Catalytic activity of bovine seminal ribonuclease is essential for its immunosuppressive and other biological activities

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Bovine seminal ribonuclease (BS-RNase) is a homologue of RNase A with special biological properties, including potent immunosuppressive activity. A mutant BS-RNase was created in which His-119, the active-site residue that acts as a general acid during catalysis, was changed to an aspartic acid. H119D BS-RNase formed a dimer with quaternary structure similar to that of the wild-type enzyme but with values of $k_{cat.}$ and $k_{cat.}/K_m$ for the cleavage of UpA [uridylyl(3′ → 5′)adenosine] that were 4×10^3-fold lower. The mutant protein also demonstrated dramatically decreased immunosuppressive, anti-tumour, aspermatogenic, and embryotoxic activities. The catalytic activity of BS-RNase is therefore necessary for its special biological properties.

INTRODUCTION

Bovine seminal ribonuclease (BS-RNase; EC 3.1.27.5) is an unusual member of the superfamily of secretory ribonucleases. Unlike bovine pancreatic ribonuclease A (RNase A), the prototype of the superfamily with which BS-RNase shares 80% amino acid sequence identity, BS-RNase displays extraordinary biological properties related to cytotoxicity. These properties, which include specific aspermatogenic, anti-tumour, and immunosuppressive activities [1–5], give therapeutic potential to BS-RNase [6]. Among these biological properties, immunosuppression is most likely to be of physiological relevance as it may be required to inhibit the immune response of cows against components of bull seminal plasma [7].

BS-RNase belongs to a family of proteins known as RISBASEs (**ri**bonucleases with **s**pecial **bi**ological **a**ction) [8,9]. RISBASEs come from a variety of species and have diverse biological actions. For example, human angiogenin promotes blood vessel formation [10]. Onconase from the frog *Rana pipiens* is a potent anti-tumour agent in phase II human clinical trials in the U.S.A. [11,12]. S-RNases from plants are responsible for self-incompatibility [13,14]. The active-site residues in each RISBASE are conserved, and each RISBASE is able to catalyse the cleavage of RNA.

Is the catalytic activity of a RISBASE essential for its special biological action? Mutating the active-site histidine residues makes angiogenin incapable of promoting neovascularization [15]. Chemically modified onconase [12] and eosinophil-derived neurotoxin [16] lose their cytotoxic effects. Still, chemical modification experiments often lead to inconclusive results because the modification is incomplete or perturbs structure rather than function [17]. For example, chemical modification of the catalytic histidine residues in dimeric RNase A (which is similar in form and function to BS-RNase) results in an inactive catalyst that has full anti-tumour activity [18,19]. Yet, the chemical inactivation of BS-RNase itself has been reported to result in the loss of its immunosuppressive activity [20] and its anti-tumour activity [21].

Previously, we reported the efficient expression of a synthetic gene for BS-RNase in *Escherichia coli* [22]. We have now used this system to prepare a mutant BS-RNase in which His-119, an active-site residue, was replaced with an aspartic acid. Here, we report on the consequences of this mutation on the enzymic and biological activities of BS-RNase.

EXPERIMENTAL

Materials

Wild-type BS-RNase was purified from bull seminal plasma as described [1,22]. Recombinant wild-type BS-RNase was also produced in *E. coli* strain BL21(DE3) by using expression vector pLSR1 as described [22]. Plasmid pLSR119 [23] codes for a mutant BS-RNase in which His-119 has been changed to an aspartic acid residue. H119D BS-RNase was produced in *E. coli* strain BL21(DE3)/pLSR119, purified from cellular extract, and folded/oxidized as described [23]. UpA [uridylyl(3′ → 5′)adenosine] was synthesized by J. E. Thompson using the methods of Ogilvie and co-workers [24] and Beaucage and Caruthers [25]. Other materials were obtained as described elsewhere [26].

