PROTEIN ENGINEERING TO EXPLOIT AND EXPLORE BOVINE SECRETORY RIBONUCLEASES

by

JIN-SOO KIM

A thesis submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
(Biochemistry)

at the
UNIVERSITY OF WISCONSIN–MADISON
1994

ACKNOWLEDGEMENTS

I would like to thank Dr. Ronald T. Raines for his advice and support. His scientific insight has been very helpful throughout this work. I would also like to thank the entire Raines group for their friendship and companionship. I am grateful to Dr. J. Soucek and Dr. J. Matousek for their collaboration with us, which has been a valuable part of the BS-RNase research. I thank Dr. M. Karpeisky for suggesting the protein fusion project, and Dr. G. D'Alessio and Dr. L. Mazzarella for providing the coordinates of BS-RNase. I have been generously supported by Steenbock predoctoral fellowship from the Department of Biochemistry. Finally, I thank my parents, who have encouraged (or at least not discouraged) me to pursue a career in science since I was a kid.

ABSTRACT

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Jin-Soo Kim

Under the supervision of Dr. Ronald T. Raines

at the University of Wisconsin–Madison

Ribonuclease S-peptide (residues 1–20) and S-protein (residues 21–124) are the enzymatically inactive products of the limited digestion of bovine pancreatic ribonuclease A (RNase A) by subtilisin. S-Peptide binds S-protein with high affinity to form RNase S, which has full enzymatic activity. We developed a novel protein fusion system for the purification and detection of proteins produced in *Escherichia coli* by employing S-peptide as an affinity tag. Any protein fused to S-peptide through recombinant DNA techniques can be purified by one-step affinity chromatography using an immobilized S-protein resin, and assayed by measuring ribonuclease activity following activation with S-protein.

Bovine seminal ribonuclease (BS-RNase) is a close homolog of RNase A, sharing 81% amino acid sequence identity. Unlike RNase A, BS-RNase is a dimer crosslinked by two disulfide bonds between Cys31 of one subunit and Cys32 of the other. At equilibrium, this dimer is a mixture of two distinct quaternary forms, **M=M** and **MxM**. In the major form, **MxM**,

the *N*-terminal tail (equivalent to S-peptide) of one subunit stretches out from the body (equivalent to S-protein) of the same subunit, and interacts with the body of the other subunit. In the other form, **M=M**, such exchange does not occur. In addition to this intriguing quaternary structure, BS-RNase has extraordinary biological properties including aspermatogenic, antitumor, and immunosuppressive activities. To illuminate structure-function relationship of BS-RNase and RNase A, we synthesized and expressed a gene for BS-RNase in *E. coli*. Next, we prepared and characterized a series of BS-RNase mutants and molecular hybrids between BS-RNase and RNase A. All the hybrids we tested showed strong biological activities, which suggests that a receptor specific for BS-RNase may not exist. In contrast, C31S and C32S BS-RNases had a reduced fraction of **MxM** at equilibrium and decreased biological activities. These results indicate that the **MxM** form, which can remain as a dimer in the reducing environment of the cytosol, is responsible for the special biological properties of BS-RNase.

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bBBr	4,6-bis(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione
BS-RNase	bovine seminal ribonuclease
CM-Cys	carboxymethyl cysteine
CM-His	carboxymethyl histidine
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
FPLC	fast protein liquid chromatography
GdnCl	guanidine hydrochloride
IPTG	isopropyl-β-D-thiogalactopyranoside
mBBr	4-bromomethyl-3,6,7-trimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione
NMR	nuclear magnetic resonance
poly(C)	poly(cytidylic acid)
RISBASE	ribonuclease with special biological actions
RNase A	bovine pancreatic ribonuclease A
S15	residues 1–15 of RNase A
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-peptide	residues 1–20 of RNase A
S-protein	residues 21–124 of RNase A
UpA	uridylyl(3',5')adenosine

