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Structural Basis for the Biological Activities of Bovine Seminal Ribonuclease*

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Bovine seminal ribonuclease (BS-RNase) is a homolog of RNase A with special biological properties that include specific antitumor, aspermatogenic, and immunosuppressive activities. Unlike RNase A, BS-RNase is a dimer cross-linked by disulfide bonds between Cys^{31} of one subunit and Cys^{32} of the other. At equilibrium, this dimer is a mixture of two distinct quaternary forms, M=M and M×M. The conversion of M=M to M×M entails the exchange of NH_2-terminal α-helices between subunits. Here, the cytotoxic activities of purified M×M were shown to be greater than those of purified M=M, despite extensive equilibration of M=M and M×M during the time course of the assays. Replacing Cys^{31} or Cys^{32} with a serine residue did not compromise the enzymatic activity of dimeric BS-RNase, but reduced both the fraction of M×M at equilibrium and the cytotoxicity. We conclude that the M×M form is responsible for the special biological properties of BS-RNase. Since cytosolic ribonuclease inhibitor binds tightly to monomeric but not dimeric BS-RNase and only the M×M form can remain dimeric in the reducing environment of the cytosol, we propose that BS-RNase has evolved its M×M form to retain its lethal enzymatic activity *in vivo*.

Bovine seminal ribonuclease (EC 3.1.27.5, BS-RNase,[1]) is a homolog of bovine pancreatic ribonuclease A (RNase A). Eighty % of the amino acid sequences of the two proteins is identical (Suzuki *et al.*, 1987). Like RNase A, BS-RNase catalyzes the cleavage of RNA after pyrimidine residues. Unlike RNase A, BS-RNase has specific antitumor, aspermatogenic, and immunosuppressive activities (Dostál and Matoušek, 1973; Matoušek, 1973; Souček *et al.*, 1986; Tamburrini *et al.*, 1990; D'Alessio *et al.*, 1991; Laccetti *et al.*, 1992; D'Alessio, 1993). These special biological properties correlate with the oligomerization state assumed by BS-RNase, which is isolated from bull seminal plasma as a dimer. For example, artificial dimers of RNase A also have antitumor activity, although to a lesser extent than does BS-RNase (Bartholeyns and Baudhuin, 1976; Vescia *et al.*, 1980). Similarly, artificial monomers of BS-RNase lack these activities (Tamburrini *et al.*, 1990).

In dimers of BS-RNase, the subunits are cross-linked by two disulfide 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 (Piccoli *et al.*, 1992). In the major form, M×M, the NH_2-terminal tail (residues 1–17) of one subunit stretches out from the COOH-terminal body of the same subunit and interacts with the body of the other subunit (Fig. 1). The noncovalent interaction between the monomers in M×M is probably similar to that between S-peptide (residues 1–20) and S-protein (residues 21–124) in RNase S (Kim *et al.*, 1992; Kim and Raines, 1993b). In the minor form, M=M, this exchange does not occur. Refolding studies have shown that BS-RNase first folds into a monomer (M), which dimerizes to form M=M and is then slowly converted to an equilibrium mixture in which M×M/M=M has been reported to be approximately 2:1 (Piccoli *et al.*, 1992). D'Alessio and co-workers (Piccoli *et al.*, 1993) have suggested that the two quaternary forms may differ in their enzymatic and biological properties.

Bovine seminal ribonuclease is the only known dimeric ribonuclease. Although no other seminal ribonucleases have been isolated, homologs of the gene that codes for BS-RNase were discovered recently in deer, giraffe, and sheep (Breukelman *et al.*, 1993).[2] The DNA sequence of each of these genes indicates that the residue corresponding to Cys^{31} in the encoded protein is replaced by a phenylalanine. This mutation eliminates one of the two intersubunit disulfide bonds.

We recently described the synthesis of a gene that codes for BS-RNase, the expression of this gene in *Escherichia coli*, and the isolation of active dimers of BS-RNase (Kim and Raines, 1993a, 1994). To illuminate the role of the two intersubunit disulfide bonds of BS-RNase, we have now prepared mutants of BS-RNase (C31S BS-RNase and C32S BS-RNase) that lack 1 of the 2 cysteine residues. We then used these mutant proteins to answer the questions: 1) can an M=M dimer form with only one intersubunit disulfide bond? 2) If so, to what extent and at what rate is this M=M dimer converted to an M×M dimer? 3) What are the consequences of these mutations on the antitumor, aspermatogenic, and immunosuppressive activities of BS-RNase?

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[1] The abbreviations used are: BS-RNase, bovine seminal ribonuclease; AcOH, acetic acid; 139 M, monomeric BS-RNase; M×M, homodimeric BS-RNase in which the NH_2-terminal tail of each subunit interacts with the COOH-terminal body of the other subunit; M=M, homodimeric BS-RNase in which the NH_2-terminal tail of each subunit interacts with the COOH-terminal body of the same subunit; RNase A, bovine pancreatic ribonuclease A; MLC, mixed lymphocyte culture; NCD, noncovalent dimer.