Analysis of quaternary structure

In dimers of BS-RNase, the subunits are cross-linked by two disulphide bonds between Cys-31 of one subunit and Cys-32 of the other subunit. These cross-linked dimers exist in two distinct quaternary forms, designated as M × M and M = M [27]. In the major form, M × M, the N-terminal tail (residues 1–17) of one subunit stretches out from the C-terminal body of the same

Abbreviations used: BS-RNase, bovine seminal ribonuclease; CpA, cytidylyl(3′ → 5′)adenosine; Mes, 4-morpholine-ethanesulphonic acid; M × M, homodimeric BS-RNase in which the N-terminal tail of each subunit interacts with the C-terminal body of the other subunit; M = M, homodimeric BS-RNase in which the N-terminal tail of each subunit interacts with the C-terminal body of the same subunit; RNase A, bovine pancreatic ribonuclease A; UpA, uridylyl(3′ → 5′)adenosine.

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subunit and interacts with the body of the other subunit. In the minor form, M = M, this exchange does not occur. The quaternary structure of dimeric H119D BS-RNase (that is, the fraction in the M × M form) was assessed by selective reduction of the two intersubunit disulphide bonds with a 10-fold molar excess of reduced dithiothreitol [28], and then separation of the resulting non-covalent dimers (which originate from M × M) and monomers (which originate from M = M) by gel-filtration chromatography [26]. The percentage M = M was determined by integration of the gel-filtration profile obtained at 280 nm.

Assay for catalytic activity

The enzyme-catalysed cleavage of UpA was monitored with an adenosine deaminase-coupled assay [29] at 25 °C in 0.1 M Mes/HCl buffer, pH 6.0, containing NaCl (0.1 M). The $\Delta\epsilon$ for this reaction was $-6000\ M^{-1}\cdot cm^{-1}$ at 265 nm [30]. Protein concentrations were determined by using an absorption coefficient of $\epsilon^{0.1\%}_{1\,cm} = 0.465$ at 278 nm for all forms of BS-RNase [31] and $\epsilon^{0.1\%}_{1\,cm} = 0.72$ at 277.5 nm for RNase A [32]. The values for $k_{cat.}$, K_m and $k_{cat.}/K_m$ were determined from initial velocity data with the program HYPERO [33].

Assays for biological activity

Immunosuppressive activity [34]

Lymphocytes from the heparinized peripheral blood of two normal allogenic humans were isolated separately on a density gradient ($d = 1.077$) of Ficoll-Paque solution gradient as described [3]. The cells from the interface were aspirated, washed three times with PBS and resuspended in RPMI 1640 medium containing inactivated pooled human AB serum (20%, v/v), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (50 μg/ml). The resulting preparations contained < 98% mononuclear cells with < 2% neutrophils or erythrocytes. Cells (1×10^5) from the two preparations were mixed 1:1. The resulting mixed lymphocyte cultures (0.2 ml) were established in microtitration plates (NUNC, U type) and cultivated at 37 °C in RPMI 1640 medium under a humidified atmosphere containing CO_2 (5%, v/v). A known concentration of RNase was added to each of three cultures. After 6 days, the ability of treated cells to proliferate was assessed by measuring the incorporation of [6-^{3}H]thymidine into newly synthesized DNA. Briefly, [6-^{3}H]thymidine (24 kBq) was added to each culture. After 4 h of additional cultivation, cells were collected with a Scatron harvester, and incorporated radioactivity was evaluated with a Beckman scintillation counter. The mean value for the three cultures containing a particular ribonuclease was compared with that for untreated cells.

Anti-tumour activity

Cultures (0.2 ml) of human erythroid leukaemia cell line K-562 were established in microtitration plates (NUNC, FB type) and cultivated at 37 °C in RPMI 1640 medium supplemented with fetal calf serum (10%, v/v) under a humidified atmosphere containing CO_2(5%, v/v). A known concentration of RNase was added to each of three cultures. After 3 days, the ability of cells to proliferate was assessed as described above. The mean value for the three cultures containing a particular ribonuclease was compared with that for untreated cells.