CHAPTER I

An Introduction to Bovine Secretory Ribonucleases

Bovine pancreatic ribonuclease A (RNase A) is a small protein (124 amino acid residues) that is highly stable and available in large quantities. These features have made this enzyme one of the favorite research objects of protein biochemists since the 1950's. The resulting studies have led to numerous important discoveries. For example, by refolding the protein that was reduced and denatured, Anfinsen and coworkers showed that the primary structure of RNase A has all the information required to form active enzyme (Anfinsen, 1973). This demonstration was followed by the successful refolding of many other proteins, which led to a major paradigm in biochemistry: the primary structure of a protein determines the tertiary structure. Since then, the question of how the three-dimensional structure of a protein can be predicted from its amino acid sequence has been one of the most challenging problems in biochemistry. RNase A was also the first enzyme for which the amino acid composition and sequence were determined. For this contribution to protein chemistry by using RNase A as a test protein, Stein and Moore shared the Nobel Prize in Chemistry with Anfinsen in 1972 (Moore and Stein, 1973).

RNase A catalyzes the cleavage of RNA in two steps: trans-phosphorylation and hydrolysis. The imidazole groups of His12 and His119 serve as a general base and general acid during catalysis. Richards and his coworkers showed that the protease subtilisin prefers to cleave a single peptide bond in RNase A (Richards and Vithayathil, 1959). The product of this cleavage, ribonuclease S (RNase S), consists of two tightly associated fragments: S-peptide (residues 1–20) and S-protein (residues 21–124). These two fragments can be separated from each other at low pH.

Neither S-peptide nor S-protein has any detectable ribonuclease activity because S-peptide lacks His119 and S-protein lacks His12. However, the noncovalent complex formed by the two fragments, RNase S, shows almost full enzymatic activity. The noncovalent interaction between S-peptide and S-protein is quite strong. The dissociation constant is estimated to be in the range of nM (Richards and Wyckoff, 1971). Thus the S-peptide: S-protein interaction is stronger than the interaction between most antigens and antibodies (μM to nM) and comparable to that between a receptor and a ligand. Numerous hydrophobic interactions and hydrogen bonds between S-peptide and S-protein are responsible for the strong noncovalent interaction. The crystal structure of RNase S is indistinguishable from that of RNase A except for the short loop region that connects S-peptide and S-protein (Wyckoff *et al.*, 1967). In this region, the *C*-terminus of S-peptide is separated from the *N*-terminus of S-protein by 27 Å.

The efficient refolding of S-protein from the denatured and reduced state requires S-peptide (Kato and Anfinsen, 1969). S-Peptide appears to provide a nucleation site for the proper folding of S-protein. S-Peptide itself adopts considerable helicity (10–50%) in water (Finkelstein *et al.*, 1991). Indeed, S-peptide is one of the smallest peptides that shows a secondary structure in solution. Due to this property, S-peptide and its analogs have been used extensively as a model peptide to study α-helical propensity.

Proteases are useful tools for probing the structure of proteins. Mild proteolysis of proteins often leads to a specific cleavage(s) between

domains (Wu *et al.*, 1994). For example, the restriction endonuclease, *Fok*I, is cleaved by trypsin into two domains (Li *et al.*, 1992). One domain recognizes a specific DNA sequence and the other domain cleaves DNA nonspecifically. The two domains, however, do not form a noncovalent complex as do RNase S-peptide and S-protein. Similarly, chymotrypsin cleaves the trp repressor dimer preferentially (Tasayco and Carey, 1992). The fragments can be separated (by denaturing the cleavage product), refolded, and reassembled to form functional dimer. The analysis of proteins by proteolysis provides valuable information on protein conformation, and is especially useful, if the structure of the proteins has not been determined by X-ray crystallography or NMR spectroscopy. Although many proteins undergo limited proteolytic fragmentation, the RNase S system is unique in that both fragments are completely inactive but the noncovalently associated product is fully active.