[2] J. J. Beintema, personal communication.

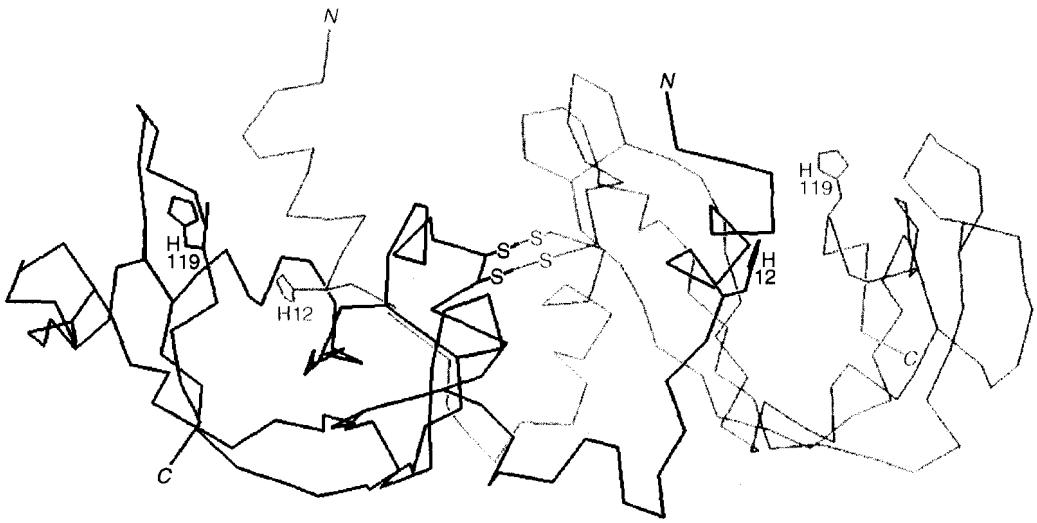

FIG. 1 **Structure of the crystalline M×M form of wild-type BS-RNase (Mazzarella *et al.*, 1987).** Only α-carbons are shown. The chains are drawn in *black* or *gray*. The intersubunit disulfide bonds, active site histidine residues, and NH_2 and COOH termini are indicated.

EXPERIMENTAL PROCEDURES

Materials

RNase A was from Boehringer Mannheim GmbH (Hamburg, Germany). *E. coli* strain BL21(DE3) was from Novagen (Madison, WI). Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA) except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Restriction endonucleases and T4 DNA ligase were from Promega (Madison, WI). T7 DNA polymerase was from New England Biolabs (Beverly, MA). [6-^{3}H]Thymidine (980 GBq/mmol) was from the Institute for the Research, Development and Application of Radioisotopes (Prague, Czech Republic). Bacto tryptone and Bacto yeast extract were from Difco (Detroit, MI). Ampicillin (sodium salt) was from International Biotechnologies (New Haven, CT). All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

Methods

DNA oligonucleotides were synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer by using the β-cyanoethyl phosphoramidite method (Sinha *et al.*, 1984). DNA sequences were determined with the Sequenase Version 2.0 kit from United States Biochemical Corp. (Cleveland, OH). Other manipulations of DNA were performed as described (Ausubel *et al.*, 1989).

Ultraviolet absorbance measurements were made on a Cary model 3 spectrophotometer equipped with a Cary temperature controller. Ribonuclease concentrations were determined by using an absorption coefficient of $\epsilon_{1\ cm}^{0.1\%} = 0.465$ at 278 nm for all forms of BS-RNase (D'Alessio *et al.*, 1972) and $\epsilon_{1\ cm}^{0.1\%} = 0.72$ at 277.5 nm for RNase A (Sela *et al.*, 1957). Enzymatic activity was assayed by the method of Kunitz (1946). Isoelectric points were determined with a model 111 Mini IEF cell from Bio-Rad and pH 8–10.5 carrier ampholytes from Pharmacia LKB Biotechnol (Piscataway, NJ).

Mutagenesis—The construction of plasmid pLSR1 for the expression of BS-RNase in *E. coli* was described previously (Kim and Raines, 1994). Site-directed mutagenesis was performed by the method of Kunkel (1987). Oligonucleotide C31S.41 (ACACTTACCTTGGGTCATCTTTCTACAAGACATCATCAAGT) was used to change the TGT codon of Cys^{31} to the TCT codon of serine. Oligonucleotide C32S.39 (ACACTTACCTTGGGTCATCTTTCTAGAACACATCATCAA) was used to change the TGT codon for Cys^{32} to the TCT codon of serine. The resulting plasmids, pLSR31 and pLSR32, were used to produce C31S BS-RNase and C32S BS-RNase, respectively.

