RNase superfamily comprises extracellular, small basic enzymes, encoded by a single exon, active on RNA substrates with a preference for phosphodiester bonds involving pyrimidines at the 3′ side. This superfamily, formerly named after its prototype (i.e. RNase A; bovine pancreatic RNase A), one of the best studied enzyme and protein models, is the only enzyme superfamily identified in the subphylum of vertebrates [1].

The superfamily includes RNases simply characterized by their RNA degrading property, as well as RNases with surprising, non-enzymatic, biological actions. Subsequent to an initial survey [2], more of these RNases have now been reported as a result of the availability of fish genomic sequences. This led to the preparation and characterization of recombinant fish RNases with angiogenic and/or host-defense properties, including RNases from zebrafish (*Danio rerio*) [3,4] and from Atlantic salmon (*Salmo salar*) [5].

Zebrafish RNases are typical members of the vertebrate superfamily [3–6]. Their genes are enclosed in single exons; they have the characteristic CKxxNTF signature in their amino acid sequences; and they contain three disulfide bonds, by contrast to mammalian, non-angiogenic RNases containing four disulfides. The two His residues and the Lys residue, which are necessary for the catalytic function of an RNase, are present in zebrafish RNases at positions corresponding to

Abbreviations
CFU, colony-forming units; LF-ZF3, large fragment from ZF-RNase-3; ZF-RNase-3, RNase-3 from zebrafish (*Danio rerio*).

those defined for the RNase A prototype and all other investigated RNases.

The present study describes the essential features of the mechanism of bactericidal action on *Escherichia coli* as a model Gram-negative bacterium of a zebrafish RNase, namely ZF-RNase-3 [4], elsewhere termed *Dr*-RNase 1 [3]. The results obtained indicate that the bactericidal activity of ZF-RNase-3 does not depend on the integrity of the RNase active site and, surprisingly, it is preserved even in the fully unfolded enzyme. When ZF-RNase-3 is added to *E. coli* cultures, it is cleaved at a specific Arg-Arg peptide bond, thus engendering two peptide fragments in an event that is essential for promoting bactericidal effects. The larger fragment (LF-ZF3, residues 31–124), produced by proteolysis as well as by reduction of a disulfide, is recognized as the actual bactericidal agent.

The protease responsible for the proteolytic attack is identified with OmpT, an outer membrane *E. coli* omptin protease. However, the most remarkable result obtained in the present study is the finding that the microbicidal action of ZF-RNase-3 can be achieved only with the suicidal cooperation of the bacterium itself.

Results

The bactericidal activity of inactivated or denatured ZF-RNase-3

In agreement with previous findings [3], ZF-RNase-3 was found to display a powerful bactericidal activity on Gram-negative bacteria such as *E. coli*. An LD_{50} value (i.e. the concentration representing the median lethal dose) of 0.75 μM was determined for ZF-RNase-3 tested on *E. coli*, which is comparable to the previously reported value of 1–1.5 μM [3]. At a concentration of 9 μM, virtually all bacterial cells were killed by ZF-RNase-3. The enzyme was also found to be cytotoxic for another Gram-negative bacterium, *Pseudomonas fluorescens*, for which an LD_{50} value of 0.5 μM was determined.

As demonstrated for other anti-pathogen RNases [7–9], and for the RNases from *Salmo salar* [5], we have determined that the RNA degrading activity of ZF-RNase-3 is not necessary for its bactericidal activity. More surprisingly, ZF-RNase-3 was found to be active as a bactericidal agent even when it was fully denatured either by treatment at high temperature (85 °C) or by reduction of the RNase disulfides followed by alkylation of the freed sulfhydryls. The S-alkylation was confirmed by MS, and the unfolded state of each denatured protein was verified through CD analyses (data not shown).