The S-peptide fragment of RNase A has had a singular role in the development of protein chemistry. Before molecular biologists were able to use recombinant DNA technology to explore protein structure-function relationships, chemists synthesized analogs of S-peptide and studied their complexes with S-protein (Richards and Wyckoff, 1971). These studies were successful in illuminating molecular aspects of enzymatic catalysis and protein-protein interaction, and were the harbinger of current work on proteins containing unnatural or otherwise mutant amino acid residues. For example, Taylor and coworkers showed that the crystal structure of modified RNase S, in which His12 in S-peptide was replaced by a 4-fluoro-histidine, is almost identical to that of the native protein,

although the modified RNase S is inactive (Taylor *et al.*, 1981). The researchers concluded that His12 is indeed an active-site residue. Zuckermann and Schultz exploited the RNase S system ingeniously to develop a sequence-selective ribonuclease. They attached a small oligodeoxynucleotide through disulfide bond to an analog of S-peptide in which Lys1 was replaced by a cysteine. Addition of S-protein to this DNA-peptide hybrid yielded a ribonuclease that cleaves RNA specifically, indicating that the oligonucleotide domain directs the hybrid enzyme to the complementary target site (Zuckermann and Schultz, 1988).

The dissociation of S-peptide from S-protein at low pH and their reassociation imply that a dimer of RNase A may form under certain conditions. In this imaginary dimer, S-peptide of one subunit stretches out from S-protein and interacts with S-protein of the other subunit. Thus, the active site residues, His12 and His119, are contributed by different polypeptide chains. Indeed, such an RNase A dimer exists. Crestfield and coworkers observed that RNase A forms aggregates when lyophilized from aqueous acetic acid (30–50% v/v) (Crestfield *et al.*, 1962). These aggregates are fully active and can be separated by gel filtration chromatography. The aggregates consist mainly of dimeric forms, although higher aggregates such as trimers and tetramers appear as well. The two active sites of a dimer appear to act independently from each other. Crestfield and coworkers demonstrated that the dimer has composite active sites as described above by preparing dimers from RNase A that was selectively carboxymethylated at either His12 or His119, which inactivates the monomeric enzyme. Homogeneous dimers (CM-His12 +

CM-His12 and CM-His119 + CM-His119) showed little catalytic activity, as expected. However 50% of specific activity was detected from a heterogeneous dimer (CM-His12 + CM-His119), which indicates that the two active-site histidine residues are contributed by each monomer and that one active site in is intact in this heterogeneous dimer (Crestfield and Fruchter, 1967). Although the dimer of RNase A is quite stable at room temperature, it dissociates into monomers by heating to 50 °C. The enzymatic activity is lost by dissociation of the hybrid dimer. This heat instability leads to an important implication in the S-protein fusion system, which is described in CHAPTER II.

The dimerization of RNase A requires regional unfolding and relocation of S-peptide. The acidic pH apparently facilitates the partial unfolding of S-peptide from S-protein while the high protein concentration during lyophilization favors oligomerization. This dimerization process is very unlikely to occur in physiological conditions. Surprisingly, D'Alessio and coworkers have isolated a naturally dimeric ribonuclease from bull seminal fluid (D'Alessio *et al.*, 1972). This protein, bovine seminal ribonuclease (BS-RNase), is a close homolog of RNase A, sharing 81% amino acid sequence identity (Suzuki *et al.*, 1987). All the active-site residues including the two histidines and eight half-cystines that form intrachain disulfide bonds are conserved at the same positions in the amino acid sequence. The crystal structures of the two ribonucleases are very similar as expected from their sequence homology, but differ largely in their quaternary structure (Capasso *et al.*, 1983; Mazzarella *et al.*, 1987).

BS-RNase is isolated as a homodimer in which the subunits are crosslinked by two disulfide bonds between Cys31 of one subunit and Cys32 of the other subunit. Furthermore, BS-RNase exists in two different quaternary forms designated as **MxM** and **M=M** (Piccoli *et al.*, 1992). In the major form, **MxM**, the *N*-terminal tail (equivalent to S-peptide) stretches out from the C-terminal body (equivalent to S-protein) of one subunit and interacts with the body of the other subunit. As a result, the active sites are composite. Thus, the quaternary structure of this form is quite similar to that of RNase A dimer formed by lyophilization from acetic acid solution. In the other form, **M=M**, such exchange of the *N*-terminal tails between the two subunits does not occur. These two forms can be distinguished by selective reduction and gel filtration chromatography. The two intersubunit disulfide bonds have a significantly lower reduction potential than do the eight intrasubunit disulfide bonds (four bonds per subunit) (D'Alessio *et al.*, 1975). Upon selective reduction, the monomers from **M=M** dissociate but those from **MxM** remain associated as a noncovalent dimer due to the interaction between the *N*-terminal tail of one subunit and the C-terminal body of the other subunit. Subsequent gel filtration chromatography allows a monomer to be separated from a noncovalent dimer. When the gel filtration chromatography is performed under denaturing conditions, only monomers are eluted because the noncovalent dimer dissociates into monomers. Refolding studies have shown that BS-RNase first folds into a monomer, which dimerizes to form **M=M**, and is then slowly converted to **MxM**. At equilibrium, the