Production and Purification of M, M=M, C31S, and C32S from E. coli—Wild-type and mutant BS-RNases were produced in *E. coli* and partially purified as described (Kim and Raines, 1993a). Folding-oxidation was performed as described (di Nigris *et al.*, 1993) with minor modification. Here, denatured and reduced protein was oxidized for 24 h at a concentration of 0.7 mg/ml in 0.1 M Tris-AcOH buffer, pH 8.5, containing glutathione (3.0 mM oxidized, 0.6 mM reduced). The resulting solution was concentrated by ultrafiltration on an Amicon YM10 membrane, and the concentrate was loaded onto a fast protein liquid chromatography HiLoad™26/60 Superdex™75 gel filtration column that had been equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing NaCl (0.1 M). Fractions containing monomeric BS-RNase (M) were collected and concentrated. Any glutathionine was removed by selective reduction with a 5-fold molar excess of dithiothreitol, after the addition of 0.1 volume of 1.0 M Tris-HCl buffer, pH 8.5.

The M form of wild-type BS-RNase was stabilized by alkylating the sulfhydryl groups of Cys^{31} and Cys^{32} with iodoacetamide as described (D'Alessio *et al.*, 1975). Alternatively, the M=M form of wild-type, C31S, and C32S BS-RNase was prepared from M that had not been treated with iodoacetamide. Then, the M form was air-oxidized by dialysis for 24 h *versus* 0.05 M Tris-AcOH buffer, pH 8.5. The dialyzed protein was concentrated and subjected to gel filtration chromatography as described above. Fractions containing dimeric BS-RNase (M=M) were collected, pooled, and stored at −70 °C.

Purification of M=M and M×M from Seminal Plasma—Wild-type BS-RNase (which is an equilibrium mixture of M=M and M×M) was purified to >95% homogeneity from bull seminal plasma as described by Dostál and Matoušek (1973) or Kim and Raines (1993a). The intersubunit disulfide bonds of the purified enzyme were reduced with a 10-fold molar excess of reduced dithiothreitol, and the resulting protein was subjected to gel filtration chromatography to separate M from the noncovalent dimer (NCD). The M=M form was prepared by air oxidation of M and purified by gel filtration chromatography. Similarly, the M×M form was prepared by air oxidation of NCD and purified by gel filtration chromatography. This method of purification yielded M=M and M×M that were >90% free of the other form, as judged by selective reduction followed by gel filtration chromatography. The purified M=M and M×M forms were stored at −70 °C, at which temperature their interconversion was undetectable.

Source of Protein for Assays—The source of the ribonuclease used in a particular assay was based on the composition (bull seminal plasma contains an equilibrium mixture of M=M and M×M while *E. coli* produces M that can be oxidized to M=M) and the yield (*E. coli* ≫ bull seminal plasma) of available protein. Thus in the conversion assay (which requires 10 mg of protein), all three M=M forms were from *E. coli*. In the biological assays, ribonucleases labeled "wild-type," "M=M," and "M×M" were prepared from bull seminal plasma, and ribonucleases labeled "C31S" and C32S" were the M=M forms prepared from *E. coli*. The buffer of the various ribonucleases was changed by gel filtration chromatography to 0.1 M Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) for the conversion assay or to 10 mM sodium phosphate buffer, pH 7.0, containing NaCl (0.1 M) for the antitumor, aspermatogenic, and immunosuppressive activity assays. Wild-type BS-RNase prepared from bull seminal plasma, and that from *E. coli* (which has an additional NH_2-terminal methionine residue) had identical enzymatic activity (Kim and Raines, 1993a; Kim *et al.*, 1995) and antitumor activity (data not shown).

Conversion of M=M to M×M—Solutions of purified M=M form (1.0 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) were incubated at 37 °C. Aliquots (1.0 mg, 36 nmol) were withdrawn at

various times and treated with enough reduced dithiothreitol (56 μg, 360 nmol) to reduce only the intersubunit disulfide bonds (D'Alessio *et al.*, 1975). The fraction of M=M that had been converted to M×M was determined by selective reduction of the intersubunit disulfide bonds with a 10-fold molar excess of reduced dithiothreitol, and separation of M from NCD by gel filtration chromatography as described above. The percent M=M was determined by integration of the gel filtration profile obtained at 280 nm.

Antitumor Activity Assay—The effect of various ribonucleases on the proliferation of cell lines K-562 and ML-1 (which derive from human erythroid leukemia cells and human myeloid leukemia cells, respectively) was assessed as follows. Cultures (0.2 ml) 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 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.

Aspermatogenic Activity Assay—The effect of various ribonucleases on the production of sperm in mice was assessed as follows (Matoušek, 1994). The left testes of CBA mice (five animals/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 hemeatoxylin 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 × testes weight/body weight), and the width of spermatogenic layers. Results were recorded as the mean ± standard error of the mean (S.E.), and compared to the untreated right testes of the same mice.

