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 \(\alpha\)-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 M-M and M×M, 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 (Dostal and Matousek, 1973; Soucek et al., 1986; Tamburrini et al., 1990; D’Alessio et al., 1991; Lacetti 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 (Bartholomeys 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 ribonuclease 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 illustrate 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.
E X P E R I M E N T A L  P R O C E D U R E S

M a t e r i a l s

RNase A was from Boehringer Mannheim GmbH (Hamburg, Germany). E. coli strain B521(DES) was from NEN/Amersham (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). [6-3H]Thymidine (960 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.

M e t h o d s

DNA oligonucleotides were synthesized on an Applied Biosystems model 392 DNA/DNA synthesizer by using the β-cyanethyl 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 (Ausbuch et al., 1986).

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 ε1%1% = 0.465 at 278 nm for all forms of BS-RNase (D’Alessio et al., 1972) and ε1%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 Biochemicals (Piscataway, NJ).

M u t a g e n e s i s—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 C31S41 (ACACTTACCTTGGGCATCTTTTCTACAGATCAAGT) was used to change the TGT codon of Cys31 to the TCT codon of serine. Oligonucleotide C32S29 (ACACTTACCTTGTCATCTTTCTACAGATCAAGT) was used to change the TGT codon for Cys32 to the TCT codon of serine. The resulting plasmids, pLSR31 and pLSR32, were used to produce C31S BS-RNase and C32S BS-RNase, respectively.

P r o d u c t i o n a n d P u r i f i c a t i o n o f M=M, M=M, a n d C 3 1 S a n d C 3 2 S f r o m E. c o l i—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 mM Tris-AcOH buffer, pH 8.5, containing glutathione (0.3 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 mM). Fractions containing monomeric BS-RNase (M) were collected and concentrated. Any glutathione was removed by selective reduction with a 5-fold molar excess of diethithreitol, after the addition of 0.1 volume of 1.0 mM Tris-HCl buffer, pH 8.5.

The M form of wild-type BS-RNase was stabilized by alkylation the sulfhydryl groups of Cys31 and Cys32 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 4 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.

P u r i f i c a t i o n o f M=M a n d M=M f r o m S e m i n a l P l a s m a—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 Dostal and Matoušek (1973) or Kim and Raines (1993a). The inter-subunit disulfide bonds of the purified enzyme were reduced with a 10-fold molar excess of reduced diethithreitol, 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.

S o u r c e o f P r o t e i n f o r A s s a y s—The source of the ribonuclease used in a particular assay was based on the composition (bull seminal plasma contains an equilibration 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 mM 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 NH2-terminal methionine residue) had identical enzymatic activity (Kim and Raines, 1993a; Kim et al., 1995) and antitumor activity (data not shown).

C o n v e r s i o n o f M=M t o M=M—Solutions of purified M=M form (1.0 mg/ml) in 0.1 mM 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 nmoi) to reduce only the intersubunit disulfide bonds (DAlessio 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 of 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 assayed as follows. Cultures (0.2 ml) were established in microtiter 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 CO2 (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 [6-3H]thymidine into newly synthesized DNA. Briefly, [6-3H]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 (Matošek, 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 removed, weighed, stained with hematoxylin and eosin, and subjected to histological examination. Aspermatogenic activity was assessed by measuring the diameter of seminiferous tubules, the index weight (which is the 10^2 x 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. Cell density gradient (d = 1.077) of Ficoll-Hypaque solution gradient as described by Soucek 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 (10^6) from the two preparations were mixed 1:1. MLC cultures (0.2 ml) were established in microtiter plates (NUNC, U type) and cultivated at 37 °C in RPMI 1640 medium under a humidified atmosphere containing CO2 (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 [6-3H]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 with that for untreated cells.

RESULTS

Preparation of Wild-type and Mutant BS-RNase—Two cytosine 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, 1992; 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 Cys31 and Cys32 (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 isolate as

![Figure 2](image_url)
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. 4A. 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. 4B. 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 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 homooligomers have active sites that are located at an interface between subunits. For example, the

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**TABLE I**

<table>
<thead>
<tr>
<th>BS-RNase</th>
<th>k1</th>
<th>k⁻¹</th>
<th>K(=k₁/k⁻¹)</th>
<th>ΔG°</th>
</tr>
</thead>
<tbody>
<tr>
<td>C31S</td>
<td>0.14</td>
<td>0.46</td>
<td>0.30</td>
<td>0.75</td>
</tr>
<tr>
<td>C32S</td>
<td>0.12</td>
<td>0.30</td>
<td>0.40</td>
<td>0.57</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.21</td>
<td>0.16</td>
<td>1.3</td>
<td>-0.18</td>
</tr>
</tbody>
</table>

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**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 + NCDI) × 100%. The gel filtration profiles for the wild-type enzyme at 0, 2, 4, 6, and 8 days are shown in Fig. 2.

**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 [6-3H]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.
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 $\alpha$-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$\times$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$\times$M. In effect, we replaced the two inter-subunit 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$\times$M form was, however, reduced significantly. Each cysteine to serine mutation made the conversion of M=M to M$\times$M approximately 2-fold slower and the conversion of M$\times$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$\times$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. 4B). The antitumor activity increased as C32S < M=M < wild-type < M$\times$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. 4A). 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 $\mu$g/mL, and monomers of wild-type BS-RNase and RNase A inhibited proliferation at a concentration of 100 $\mu$g/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$\times$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$\times$M forms of wild-type BS-RNase equilibrate with $k_{12} = \ln 2/(k_{1} + k_{-1}) = 1.9$ days. The antitumor assay takes 3 days, the immunosuppression assay 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$\times$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$\times$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$\times$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 π = 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 (Manchacio 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 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 A, may be cytotoxic for similar reasons (Youle and Raines, 1993b).

Our results make predictions about the structure and function of the mammalian seminal ribonuclease from deer, giraffe, and sheep (Breukelmann et al., 1993). Although these enzymes have yet to be isolated, they are likely to exist as dimers cross-linked by a disulfide bond between Cys³² 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.

Acknowledgments—We thank B. R. Kelemen and D. M. Nierengarten for assistance in protein purification and protein chemistry.