relative content of **M**x**M** is 60–80%. The two dimers as well as the monomer are potent ribonucleases.

Unlike RNase A, BS-RNase is not selectively cleaved by subtilisin (Parente *et al.*, 1976). The conformational difference in the loop region (residues 15–24) that connects the *N*-terminal tail and the *C*-terminal body in BS-RNase may be responsible for this resistance to selective digestion by subtilisin. The amino acid sequences of BS-RNase and RNase A differ greatly in this loop region. Interestingly, BS-RNase also forms an aggregate when lyophilized from acidic solutions (Libonati, 1969). The aggregate shows similar catalytic activity towards double-stranded RNA as does BS-RNase, and appears to be a tetramer (dimer of BS-RNase). This result demonstrates the remarkable flexibility of the structure of bovine ribonucleases.

Homologous proteins almost always share very similar structure and function (Doolittle, 1987). The two bovine secretory ribonucleases, RNase A and BS-RNase, appear to be an exception to this rule. These enzymes catalyze the cleavage and hydrolysis of RNA after pyrimidine residues with similar efficiency and specificity, and have an almost identical tertiary structure. Except for these similarities, the enzymatic and biological properties as well as the quaternary structure of BS-RNase differ greatly from those of RNase A.

Unlike RNase A, BS-RNase has the two following unusual catalytic features. First, BS-RNase can catalyze the degradation of RNA in double-stranded RNA or in a DNA–RNA hybrid under conditions where RNase A cannot (Sorrentino *et al.*, 1980). Interestingly, artificial dimers of RNase

A prepared by chemical crosslinking display increased catalytic activity against double-stranded RNA. The ability of the dimeric ribonucleases to degrade double-stranded RNA appears to arise from their higher affinity for the substrate. Secondly, BS-RNase displays nonhyperbolic saturation kinetics for the hydrolysis of 2',3'-cyclic nucleotide, the substrate of the second, rate-limiting step of the overall reaction, but not for the hydrolysis of the substrate of the first step (Piccoli *et al.*, 1988). This unusual property is also related to the quaternary structure. Artificial momomers of BS-RNase show regular hyperbolic saturation kinetics for the hydrolysis of the substrates of both steps as does RNase A. In contrast, dimers of RNase A prepared by lyophilization from acetic acid solution show nonhyperbolic kinetics for the hydrolysis of the cyclic nucleotide (Piccoli and D'Alessio, 1984). The nonhyperbolic kinetic pattern of the dimeric ribonucleases appears to result both from the composite nature of the active site and from allosterism.

BS-RNase displays unusual biological activities, although the physiological role of this protein is not known (D'Alessio *et al.*, 1991). For example, BS-RNase shows a strong cytotoxic effect on tumor cells in vivo and in vitro, but not on normal cells (Laccetti *et al.*, 1992). However, monomers of BS-RNase and chemically inactivated dimers of BS-RNase lack the antitumor activity, indicating that both the quaternary structure and the catalytic activity are required for the antitumor effect of BS-RNase (Vescia *et al.*, 1980). Artificial dimers of RNase A show an antitumor activity, although to a lesser extent (Bartholeyns and Baudhuin, 1976; Bartholeyns and Zenebergh, 1979). BS-RNase also blocks the proliferation

of activated T cells (Soucek *et al.*, 1986). This immunosuppressive activity of BS-RNase is likely to be physiologically significant, since this activity may be required to suppress the female immune response against bull seminal components (James and Hargreave, 1984). Like the antitumor activity, the immunosuppressive activity of BS-RNase is closely related to the dimeric structure and catalytic activity (Tamburrini *et al.*, 1990). BS-RNase also induces reversible infertility, when injected into sexually mature male rats (Dostal and Motousek, 1973). The physiological significance of this antispermatogenic effect of BS-RNase is not known.