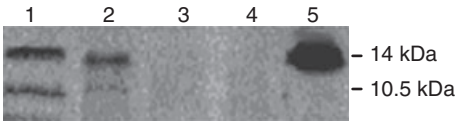

Fig. 1. Western blotting with an antibody against ZF-RNase-3 tested on extracts of *E. coli* treated with ZF-RNase-3. Lane 1, SDS-PAGE run under reducing conditions; 2, SDS-PAGE run under non-reducing conditions; 3, negative control: SDS-PAGE of an extract of untreated *E. coli* run under reducing conditions; 4, as in 3, run under nonreducing conditions; 5, untreated ZF-RNase-3.

The proteolytic cleavage of ZF-RNase-3 by *E. coli*

To monitor the fate of the protein in the bacteria under investigation, an anti-ZF-RNase-3 serum was prepared and utilized to trace ZF-RNase-3 when added to *E. coli* cultures incubated with the RNase for 6 h at a cell density of 4000 colony-forming units (CFU)·mL^{-1}. A bacterial extract was then analysed by SDS-PAGE. Under reducing conditions, two protein components were recognized in western blots with the anti-ZF-RNase-3 serum (Fig. 1). The mobility of the slow component was identical to that of unreacted ZF-RNase-3, whereas the mobility of the faster component was equivalent to that of a 10.5 kDa protein. When the SDS-PAGE run was performed in the absence of reducing agents, the protein ran with the electrophoretic mobility of the untreated protein (Fig. 1). This indicated that a disulfide(s) bond had to be cleaved for the production of the fast protein band.

When the experiment was repeated in the presence of a cocktail of protease inhibitors (see Experimental procedures), the fast component was not produced upon incubation of ZF-RNase-3 with the *E. coli* culture, and only wild-type ZF-RNase-3 was detectable in the bacterial protein extract upon SDS-PAGE followed by western blotting (data not shown). This suggested that the fast moving protein recovered from the *E. coli* culture was a product of digestion of ZF-RNase-3 added to the culture, and that the fast component was engendered not only by cleavage of disulfide(s), but also by proteolysis.

The fast moving component, henceforth termed Large Fragment from ZF-RNase-3 (LF-ZF3), as identified by SDS-PAGE run in the presence of a reducing agent, was removed from the blotted gel and subjected to N-terminal sequencing (see Experimental procedures). The sequence of the first eight residues was determined to be: NH_2-R-I-T-R-F-P-T-G-. As shown in Fig. 2, this sequence identified unequivocally the scissile bond as the Arg-Arg bond at positions 30–31 of the ZF-RNase-3 sequence [4], and indicated that

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                                   ↓
ZF-RNase-3  NH2-EIRRRYEHFLTQHVYGGITEQTCDRVMRQRRITRFPTGNDCKEVNTFIQANGNHVRTVCT
                GGGTRQTDNRDLYMSNNQFTVITCTLRSGERHPNCRYRGKESSRKIVVACEGEWPTHYEK
                GVI-COOH
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Fig. 2. Amino acid sequence of ZF-RNase-3. The eight residues identified by automated Edman degradation in the fragment LF-ZF3 are shown in bold and underlined. The arrow indicates the Arg-Arg scissile bond.

LF-ZF3 was the segment of ZF-RNase-3 with the N-terminal end at position 31 of the protein.

These findings indicated that LF-ZF3 is a proteolytic product of ZF-RNase-3 resulting from its treatment with *E. coli*, and that the scissile bond was between Arg-30 and Arg-31. Furthermore, because ZF-RNase-3 contains a Cys residue at position 23, engaged in a disulfide bridge, as are all Cys residues of ZF-RNase-3 [4], the production of free LF-ZF3 was a result not only of proteolysis, but also cleavage of the disulfide bond linking Cys-23 to Cys-84, as identified from the 3D structure recently reported for ZF-RNase-3 [6].

It should be noted that the LF-ZF3 fragment, fully active as a bactericidal agent with an LD_{50} of 0.75 μM, was found to have no ordered structure upon inspection of its CD spectra (data not shown).