Immunosuppressive Activity Assay—The effect of various RNases on the proliferation of human lymphocytes stimulated by MLC was assessed as follows (Berger, 1979). 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 by Souček *et al.* (1986). The cells from the interface were aspirated, washed three times with phosphate-buffered saline, 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 (10^5) from the two preparations were mixed 1:1. MLC 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, as described in the antitumor activity assay. The mean value for the three cultures containing a particular ribonuclease was compared that for untreated cells. The immunosuppressive activity assay was also performed on a preparation of human lymphocytes stimulated with either phytohemagglutinin or concanavalin A.

RESULTS

Preparation of Wild-type and Mutant BS-RNase—Two cysteine residues form intersubunit disulfide bonds in the native dimer of BS-RNase. Oligonucleotide-mediated site-directed mutagenesis was used to change the codon for each of these residues to a codon for serine. Monomers of wild-type, C31S, and C32S BS-RNase were prepared from *E. coli* as described (Kim and Raines, 1993a; de Nigris *et al.*, 1993).

Interconversion of M=M and M×M—M=M form of wild-type, C31S, and C32S BS-RNase was prepared in a two-step oxidation. In the first oxidation step, protein was refolded and oxidized in the presence of glutathione to yield monomer (M). Since glutathione can form a mixed disulfide with the sulfydryl group of Cys^{31} and Cys^{32} (Smith *et al.*, 1978), each M was treated with a 5-fold molar excess of dithiothreitol and then dialyzed at pH 8.5 to allow for dimer formation by air oxidation. After dialysis for 24 h, >70% of each BS-RNase was isolable as

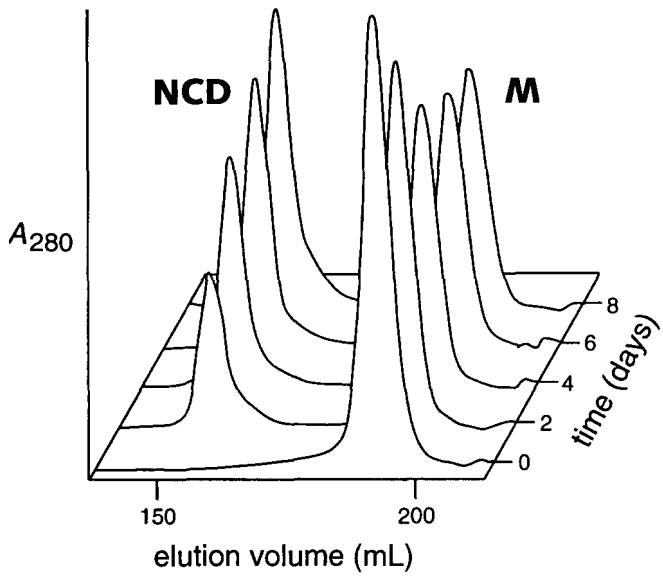

FIG. 2. **Analysis of the quaternary structure of BS-RNase by gel filtration chromatography.** The M=M form of wild-type BS-RNase was allowed to equilibrate at 37 °C with the M×M form. At various times, aliquots were withdrawn, and the intersubunit disulfide bonds were reduced selectively, thereby converting the M=M form to monomer (M) and any M×M form to NCD. These forms were then separated by gel filtration chromatography and quantified by A_{280}.

an M=M (>90%) or M×M (<10%) dimer. The ability of dimeric C31S and C32S BS-RNase to catalyze the degradation of RNA was identical (±5%) to that of the wild-type enzyme.

In dimers of wild-type BS-RNase, the two intersubunit disulfide bonds have a higher (that is, less negative) reduction potential than do the eight intrasubunit disulfide bonds (D'Alessio *et al.*, 1975). The intersubunit bonds can be reduced selectively by treating the dimers with a 10-fold molar excess of reduced dithiothreitol. Upon reduction, the monomers from M=M dissociate but those from M×M remain associated as a NCD due to extensive noncovalent interactions between the two subunits. The interaction between the monomers in NCD is probably similar to that between S-peptide (residues 1–20) and S-protein (residues 21–124) in RNase S (Kim *et al.*, 1992; Kim and Raines, 1993b). Selective reduction followed by gel filtration chromatography thus allowed us to distinguish M=M from M×M, as shown in Fig. 2. The M=M and M×M forms comigrated on an isoelectric focusing gel, with pI = 10.3.