Due to these unusual biological properties, BS-RNase belongs to a special group of ribonucleases termed RISBASEs (*RI*bonucleases with *S*pecial *B*iological *A*ctions) (D'Alessio *et al.*, 1991; D'Alessio, 1993). This group includes ribonucleases with diverse biological properties from various sources. Two members of this group are particularly interesting due to their biological properties related to those of BS-RNase. Onconase from frog, *Rana pipiens*, exhibits a strong antitumor effect (Ardelt *et al.*, 1991). The amino acid sequence of onconase is 27% and 30% identical to those of BS-RNase and RNase A, respectively. All the key catalytic residues are conserved among the three proteins. When its ribonuclease activity is blocked by chemical modification, onconase loses its antitumor activity. Thus the antitumor activity of both BS-RNase and onconase requires the ribonuclease activity. Human angiogenin is involved in blood vessel formation (Shapiro *et al.*, 1986). The amino acid sequence of this protein is 33% identical to that of RNase A. The ribonuclease activity of angiogenin is also essential for its angiogenic activity. Angiogenesis is

closely related to tumor cell growth. Indeed, angiogenin was first isolated from human tumor cells. It is intriguing that both angiogenin and BS-RNase bind actin and induce its polymerization (Simm *et al.*, 1987; Hu *et al.*, 1993). The actin binding activity of angiogenin may be related to angiogenesis and thus to tumor cell growth. BS-RNase may block the growth of tumor cells by competing with angiogenin for binding to actin.

Other members of this group include neurotoxic ribonucleases such as eosinophil-derived toxin (EDN) and eosinophil cationic protein (ECP) from human, and S-RNases from flowering plants, which are responsible for self-incompatibility (D'Alessio *et al.*, 1991). Although RISBASEs show very diverse biological properties, they have one thing in common: the ribonuclease activity of RISBASEs is intrinsic to their biological actions. When the enzymatic activity of RISBASEs is blocked by chemical modification or site-directed mutagenesis or ribonuclease inhibitors, RISBASEs lose their biological activities as well.

Two different hypotheses on the mechanism of RISBASEs have been proposed: extracellular and intracellular mechanisms. Since all RISBASEs are secreted, RISBASEs may degrade 'extracellular RNAs' that may function as communicators between cells (Benner, 1988). This mechanism predicts the existence of communicator RNAs, which have yet to be isolated. In contrast, the other mechanism predicts the existence of cell-specific receptors for RISBASEs (D'Alessio, 1993). By this mechanism, RISBASEs bind to specific receptors, enter the cells through endocytosis, and then degrade RNAs in the cells. Several receptors specific for RISBASEs have been reported. The cell-type specific action of BS-

RNase appears to favor the intracellular mechanism. For example, BS-RNase is cytotoxic to tumor cells but not to normal cells. The receptor(s) for BS-RNase has yet to be isolated.

The two closely related bovine ribonucleases, RNase A and BS-RNase, are exquisite objects for studies that use the techniques of recombinant DNA. Both ribonucleases are well characterized and their three-dimensional structures are known. The intriguing structure and function of the two ribonucleases are exploited and explored by protein engineering studies in this dissertation. CHAPTER II contains the development of a novel protein fusion system that employs ribonuclease S-peptide derivatives as affinity tags. The expression and purification of BS-RNase from a synthetic gene is described in CHAPTER III. CHAPTER IV contains the characterization of a misfolded but active dimer of BS-RNase by molecular genetic and biophysical methods. The relationship between the quaternary structure of BS-RNase and its biological properties is explored in CHAPTER V. The characterization of an inactive BS-RNase mutant and hybrids between RNase A and BS-RNase is described in CHAPTER VI. Finally, CHAPTER VII contains the methods for all the experiments of this thesis.

CHAPTER II

Ribonuclease S-Peptide as a Carrier in Fusion Proteins

Originally published as

Kim, J.-S. and Raines, R. T. (1993) Ribonuclease S-peptide as a carrier in fusion proteins. *Protein Sci.* **2**, 348-356.