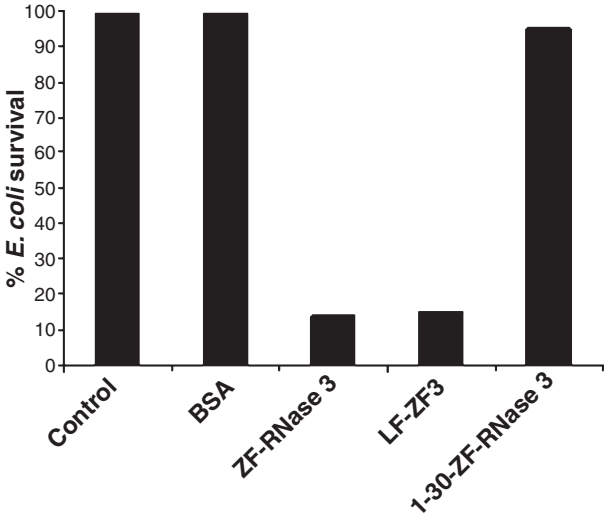

Fig. 3. Bactericidal activity of ZF-RNase-3, the large peptide fragment LF-ZF3, and synthetic peptide 1–30 of ZF-RNase-3. After incubation of proteins and peptide (final concentration of 3 μM) with *E. coli* cells for 6 h at 37 °C, cell survival was determined as a percentage of untreated control. Bovine serum albumin (BSA, 3 μM) was used as a negative control. The average of three measurements is shown. The standard error was lower than 5%.

The bactericidal component of ZF-RNase-3

To investigate the role of LF-ZF3 in the cytotoxic action of ZF-RNase-3 on *E. coli*, a Δ1-30 recombinant variant of the RNase was cloned by PCR from the cDNA encoding the wild-type protein and expressed, as described in Experimental procedures. When this ZF-RNase-3 variant was purified and tested on *E. coli*, it was found to be as active as a bactericidal agent as native ZF-RNase-3 (Fig. 3). Thus, the large product of proteolysis of ZF-RNase-3, produced by a protease(s) from *E. coli*, appears to be the cytotoxic agent responsible for the RNase bactericidal action. It should be noted that the small proteolytic fragment (peptides 1–30 of ZF-RNase-3 sequence) was found to have no bactericidal activity (Fig. 3).

The question of the origin of the activity responsible for the proteolytic cleavage of ZF-RNase-3, and hence for the bactericidal event, was first investigated by testing for proteolytic activity on ZF-RNase-3: (a) the culture medium and (b) the contents of the cell periplasm. The supernatant of an *E. coli* culture in stationary phase was concentrated 25-fold in a Savant centrifuge (Savant Instruments Inc., Holbrook, NY, USA), and dialyzed against 50 mM Tris-HCl at pH 7.5. When tested on ZF-RNase-3, the medium was found to be devoid of any proteolytic activities (data not shown). Identical, negative results were obtained when the periplasmic fraction of the same bacterial culture was tested. This led to the conclusion that the protease producing the cleavage of ZF-RNase-3 was not a secreted protein but was embedded within the bacterium body.

Then, a bacterial lysate of *E. coli* cells previously treated with ZF-RNase-3 for 6 h at 37 °C was fractionated (see Experimental procedures) and examined by western blotting after SDS-PAGE. Under nonreducing conditions, a single protein was detectable in the membrane fraction, with the mobility of intact ZF-RNase-3 (Fig. 4A, lane 1). However, this protein was not intact ZF-RNase-3 but comprised the two proteolytic fragments held together by a disulfide bridge, because, when the SDS-PAGE was run under reducing conditions (Fig. 4A, lane 2), two protein bands were visible: that of intact ZF-RNase-3 and that of the large fragment LF-ZF3. In the cytosolic fraction, a single band was found instead with the mobility of fragment LF-ZF3, regardless of whether the run