We used the selective reduction-gel filtration chromatography method to follow the time course of the interconversion of the M=M and M×M forms of wild-type, C31S, and C32S BS-RNase. The M=M form of wild-type BS-RNase equilibrated with the M×M form over several days, as shown in Fig. 3. Numerical analysis of the data in Fig. 3 gave the kinetic and thermodynamic parameters in Table I. At equilibrium, 57% (= $k_1/(k_1 + k_{-1})$) of the wild-type dimer was present as the M×M form, indicating that the M×M form is slightly more stable than is the M=M form of wild-type BS-RNase. The M=M form of the C31S and C32S enzymes interconverted more slowly with the M×M form than did the wild-type enzyme. At equilibrium, only 23% of the C31S dimer and 29% of the C32S dimer were present as the M×M form, indicating that the M=M form is more stable than is the M×M form in the C31S and C32S enzymes.

Antitumor Activity—Previously, we reported that wild-type BS-RNase inhibits the proliferation of 20 human tumor cell lines to different extents (Souček and Matoušek, 1993). Now, we have determined the ability of various forms of BS-RNase (monomeric BS-RNase, C31S BS-RNase, C32S BS-RNase,

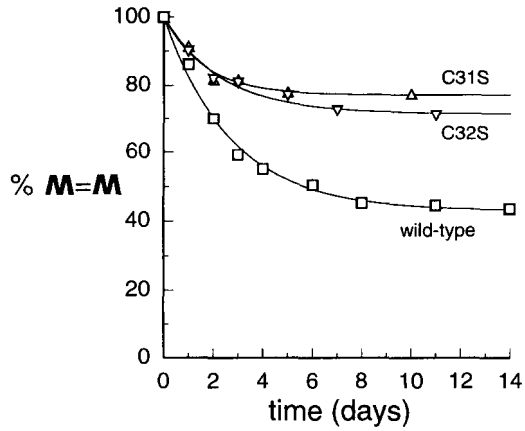

FIG. 3. **Time course for the equilibration of the M=M and M×M forms of wild-type (*G*), C31S (*C*), and C32S (*S*) BS-RNase at 37 °C.** The two forms were separated by selective reduction of the intersubunit disulfide bond(s) followed by gel filtration chromatography. The *ordinate* is (% M=M) = [M/(M + NCD)] × 100%. The gel filtration profiles for the wild-type enzyme at 0, 2, 4, 6, and 8 days are shown in Fig. 2.

TABLE I

Kinetic and thermodynamic parameters for the $M = M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M \times M$ *interconversion of wild-type and mutant BS-RNase at 37 °C*

Parameters were calculated by fitting the data in Fig. 2 to the equation $(\%\ M{=}M)_t = (k_1 + k_1 e^{-(k_1+k_{-1})t})/(k_1 + k_{-1}) \times 100$. The values of r^2 for this fit were 0.99, wild-type; 0.98, C31S; and 0.98, C32S.

BS-RNase	k_1	k_{-1}	$K (= k_1/k_{-1})$	$\Delta G°$
	days^{-1}			*kcal/mol*
C31S	0.14	0.46	0.30	0.75
C32S	0.12	0.30	0.40	0.57
Wild-type	0.21	0.16	1.3	−0.18

M=M BS-RNase, M×M BS-RNase, and wild-type BS-RNase (which is an equilibrium mixture of the M=M and M×M forms)) and of RNase A to inhibit the proliferation of two of these lines: K-562 and ML-1. C32S BS-RNase, M=M BS-RNase, wild-type BS-RNase, and M×M BS-RNase were highly toxic to K-562 cells, as shown in Fig. 4*A*. The C31S BS-RNase had a modest cytotoxic effect. Monomeric BS-RNase and RNase A showed no measurable effect on the proliferation of K-562 cells.

All ribonucleases had a weaker effect on the proliferation of ML-1 cells than on that of K-562 cells, as shown in Fig. 4*B*. The weaker response of the ML-1 cell line allowed us to better compare the toxicity of the various forms of BS-RNase. The cytotoxic effect of the various other forms of BS-RNase increased in the order C31S < C32S < M=M < wild-type < M×M. Again, monomeric BS-RNase and RNase A showed no measurable effect on cell proliferation.

Aspermatogenic Activity—Wild-type BS-RNase induces reversible infertility in rodents (Dostál and Matoušek, 1973). We determined the aspermatogenic activity of various forms of BS-RNase and of RNase A by injecting the ribonucleases into one testis of five mice and recording after 10 days the diameter of seminiferous tubules, the weight of the testes, and the width of spermatogenic layers. The mean values of these three parameters are reported in Fig. 5 relative to that of the non-injected testes of the same mice. Wild-type BS-RNase and the M×M form were the most aspermatogenic of the ribonucleases tested. C32S BS-RNase and M=M had modest aspermatogenic activity. RNase A, monomeric BS-RNase, and C31S RNase A displayed no significant activity.