Kim, J.-S. and Raines, R. T. (1994) Peptide tags for a dual affinity fusion system. *Anal. Biochem.* **219**, 165-166.

Summary

S-Peptide (residues 1–20) and S-protein (residues 21–124) are the enzymatically inactive products of the limited digestion of ribonuclease A by subtilisin. S-Peptide binds S-protein with high affinity to form ribonuclease S, which has full enzymatic activity. Recombinant DNA technology was used to produce a fusion protein having three parts: carrier, spacer, and target. The two carriers used were the first 15 residues of S-peptide (S15) and a mutant S15 in which Asp14 had been changed to Asn (D14N S15). The spacer consisted of three proline residues and a four residue sequence recognized by factor X_a protease. The target was β-galactosidase. The interaction between the S-peptide portion of the fusion protein and immobilized S-protein allowed for affinity purification of the fusion protein under denaturing (S15 as carrier) or nondenaturing (D14N S15 as carrier) conditions. A sensitive method was developed to detect the fusion protein after SDS–PAGE by its ribonuclease activity following activation with S-protein. S-Peptide has distinct advantages over existing carriers in fusion proteins in that it combines a small size (≥15 residues), a tunable affinity for ligand ($K_d \geq 10^{-9}$ M), and a high sensitivity of detection ($\geq 10^{-16}$ mol in a gel).

Introduction

Proteins are essential components of all forms of living organisms. Many proteins have been isolated and used for biomedical and research purposes. The progress of genetics and the human genome project provides an endless list of new genes that encode proteins with unknown but potentially important functions.

In order to study a newly identified gene, biologists usually need to clone and express the gene in a heterologous organism. Next, the protein of interest must be purified from other cellular proteins for further study. Conventional methods of protein purification are based on the size, charge and solubility of a protein. Since these properties are specific to each protein, a new purification strategy must be developed for each new protein, which can be idiosyncratic and problematic. Furthermore, if the protein does not display any detectable properties (such as an enzymatic activity), its purification can be especially difficult and tedious.

Fortunately, this problem can be solved by making a fusion protein, through recombinant DNA techniques, in which a fusion tag or a carrier is attached to a target protein (Uhlén and Moks, 1990; Ford *et al.*, 1991; Nilsson *et al.*, 1992). The fusion tag can be a whole protein or an artificial peptide or a small portion of a protein, all of which can bind to a specific ligand, such as a substrate or metal ion or another protein.

Several fusion protein systems have been developed in recent years. For example, glutathione S-transferase (GST) fusion system exploits its specific binding to immobilized glutathione (Smith and Johnson, 1988).

The enzyme activity of the GST moiety can also be used to detect fusion proteins. Likewise, poly(His) tag can serve as a carrier that binds to an immobilized metal affinity resin (Hochuli *et al.*, 1988). The advantage of this system over the GST system, which employs a whole protein as a fusion tag, is that the size of the carrier is much smaller: only 6 histidine residues are necessary. Small carriers are less likely than large carriers to alter the properties of a fusion protein or to perturb proper folding. However, poly(His) tag does not possess any assayable properties. Unfortunately, no fusion system has yet been developed in which a fusion tag is both small and assayable.

Our protein fusion system employs ribonuclease S-peptide as a fusion tag. S-Peptide (residues 1–20) and S-protein (residues 21–124) are the enzymatically inactive products of the limited digestion of ribonuclease A by subtilisin (Richards, 1955; Richards and Vithayathil, 1959). S-Peptide binds S-protein with high affinity to form ribonuclease S, which has full enzymatic activity. This specific interaction allows one-step purification and sensitive assay of S-peptide fusion proteins. S-peptide has distinctive advantages over existing carriers in that it combines a small size and a high sensitivity of detection.

Results

Design of fusion system

A fusion protein has three parts: carrier, spacer, and target. The carriers used in this work were derived from ribonuclease S-peptide. These carriers met two other design criteria.