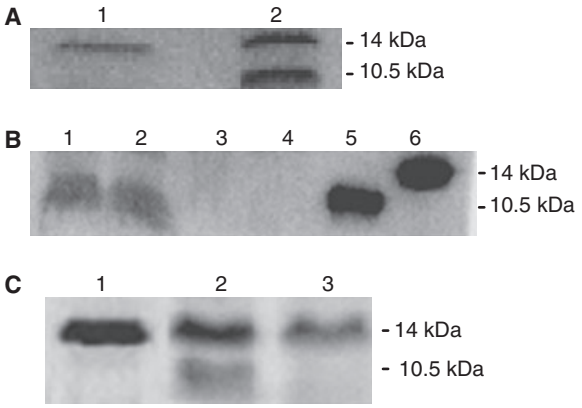

Fig. 4. Western blotting with an antibody against ZF-RNase-3. (A) Lane 1, membrane fraction of a protein extract of *E. coli* first treated with ZF-RNase-3, run under nonreducing conditions; lane 2, membrane fraction of protein extract of *E. coli* after ZF-RNase-3 treatment, run under reducing conditions. (B) Lane 1, cytosolic fraction of a protein extract of *E. coli* treated with ZF-RNase-3, run under nonreducing conditions; lane 2, cytosolic fraction of a protein extract of *E. coli* treated with ZF-RNase-3, run under reducing conditions; lanes 3 and 4, cytosolic fraction of a protein extract of *E. coli* loaded under nonreducing or reducing conditions, respectively; lane 5, untreated LF-ZF3; lane 6, untreated ZF-RNase-3. (C) Lane 1, untreated ZF-RNase-3; lane 2, membrane fraction of *E. coli* treated with ZF-RNase-3 under reducing conditions; lane 3, cytosolic fraction of *E. coli* treated with ZF-RNase-3 under reducing conditions.

was performed under reducing or nonreducing conditions (Fig. 4B, lanes 1 and 2). Clearly, in the cytosolic reducing environment, the disulfide linking the two fragments was cleaved, and LF-ZF3 was severed from the small fragment.

The apparent conclusion drawn from these data was that the protease comprised a component of the bacterium membrane(s), and that the LF-ZF3 fragment, upon proteolysis, could permeate the bacterium cytosol. Then, the reducing environment of the cytosol could readily cleave the Cys-23/Cys-84 disulfide bridge.

To definitely localize the protease, we prepared membrane and cytosolic fractions of *E. coli*, and treated them separately with ZF-RNase-3 for 2 h at 37 °C. By western blotting, we observed that ZF-RNase-3 was processed only in the presence of the membrane fraction, whereas no proteolysis occurred in the presence of the cytosolic fraction (Fig. 4C).

OmpT is the protease that initiates the bactericidal action of ZF-RNase-3

With respect to the identity of the protease that cleaves ZF-RNase-3, the possibility that it was a classical serine protease, as suggested by the presence of an Arg residue at the N-terminal side of the scissile bond, was excluded based on the lack of any effects of a serine protease inhibitor, such as phenylmethanesulphonyl fluoride, on the proteolytic activity of the *E. coli* membrane fraction (Fig. 5). Similalrly, the possibility that it was a metalloprotease was also excluded based on the lack of inhibition by EDTA of the proteolytic activity at hand (Fig. 5). Instead, $ZnCl_2$ and $CuCl_2$ at a concentration of 1 mM were found to be significant inhibitors of the proteolytic activity, whereas other cations ($MgCl_2$, $MnCl_2$, $CaCl_2$) were found to exert no inhibitory effects (Fig. 5). These results, especially the strong inhibition of proteolysis by $ZnCl_2$, the localization of the protease activity on the membrane fraction, and the recognition of Arg-Arg as the scissile bond, suggested that the protease responsible for the selective cleavage of ZF-RNase-3 was a protease from the *E. coli* omptin family, such as OmpT.

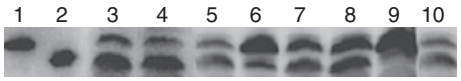

Fig. 5. Western blotting with an antibody against ZF-RNase-3 tested on: lanes 1 and 2, untreated ZF-RNase-3 and untreated LF-ZF3 as positive controls; lane 3, membrane fraction of *E. coli* treated with ZF-RNase-3; lanes 4–10, membrane fraction of *E. coli* treated with ZF-RNase-3 in the presence of 1 mM EDTA (lane 4); $CaCl_2$ (lane 5); $CuCl_2$ (lane 6); $MgCl_2$ (lane 7); $MnCl_2$ (lane 8); $ZnCl_2$ (lane 9); and phenylmethanesulphonyl fluoride (lane 10).