Aspermatogenic activity [35]

The left testes of CBA mice (five animals per group) were injected with an RNase (50 μl of a 1.0 mg/ml solution). After 10 days, the testes were isolated, weighed, stained with haematoxylin and eosin, and subjected to histological examination. Aspermatogenic activity was assessed by measuring the diameter of seminiferous tubules, the index weight (which is the $10^3 \times$ testes weight/body weight), and the width of spermatogenic layers. Results were recorded as the mean ± S.E.M. and compared with the untreated right testes of the same mice.

Embryotoxic activity

Bovine embryos were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (20%, v/v). The embryos were cultivated in Falcon plates covered with paraffin oil under an atmosphere of N_2 (90%, v/v), O_2 (5%, v/v), and CO_2 (5%, v/v). The mortality of the embryos was assessed 6, 24, 48, and 72 h after the addition of a ribonuclease (10 μg/ml).

RESULTS AND DISCUSSION

H119D and wild-type BS-RNase were produced in *E. coli* and purified to homogeneity. A critical quality of these preparations was the fraction of each dimeric protein that existed in the M × M form. In this form, the N-terminal α-helices of the subunits are exchanged, thereby stabilizing the dimer to conditions that reduce the two intersubunit disulphide bonds. The M × M form appears to be necessary for the special biological actions of BS-RNase, perhaps because it is not bound tightly by cytosolic ribonuclease inhibitor [26]. The fraction of the H119D and wild-type BS-RNases from *E. coli* that were in the M × M form at equilibrium were 60% and 57% respectively. Thus any difference in enzymic or biological activity is unlikely to arise from a difference in quaternary structure.

BS-RNase, like other ribonucleases, catalyses the transphosphorylation of RNA [36]. During catalysis, the imidazole group of His-119 in BS-RNase is likely to serve as a general acid, as does the imidazole group of His-119 of RNase A [37]. Replacing His-119 with an aspartic acid residue in effect tests whether a carboxylic acid group can also serve as a general acid during catalysis.

The steady-state kinetic parameters for UpA cleavage by the wild-type and H119D BS-RNases are shown Table 1. Catalysis by the wild-type enzyme from *E. coli* and from bull seminal plasma were indistinguishable. Apparently, the additional methionine residue at the N-terminus of the enzyme from *E. coli* [22] does not affect catalysis of UpA cleavage. In contrast, catalysis by H119D BS-RNase displayed a 4×10^3-fold decrease in $k_{cat.}$ and in $k_{cat.}/K_m$.

Does Asp-119 act as a general acid during catalysis by H119D BS-RNase? If an enzyme-catalysed reaction depends only on the

Table 1 Steady-state kinetic parameters for cleavage of UpA by wild-type and mutant bovine seminal ribonucleases

Data were obtained at 25 °C in 0.1 M Mes/HCl buffer, pH 6.0, containing NaCl (0.1 M).

Bovine seminal ribonuclease	$k_{cat.}$ (ms^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($\mu M^{-1}\cdot s^{-1}$)
H119D from *E. coli*	0.0012 ± 0.0002	1.2 ± 0.3	0.0010 ± 0.0001
Wild-type from *E. coli*	4.8 ± 0.5	1.3 ± 0.2	3.7 ± 0.2
Wild-type from seminal plasma	6.4 ± 0.8	1.5 ± 0.2	4.3 ± 0.2