Immunosuppressive Activity—Previously, we reported that

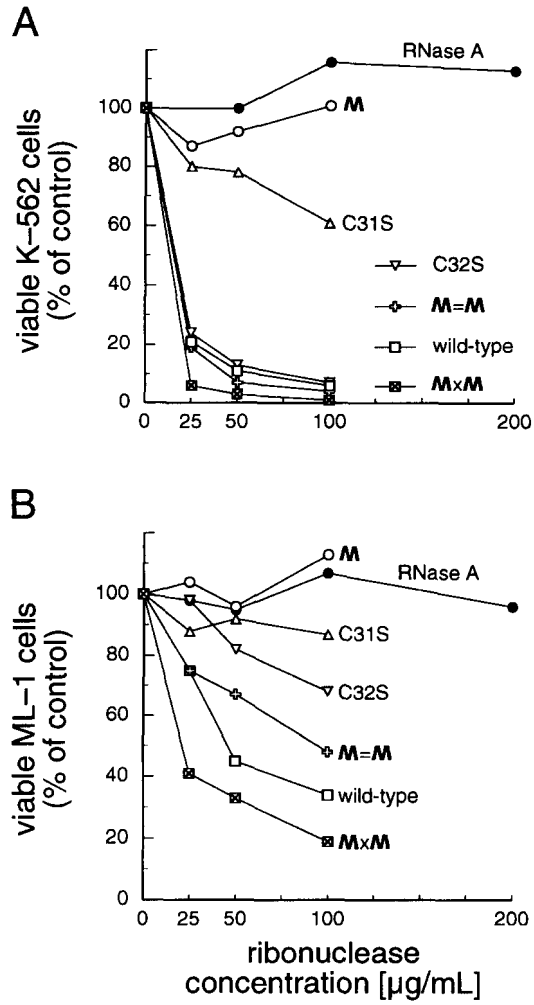

FIG. 4. **Effect of various forms of BS-RNase and of RNase A on the proliferation in culture of human tumor cell lines K-562 (*A*) and ML-1 (*B*).** Proliferation was evaluated by the incorporation of [6-^{3}H]thymidine into cellular DNA. Values are the mean from three cultures and are reported as a percent of the control, which was the mean value from medium containing no exogenous ribonuclease. Data were recorded 3 days after addition of ribonuclease to the culture.

wild-type BS-RNase displayed a remarkable immunosuppressive activity both *in vitro* and *in vivo* (Souček *et al.*, 1983, 1986). Now, we have determined the ability of various forms of BS-RNase and of RNase A to inhibit the proliferation of normal human lymphocytes stimulated in MLC. The inhibitory effect of BS-RNase mutants on the proliferation of lymphocytes was much more pronounced than that on the proliferation of tumor cells. A concentration of only 12.5 μg/ml of M=M BS-RNase, wild-type BS-RNase, or M×M BS-RNase caused approximately 90% inhibition, as shown in Fig. 6. The effects of C32S and C31S BS-RNase were more modest, whereas monomeric BS-RNase and RNase A were ineffective. At higher concentrations, monomeric ribonucleases did cause inhibition, but this inhibition was at least 10-fold weaker than that exhibited by the various dimeric forms. The relative inhibitory effect of the ribonucleases on the proliferation of phytohemagglutinin- or concanavalin A-stimulated human lymphocytes (data not shown) was similar to that on cells in an MLC: (M, RNase A) < C31S < C32S < (M=M, wild-type, M×M).

DISCUSSION

Many enzymes exist as multimers composed of identical subunits. Some of these homomultimers have active sites that are located at an interface between subunits. For example, the

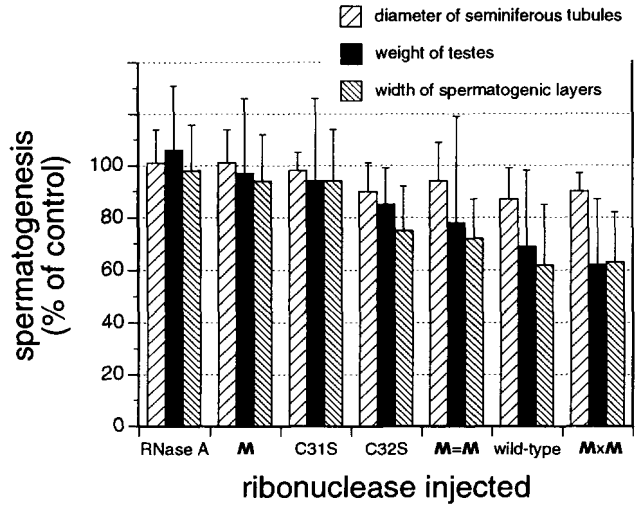

FIG. 5. **Effect of various forms of BS-RNase and of RNase A on mouse spermatogenesis.** Each value is an average from five injected testes and is reported as a percent of the control, which was from the non-injected testes of the same five mice. Data were recorded 10 days after injection.