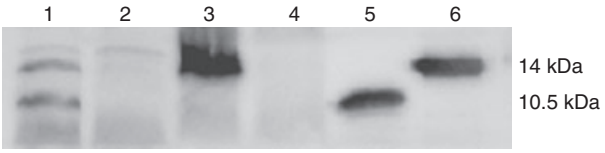

Fig. 6. Western blotting analyses with anti-ZF-RNase-3 tested on extracts of *E. coli* BL21(DE3) (ompT+) treated with: lane 1, ZF-RNase-3; lane 2, untreated *E. coli* BL21(DE3) (ompT+); lane 3, *E. coli* BL21(DE3) treated with ZF-RNase-3; lane 4, untreated *E. coli* BL21(DE3); lane 5, recombinant LF-ZF3; lane 6, recombinant ZF-RNase-3.

A conclusive identification was then obtained when ZF-RNase-3 was tested on *E. coli* BL21(DE3) strain, which does not possess the *ompT* gene, and on *E. coli* BL21(DE3) complemented with the *ompT* gene. No proteolysis was demonstrated when ZF-RNase-3 was added to cultures of BL21(DE3) bacterial cells (Fig. 6), nor was any cytotoxicity (80% survival). Instead, the BL21 (DE3) (ompT+) strain was sensitive to ZF-RNase-3 and was able to cleave it in the same way as DH5α with approximately 10% survival (Fig. 6).

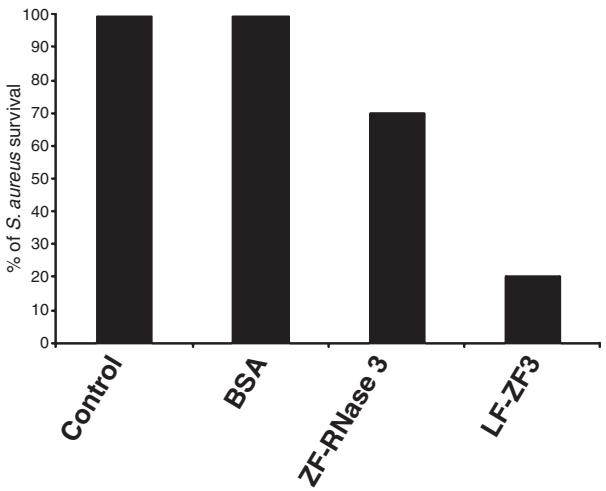

Fig. 7. Bactericidal activity of 3 μM (final concentration) ZF-RNase-3 or LF-ZF3 incubated with *S. aureus* cultures for 6 h. Cell survival was determined as a percentage of untreated control. Bovine serum albumin (BSA), was tested at 3 μM as a negative control. The average of three measurements is shown. The standard error was lower than 5%.

The effects of LF-ZF3 on Gram-positive bacteria

ZF-RNase-3 has been previously found to exert only mild cytotoxic activity on Gram-positive bacteria, such as *Staphylococcus aureus* [3]. As could be expected from the absence of the OmpT protease in Gram-positive bacteria, no proteolytic effects on ZF-RNase-3 were detected when the RNase was tested on *S. aureus* cells. On the other hand, the recombinant form of the LF-ZF3 fragment (i.e. the Δ1-30-ZF-RNase-3 variant) was also found to be active on Gram-positive *S. aureus* (Fig. 7). Thus, the LF-ZF3 fragment, as generated by a Gram-negative bacterium, can penetrate a Gram-positive bacterium and exert its cytotoxicity.

These results confirm that ZF-RNase-3 is bactericidal only on the condition that the host bacterium contains OmpT; on the other hand, they substantiate the conclusion that the real bactericidal agent in ZF-RNase-3 is the 31–124 LF-ZF3 fragment of the RNase.