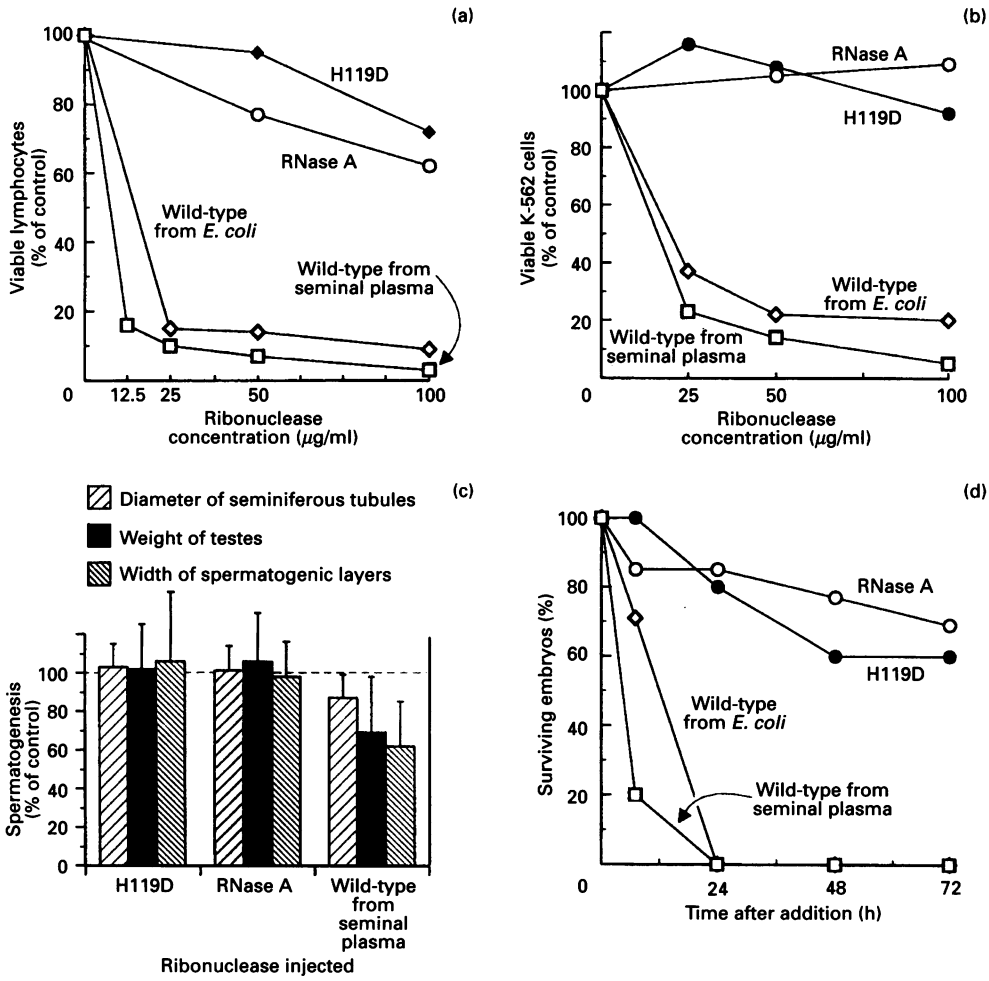

Figure 1 Biological activities of wild-type and mutant ribonucleases

Assays were performed of the (**a**) immunosuppressive, (**b**) anti-tumour, (**c**) aspermatogenic, and (**d**) embryotoxic activity of H119D BS-RNase from *E. coli* (**a**, ◆; **b** and **d**, ●), wild-type BS-RNase from *E. coli* (◇), wild-type BS-RNase from bovine seminal plasma (□), and RNase A from bovine pancreas (○). Controls were medium (**a** and **b**) or testes (**c**) containing no added ribonuclease.

side-chain of a particular residue being protonated, then its velocity (v) is given by the proportion:

$$v \propto \frac{1}{1+10^{pH-pK_a^{wt}}} \quad (1)$$

Similarly, if this residue in the wild-type enzyme (with pK_a^{wt}) is replaced with another (with pK_a^{mut}), then the ratio of their velocities is given by the equation:

$$\frac{v^{wt}}{v^{mut}} = \frac{1+10^{pH-pK_a^{mut}}}{1+10^{pH-pK_a^{wt}}} \quad (2)$$