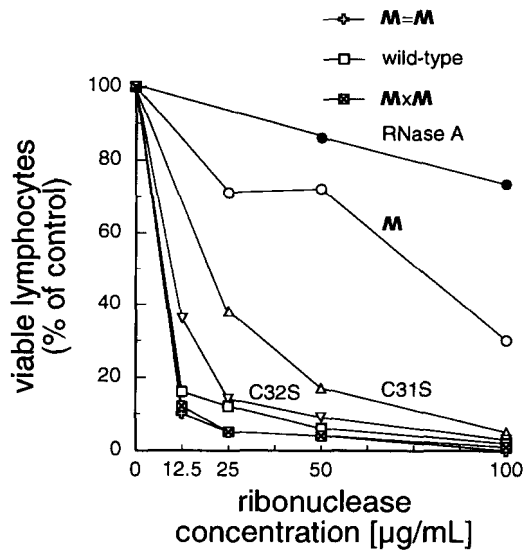

FIG. 6. **Effect various forms of BS-RNase and of RNase A on the proliferation in culture of MLC-stimulated human lymphocytes.** Proliferation was evaluated by the incorporation of [6-^{3}H]thymidine into cellular DNA. Values are the mean from three cultures and are reported as a percent of the control, which was the mean value from cultures containing no exogenous ribonuclease. Data were recorded 6 days after addition of ribonuclease to the culture.

active site residues in dimers of HIV-1 protease are contributed by different subunits (Wlodawer *et al.*, 1989). This enzyme loses all catalytic activity upon dimer dissociation. The active site residues in dimers of triosephosphate isomerase are contributed by one subunit, but residues adjacent to those in the active site are contributed by the other subunit (Banner *et al.*, 1975). Like HIV-1 protease, triosephosphate isomerase loses all activity upon dimer dissociation. (Recently, Borchert *et al.* (1994) created a mutant of triosephosphate isomerase that loses only 10^3-fold in activity upon dimer dissociation.)

BS-RNase differs in a significant way from HIV-1 protease, triosephosphate isomerase, and all other multimeric enzymes with composite active sites. BS-RNase exists in two distinct quaternary forms. The interconversion of these two forms requires regional unfolding and movement of the NH_2-terminal α-helix of each monomer. Under severe conditions (such as lyophilization from 50% v/v acetic acid), RNase A forms a dimer in which the active sites are composite and thus similar to the active sites in the M×M form of BS-RNase (Crestfield *et al.*, 1962). In contrast, BS-RNase undergoes this conversion in physiological conditions. Here, we have illuminated the molecular basis and biological consequences of this conversion.

Site-directed mutagenesis was used to alter the relative free energy of M=M and M×M. In effect, we replaced the two intersubunit disulfide bonds of native BS-RNase (C31-C32′ and C32-C31′) with a single nonnative disulfide bond (C31-C31′ in C32S BS-RNase or C32-C32′ in C31S BS-RNase). The resulting mutant enzymes retained full enzymatic activity. The extent of their conversion to the M×M form was, however, reduced significantly. Each cysteine to serine mutation made the conversion of M=M to M×M approximately 2-fold slower and the conversion of M×M to M=M approximately 2-fold faster (Table I).

We used wild-type and mutant BS-RNases, which differ in their equilibrium populations of the M×M form, to correlate quaternary structure with cytotoxic activity. In previous experiments, we found that individual cell lines respond differently to wild-type BS-RNase (Souček and Matoušek, 1993). For example, the proliferation of myeloid tumor cells was less inhibited than was that of lymphoid tumor cells. In the present study, we used the less sensitive myeloid cell line ML-1 to distinguish between the antitumor activity of various forms of BS-RNase (Fig. 4*B*). The antitumor activity increased as C32S < M=M < wild-type < M×M. We were not able to use the more sensitive lymphoid cell line K-562 to distinguish between the various forms of BS-RNase (Fig. 4*A*). Still, monomers of wild-type BS-RNase and RNase A were ineffective inhibitors of proliferation, and C31S BS-RNase showed only marginal activity.

The immunosuppressive activity of the various forms of BS-RNase and of RNase A exceeded the antitumor activity. All dimeric forms were active inhibitors of lymphocyte proliferation at a concentration of 12.5 μg/ml, and monomers of wild-type BS-RNase and RNase A inhibited proliferation at a concentration of 100 μg/mL (Fig. 6). This result is consistent with our previous finding that angiogenin, a monomeric homolog of RNase A, exhibits an immunosuppressive activity but not an antitumor or aspermatogenic activity (Matoušek *et al.*, 1992). These results intimate a physiological role for BS-RNase. The marked immunosuppressive activity of BS-RNase, which constitutes 3% of the protein in bull seminal plasma, may protect sperm cells from the cow immune system (James and Hargreave, 1984). In humans, immunosuppression by humanized BS-RNase could be of use in evoking tissue tolerance during transplantation surgery.