Discussion

The main, significant finding of the present study is that when the zebrafish RNase ZF-RNase-3 is added to a culture of *E. coli*, it is proteolytically cleaved at a specific peptide bond, thus generating a proteolytic fragment termed LF-ZF3 (residues 31–124 of the RNase sequence). This fragment, resulting both from proteolysis and cleavage of a disulfide bridge (Cys-23-Cys-84), is identified as the actual ZF-RNase-3 bactericidal agent for *E. coli*. It should be noted that also in cultures of another Gram-negative bacterium, such as *P. fluorescens*, ZF-RNase-3 is cleaved, thus generating an LF-ZF3 fragment and cytotoxicity (data not shown).

Other cases of antimicrobial activity initiated by limited proteolysis of a protein have been reported and reviewed [10]. The preliminary results obtained in our laboratories indicate that human angiogenin, previously reported to be antimicrobial [11], is bactericidal when added to *E. coli* DH5α cultures, with an LD_{50} of 2 μM, and is proteolytically degraded just like ZF-RNase-3 (A. Zanfardino, E. Pizzo, A. Di Maro, M. Varcamonti & G. D'Alessio, unpublished data).

The protease responsible for the proteolytic cleavage of ZF-RNase-3 is identified with OmpT, an *E. coli* enzyme from the omptin family, first classified as a non-ortodox serine protease, and recently recognized as an aspartic protease [12,13]. The identification is based on certain criteria: (a) the activity is localized in the membrane compartment; (b) the specificity of cleavage, occurring at an Arg-Arg bond, corresponds to that described for omptins [14–16]; (c) no proteolysis occurs when the toxic RNase is tested on Gram-positive bacteria, or on the *E. coli* BL21(DE3) strain (i.e. on bacteria that do not possess the *ompT* gene); and (d) proteolysis is restored upon *ompT* complementation of the BL21(DE3) strain.

Of interest, although ZF-RNase-3 is not bactericidal for Gram-positive bacteria, nor for cultures of BL21(DE3) *E. coli* strain, the toxic component of the RNase, namely the LF-Z3 fragment, was found to be active on both bacteria. This confirms the key role played by the protease in the mechanism of bactericidal action of ZF-RNase-3, and reveals the surprising suicidal nature of the response of Gram-negative bacteria to ZF-RNase-3. Without the collaboration of the bacterium itself and its surface protease, no bactericidal action could be exerted by ZF-RNase-3.

The essential first step in the mechanism of bactericidal action of ZF-RNase-3 is the effective binding of RNase to the bacterium. Apparently, this is electrostatic, as suggested by the basic features of the protein, with a calculated pI of 9.38, and a high content of 16 Arg residues (13% of the ZF-RNase-3 sequence). Furthermore, the importance of these interactions in the cytotoxic action of ZF-RNase-3 was demonstrated by the inhibitory effect of NaCl on the cytotoxic action of ZF-RNase-3. At a concentration of 500 mM, NaCl was found to inhibit the bactericidal activity of ZF-RNase-3 by almost 100%. It should be noted that this inhibition may likely be the result of an antagonizing effect of NaCl on LF-ZF3, although the

possibility that the protease activity is inhibited cannot be excluded.

The second step is the cleavage of the RNase by the outer membrane OmpT protease. Then, the excised fragment is delivered to the cytosol, where the Cys-23/Cys-84 disulfide linking the N-terminal fragment 1–30 to the large 31–124 fragment is readily cleaved.

The final, conclusive step in the mechanism (i.e. leading to bacterial cell death) needs further investigation. Certainly, it is not a result of the RNA degrading activity of ZF-RNase-3. Catalytically inactivated ZF-RNase-3 is as bactericidal as the native enzyme. Furthermore, the active bactericidal component of the RNase (i.e. the LF-Z3 fragment) cannot have any RNase activity because it is deprived of one of the His residues (His-13) essential for catalysis. This may not be surprising because the toxic action of other antimicrobial RNases is exerted in the absence of RNase catalytic activity [5,7–9].