In RNase A, the imidazolium side-chain of His-119 has a $pK_a^{wt} = 6.2$ [38], which is a value typical for histidine residues. If the carboxylic acid side-chain of Asp-119 has a $pK_a^{mut} = 4.0$, which is a value typical for aspartic acid residues, then $v^{wt}/v^{mut} = 60$ at pH 6.0. Since we observe $v^{wt}/v^{mut} = 4 \times 10^3$, we conclude that Asp-119 does not act as a general acid during catalysis by the mutant BS-RNase. Additional support for this conclusion comes from studies of H119A RNase A, which has $(k_{cat.}/K_m)^{wt}/(k_{cat.}/K_m)^{mut} = 1.6 \times 10^4$ [37] for UpA cleavage, and H114N angiogenin, which has $(k_{cat.}/K_m)^{wt}/(k_{cat.}/K_m)^{mut} > 3.3 \times 10^3$ for CpA [cytidylyl(3′ → 5′)adenosine] cleavage [15]. In these homologues of BS-RNase, His-119 is replaced with an uncharged residue that cannot act as a general acid. Since secretory ribonucleases with an alanine, asparagine, or aspartic acid residue at position 119 have similar abilities to cleave diribonucleotide substrates, the side-chains of these residues are unlikely to participate in catalysis.

Is the high catalytic activity of wild-type BS-RNase essential for its biological activities? We assessed various cytotoxic activities of wild-type BS-RNase, H119D BS-RNase, and RNase A. First, we determined the ability of the ribonucleases to inhibit the growth of human lymphocytes in a mixed lymphocyte culture. Although a low concentration of wild-type BS-RNase from either seminal plasma or *E. coli* had a strong inhibitory effect on lymphocyte growth, a high concentration of H119D BS-RNase or RNase A had only a modest effect (Figure 1a). Secondly, anti-tumour activity was assessed with cultures of human tumour cell line K-562. The wild-type BS-RNases showed a strong inhibitory

effect on the growth of the tumour cells, but H119D BS-RNase and RNase A showed no such effect (Figure 1b). Thirdly, we examined the effect of the ribonucleases on spermatogenesis in mice by injecting the ribonucleases into one testis of each of five mice and recording, after 10 days, the diameter of seminiferous tubules, the weight of the testes, and the width of spermatogenic layers. Although wild-type BS-RNase was highly aspermatogenic, neither H119D BS-RNase nor RNase A had a significant effect (Figure 1c). Finally, the embryotoxicity of the ribonucleases was tested by adding the proteins to the medium of cultured bovine embryos and recording mortality. Wild-type BS-RNase was 100 % lethal to bovine embryos 1 day after addition (Figure 1d). In contrast, RNase A and H119D BS-RNase was only slightly toxic to the embryos 72 h after addition.

In all of the biological assays, wild-type BS-RNase from seminal plasma was slightly more active than was the wild-type protein from *E. coli*. This result is not unexpected because BS-RNase is produced from *E. coli* in the relatively inactive M = M form [26]. This form largely, but not completely, equilibrates ($t_{\frac{1}{2}} = 1.9$ days) with the active M × M form during the time course of the biological assays. (Immunosuppressive activity is assessed after 6 days; anti-tumour activity after 3 days; and aspermatogenic activity after 10 days.) BS-RNase is isolated from seminal plasma as an equilibrium mixture in which 60–80 % of the protein is in the M × M form.

Thus all four biological assays yielded consistent results: H119D BS-RNase was biologically inactive. Previous studies used chemically inactivated protein to claim that the catalytic activity of dimeric ribonucleases is either essential for its anti-tumour and immunosuppressive activities [20,21] or unimportant for its anti-tumour activity [18,19]. Here, we have used a BS-RNase prepared by site-directed mutagenesis to show that catalytic activity is indeed required for its immunosuppressive and anti-tumour activities, as well as for its aspermatogenic and embryotoxic activities.

We thank J. E. Thompson for providing UpA. Work in Madison was supported by grant GM44783 (NIH). Work in Prague was supported by grant 506/93/1133 from the Grant Agency of the Czech Republic. J.-S.K. was a Steenbock predoctoral fellow. R.T.R. is a Presidential Young Investigator (NSF), Searle Scholar (Chicago Community Trust), and Shaw Scientist (Milwaukee Foundation).

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