First, a carrier should be as small as possible because small carriers are less likely than large carriers to alter the properties of a fused protein or to be immunogenic. The truncated RNase S formed from the first 15 residues of S-peptide (S15) and S-protein has the same enzymatic activity and dissociation constant as does RNase S (Potts *et al.*, 1963). The structure of this truncated RNase S has been determined by X-ray diffraction analysis (Fig. II-1) (Wyckoff *et al.*, 1967). This analysis indicates that the C-terminus of the S15 fragment is accessible to solvent. Thus, the attachment of a target protein to the C-terminus of S15 should not prevent its interaction with S-protein to form a functional ribonuclease. This hypothesis is supported by the structure of bovine seminal ribonuclease, a dimeric homolog of RNase A in which the S-peptide fragments of two monomers are exchanged (D'Alessio *et al.*, 1991). A fusion protein in which a target protein is fused to the C-terminus of S15 should therefore be of minimal size but retain its ability to bind properly to S-protein.

Secondly, a fusion system should allow for tight binding to ligand (for protein immobilization) or weak binding to ligand (for protein purification). The dissociation constant of RNase S is about 10^{-9} M. This value is small enough to immobilize a fusion protein having an S-peptide

carrier to an S-protein affinity resin, but it is too small to allow for the purification of the fusion protein under nondenaturing conditions. All of the forty known amino acid sequences for pancreatic ribonucleases have Asp as residue 14 (Beintema, 1987). In RNase A, the carboxylate group of Asp14 forms a hydrogen bond with the phenolic hydroxyl group of Tyr25. This interaction increases the acidity of the carboxyl group of Asp14, which has pK_a = 3.8 in S15 but pK_a = 2.4 in the complex of S15 with S-protein (Niu *et al.*, 1979; Cohen *et al.*, 1980). The mutant RNase S in which this interaction is weakened by changing Asp14 to Asn has the same enzymatic activity as does RNase S but a dissociation constant that is 20-fold higher (Filippi *et al.*, 1975). A protein fused to D14N S15 should therefore have a dissociation constant more appropriate for affinity purification under native conditions.

Thus, the two carriers used in this work were S15, which is a truncated but otherwise wild-type S-peptide, and D14N S15, which is a truncated and mutated S-peptide. The sequences of these two carriers are

S15: Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser

D14N S15: Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asn-Ser

Recombinant DNA was used to produce proteins in which a target protein was fused to the C-terminus of these carriers (Fig. II-2).

The spacer used in this work consisted of three proline residues followed by the sequence of four residues recognized by blood coagulation factor X_a, a commercial protease. This spacer was designed (1) to minimize

interaction between the carrier and target because polyproline tends to adopt a rigid, extended structure (Katchalski *et al.*, 1963), and (2) to allow the intact target to be liberated from the remainder of the fusion protein because factor X_a catalyzes the cleavage of the peptide bond after the sequence Ile–Glu–Gly–Arg (Nagai and Thϕgersen, 1987). The target used in this work was β-galactosidase, an enzyme with a catalytic activity that is easy to assay and that has been both the carrier and target in many other fusion proteins.

Production of fusion protein

Plasmids pSG601 and pSG919 directed the synthesis of a fusion protein with an apparent molecular weight of 118 kD in *E. coli* strain CSH27 (Fig. II-3). The amount of fusion protein produced from an IPTG-induced culture of CSH27/pSG919 was two- to three-fold lower than that from an IPTG-induced culture of CSH27/pSG601.

In crude extracts prepared from uninduced and IPTG-induced cultures of CSH27/pSG601, β-galactosidase activity was measured and, after the addition of various amounts of S-protein, ribonuclease activity was measured. Only the crude extract from the induced culture showed significant β-galactosidase or ribonuclease activity (Fig. II-4). This ribonuclease activity appeared only after the addition of S-protein.

Purification of fusion protein

An affinity resin for S-peptide fusion proteins was produced in which S-protein was attached covalently to CNBr-activated Sepharose 4B.

Batch adsorption to this resin of fusion protein having S15 (data not shown) or D14N S15 (Fig. II-5) as carrier was complete at 4 °C in 20 mM sodium phosphate buffer, pH 7.0. Resin produced by direct coupling of S-protein had a ten-fold higher binding capacity for fusion protein than did resin produced *in situ* by treating RNase A~Sepharose with subtilisin. Sepharose alone did not absorb the fusion proteins.