Upon binding to the host bacterium through electrostatic interactions, the hydrophobic content of toxins drives the cytotoxic action through a disruption of cell homeostasis. In the case at hand, it may be relevant that both unstructured ZF-RNase-3 and LF-ZF3 maintain their cytotoxicity. This uncoupling between the biological, toxic action of a host-defense protein and its structure also has been found for bactericidal lysozyme [17,18]. An alternative possibility for explaining the toxic action of the unfolded RNase may be based on the effects of clusters of positively charged residues present in the RNase primary structure [7]. These could easily assemble even in the absence of an ordered structure. In the case of LF-ZF3, the rich content of Arg residues (11% of the protein sequence) could have a disturbing, lethal action on the cytosolic content of the bacterium.

Experimental procedures

Materials

Unless otherwise indicated, all reagents, including proteins and enzymes, were obtained from Sigma (St Louis, MO, USA), including RNase A (type XII-A). TAQ DNA polymerase High Fidelity and *E. coli* strains were obtained from Invitrogen (Carlsbad, CA, USA). Restrictases and plasmid pET22b$^{(+)}$ were obtained from New England Biolabs (Ipswich, MA, USA).

Cloning, expression and isolation experiments

Recombinant ZF-RNase-3 was obtained as described previously [4]. In the preparation of LF-ZF3, the LF-ZF3 coding sequence was amplified using the ZF-RNase 3 coding sequence as a template. The primers used were: LF-ZF3, forward: 5′-TTC**catatg**CGCATCACACGATTTC-3′; LF-ZF3, reverse: 5′-CCC**aagctt**AAATAACACCTTTTTCATAGT-3′. Bold lowercase letters indicate restriction sites, *Nde*I and *Hind*III, respectively, added to perform cloning. A PCR with TAQ DNA polymerase (Invitrogen) was performed with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 45 s at 95 °C, 30 s at 56 °C and 60 s at 72 °C. PCR products were isolated by electrophoresis on low melting 1% agarose gel, and purified using the Product Purification Kit (Roche Applied Science, Basel, Switzerland). Purified DNA, treated with *Nde*I and *Hind*III restrictases, was inserted into the pET22b$^{(+)}$ expression vector. All cloned, purified DNAs were certified through sequencing (MWG Biotech, Edersberg, Germany) before processing. The recombinant expression plasmid was used to transform competent *E. coli* strain BL21(DE3). Cells were grown at 37 °C until A_{600} of 1 was reached, and then induced with 0.1 M isopropyl-1-thio-D-galactopyranoside and grown overnight. The expressed recombinant LF-ZF3 protein was purified from inclusion bodies in accordance with standard protocols of denaturation/renaturation steps, followed by cation exchange chromatography and HPLC, as described previously [4].

For cloning the ompT coding sequence in pET22b$^{(+)}$, the primers used were: forward: -5′-ttccatatgggagcaatatgta attgactc-3′; reverse: -5′-cccaagcttggctagttattccccggg-3′. The expression was driven by the ompT promoter linked to the ompT coding sequence.

Assays of bactericidal activity

Antibacterial tests were performed as described previously [8] on *E. coli* DH5α or BL21(DE3), *P. fluorescens* (ATCC 13525) and *S. aureus* (ATCC 6538P).

BL21 (DE3) was also used as recipient strain of the plasmid (pET22b) bearing a PCR-amplified copy from the DH5a chromosome of the *ompT* gene. This strain was used for the complementation experiments.

Bacteria were grown overnight, diluted 1 : 1000 in 20 mM sodium phosphate buffer (pH 7.0) and incubated with the protein under test. Recombinant ZF-RNase-3 and LF-ZF3 were used at a final concentration of 3 μM, unless dose–response curves or maximal effects were determined. Bacteria were used at a density of 4000 CFU·mL^{-1}. After 6 h at 37 °C, serial dilutions of each protein/bacteria mix were prepared and plated, and the CFU·mL^{-1} remaining after each treatment were determined. Negative controls were carried out with proteins from *E. coli* strain BL21(DE3) transformed with an empty pET22b$^{(+)}$ vector. For each experiment, carried out in duplicate, triplicate assays were performed. Standard deviations were always < 5% or as detailed for each experiment. When indicated, one of the

following were added: Protease Inhibitor Cocktail (P8465; Sigma), NaCl 250 or 500 mM, EDTA, phenylmethanesulphonyl fluoride, $CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ at a concentration of 1 mM.