The results of our biological assays suggest that the M×M form of BS-RNase has significantly more cytotoxic activity than does the M=M form (Figs. 4–6). Still, these results are difficult to interpret. The problem is that the M=M and M×M forms of wild-type BS-RNase equilibrate with $t_{1/2} = \ln 2/(k_1 + k_{-1}) = 1.9$ days. The antitumor assay takes 3 days, the immunosuppression assays takes 6 days, and the aspermatogenesis assay takes 10 days. The two forms of BS-RNase therefore largely equilibrate during the course of the biological assays. The mutant enzymes enabled us to solve this dilemma. The C31S and C32S enzymes contain far less M×M at equilibrium than does wild-type BS-RNase (Fig. 3 and Table I). In the biological assays, these mutant enzymes are the best available representatives of the M=M form. The cytotoxic activities of the mutant enzymes increased as C31S < C32S < wild-type (Figs. 4–6). This same order described the fraction of the enzymes that existed at equilibrium as the M×M form. This correlation suggests that the diminished cytotoxicity of C31S and C32S BS-RNases arises from a decreased fraction of the more potent M×M form.

What is the molecular basis for the different cytotoxicity of the two quaternary forms of BS-RNase? The M×M and M=M forms are identical in amino acid sequence and disulfide bonding pattern. Each form has a p*I* = 10.3, which is the same as that of BS-RNase isolated from seminal plasma (D'Alessio *et al.*, 1972). The two forms comigrate during gel filtration chromatography (Kim and Raines, 1993a, 1994), suggesting that the native proteins have similar Stokes radii. Thus, the overall physical properties of the M×M and M=M forms are indistinguishable. Further, the existence of a cellular receptor, which could in theory distinguish the two forms of BS-RNase, is unlikely (Mancheño *et al.*, 1994).[3]

We suggest that the difference in the cytotoxicity of the two forms derives from their different fate inside the cell. The basis of our separation of M×M and M=M is the ability of M×M but not M=M to remain dimeric in the presence of a reducing agent. The reduction potential in the cytosol of mammalian cells (Hwang *et al.*, 1992) quickly reduces the intersubunit but not the intrasubunit disulfide bonds of BS-RNase (data not shown). In this environment, M×M but not M=M will remain dimeric. The M×M form of BS-RNase, with its extensive noncovalent intersubunit interactions (Fig. 1), can therefore maintain the enzyme as a dimer *in vivo*.

The dimeric form of BS-RNase has a distinct attribute that is likely to be critical for its cytotoxic activity. The cytosolic ribonuclease inhibitor protein (Kobe and Deisenhofer, 1995) binds tightly to monomers but not dimers of BS-RNase (Murthy and Sirdeshmukh, 1992). We therefore propose that the ability of BS-RNase (and of an RNase A mutant (Di Donato *et al.*, 1994)) to maintain a dimeric form *in vivo* leads to its special biological properties because only the dimer is resistant to cytosolic ribonuclease inhibitor. Onconase, a monomeric homolog of RNase A, may be cytotoxic for similar reasons (Youle *et al.*, 1993).

Other workers have proposed that the unusual features of catalysis by BS-RNase are responsible for its special biological properties. Dimers of BS-RNase or RNase A, but not monomers of RNase A, catalyze the cleavage of double-stranded RNA (Libonati and Floridi, 1969; Libonati *et al.*, 1976). Additionally, catalysis of the hydrolysis of nucleoside 2′,3′-cyclic phosphodiesters by dimers but not monomers of BS-RNase or RNase A is regulated allosterically (Piccoli and D'Alessio, 1989; Piccoli *et al.*, 1988; Tamburrini *et al.*, 1989). The physiological significance of these enzymatic properties has been the object of much speculation (D'Alessio *et al.*, 1991). Most recently, Eisenberg and co-workers (Bennett *et al.*, 1994) suggested that the M×M form of BS-RNase evolved because of its unique allosteric properties. In contrast, we believe that the unusual features of catalysis by BS-RNase are only artifacts of its quaternary structure. All of the special biological properties of BS-RNase result from its cytotoxicity, and the uninhibited degradation of cellular RNA (whether single-stranded or double-stranded) by an invasive enzyme (whether nonallosteric or allosteric) is likely to be cytotoxic (Saxena *et al.*, 1991; Newton *et al.*, 1994).

Our results make predictions about the structure and function of the mammalian seminal ribonucleases from deer, giraffe, and sheep (Breukelman *et al.*, 1993).[2] Although these enzymes have yet to be isolated, they are likely to exist as dimers cross-linked by a disulfide bond between Cys^{32} of each subunit. Since these enzymes lack a cysteine residue at position 31, the major form of these enzymes is likely to be M=M. Accordingly, these enzymes may display only a fraction of the special biological properties of BS-RNase.

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[3] J.-S. Kim and R. T. Raines, unpublished results.

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