Proteolysis assays

Bacterial cells (10 mL), grown overnight at 37 °C, were centrifuged at 6000 ***g*** for 5 min at 4 °C. The cell pellet was resuspended in the initial volume of 20 mM sodium phosphate buffer (pH 7.0), then the protein under test was added at a final concentration of 3 μM. As a negative control, untreated cells were used. Samples were incubated at 37 °C for 6 h, and proteins, extracted by sonication, were run for SDS-PAGE and blotted on polyvinylindene fluoride filters to perform western analyses.

Cell fractionation

Cell fractionation was carried out as described previously [19]. Briefly, 10 mL of cells, grown until a D_{600} of 1.0 was reached, were pelleted and resuspended in 0.5 mL of periplasting buffer (20% sucrose, 1 mM EDTA, 30 000 $U \cdot mL^{-1}$ of Ready-Lyse lysozyme (Epicentre Technologies, Madison, WI, USA). After incubation on ice for 5 min, spheroplasts were pelleted by centrifugation at 12 000 ***g*** for 2 min. The supernatant was preserved as the periplasmic fraction. The pelleted spheroplasts were lysed in 1 mL of water containing 400 $U \cdot mL^{-1}$ of Omnicleave Endonuclease (Epicentre Technologies). The samples, incubated for 5 min at room temperature, were briefly sonicated at 30–40% amplitude (maximum power 300 W) with a Sonics vibracell VCX-130 (Sonics & Materials, Inc., Newtown, CT, USA). Intact cells were removed by centrifugation at 12 000 ***g***. The supernatant, centrifuged at 138 000 ***g*** for 1 h, was taken as the cytoplasmic fraction, and the pellet containing the membrane fraction was resuspended in 0.5% Sarkosyl (Ciba-Geigy Corp., Summit, NJ, USA), 10 mM Tris-HCl and 5 mM EDTA.

Preparation of chemically modified ZF-RNase-3

Catalytically inactivated ZF-RNase-3 was prepared as described previously [5]. Reduced and alkylated ZF-RNase-3 was obtained first by treating ZF-RNase-3 (1 $mg \cdot mL^{-1}$) for 2 h at 37 °C with a ten-fold molar excess of dithiothreitol in 200 mM 2-(*N*-morpholino)ethanesulfonic acid-NaOH pH 6.0. Carboxymethylation of the exposed sulphydryls was then carried out by adding a 20-fold molar excess (with respect to the total -SH concentration) of iodoacetamide. After 60 min at room temperature in the dark, the protein was freed of excess reagents and by-products by gel filtration through a PD10 column (GE Healthcare Bioscience AB, Uppsala, Sweden) equilibrated in 0.1 M ammonium acetate (pH 5) and lyophilized. MS analyses revealed an increase of molecular weight consistent with the effects of the carboxymethylation reaction.

Edman degradation analysis and mass spectrometry of LF-ZF3

Sequence analyses were performed by automated Edman degradation as previously described [4], using a Procise sequencer, Model 491C (Applied Biosystems, Foster City, CA, USA).

Western blotting analyses

Recombinant LF-ZF3 and proteolytic cleavage of ZF-RNase-3 were identified by SDS-PAGE [20] followed by western blotting using a rabbit polyclonal anti-ZF-RNase-3 serum (IGTECH, Salerno Italy). Immunopositive species were detected by a chemiluminescence detection system (Super Signal® West-Pico, Pierce, Rockford, IL, USA) using a Phosphoimager (Bio-Rad, Hercules, CA, USA).

Acknowledgements

The research conducted in the present study was partly supported by PRIN grants from the Ministry of Education, Italy.

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