Nondenaturing conditions effected the elution from the affinity resin of fusion protein having D14N S15 as carrier. Raising the temperature to 37 °C of the loaded resin suspended in 20 mM Tris-HCl buffer (pH 7.4) containing NaCl (0.5 M) produced fusion protein that was a single band after SDS–PAGE (Fig. II-5). The *N*-terminal amino acid sequence of this protein was as expected. A summary of the purification of the fusion protein having D14N S15 as carrier is given in Table II-1.

Denaturing conditions (specifically, NaSCN (3 M)) were necessary to effect the elution of the fusion protein having S15 as carrier. Since target proteins vary in the efficiency with which they can be renatured, the fusion protein having S15 as carrier is more useful for immobilization than for purification of fusion proteins.

Detection of fusion protein

Fusion protein was detected by electrophoresis in a zymogram, which was an SDS–polyacrylamide gel impregnated with poly(C), a ribonuclease substrate. Ribonuclease activity was produced in the gel after SDS was extracted with aqueous isopropanol and the fusion protein was

activated with S-protein. The presence of the fusion protein was revealed after intact poly(C) was stained with toluidine blue O.

Zymogram electrophoresis of protein fused to D14N S15 produced a clear band on a dark background (Fig. II-5 and 6). As few as 10 pg (10^{-16} mol) of fusion protein was detectable after zymogram electrophoresis (Fig. II-6, lane 4). This sensitivity decreased if activation with S-protein was performed above 4 °C or if BSA was omitted from the activation solution.

Overloading fusion protein in a zymogram revealed the presence of several fragments (Fig. II-7, lane 3) in a sample that appeared to be homogeneous by detection with protein stains (Fig. II-5, lane 7). These fragments were still present in fusion protein prepared from the protease-deficient *E. coli* strains: CAG 597 and CAG 629 (data not shown).

Liberation of target protein

Purified target protein was liberated from denatured, but not native, fusion protein by treatment with factor X_a. SDS–PAGE indicated that this protease cleaved the fusion protein at several sites (data not shown).

Target protein was liberated from native fusion protein by treatment with trypsin, which cleaves proteins after Lys and Arg residues (Fig. II-7). The presence of S-peptide carrier in the fusion protein was monitored by zymogram. This amount decreased with time (0-17 h). During treatment with trypsin, a peptide that co-migrated with authentic S15 appeared (0-1 h) and then disappeared (4-17 h). The quantity and migration of the protein with an apparent molecular weight >100 kD appeared to be approximately constant. The *N*-terminal sequence of this

protein indicated that cleavage had occurred primarily after Arg10 of the S15 carrier.

Peptide tags for a dual affinity system

One-step affinity chromatography under nondenaturing conditions yielded fusion protein that was >95% pure. Nevertheless, an extremely sensitive gel assay, zymogram electrophoresis, revealed that many truncated polypeptides carrying the D14N S15 tag were copurified with the full-length fusion protein. This result was not unexpected, as the heterologous production of proteins in *E. coli* often generates such truncated proteins, which are likely to be the products of proteolytic degradation.

In a dual affinity fusion system, a different tag is attached to each end of the target protein. Affinity purification using each of these tags eliminates contamination from truncated polypeptides that contain only one tag. This approach has been described with protein tags (Hammarberg *et al.*, 1989; Jansson *et al.*, 1989). Proteins are undesirable tags, however, because their large size is likely to perturb properties of a target protein. Accordingly, two peptide tags, D14N S15 (15 residues) and poly(His) (His_6; 6 residues), were attached to the *N*- and *C*-termini of β-galactosidase respectively (Fig. II-8). The D14N S15~β-galactosidase~His_6 fusion protein was purified from *E. coli* extract by column chromatography on S-protein~Sepharose resin or Ni^{++} chelation resin or both (Table II-2). Each of these procedures yielded >95% pure protein as judged by SDS–PAGE (Fig. II-9, top). The chromatography products were also analyzed for the

presence of a D14N S15 tag by zymogram electrophoresis (Fig. II-9, bottom). Both the unpurified sample (Fig. II-9, bottom, lane 3) and the sample purified by S-protein affinity chromatography (Fig. II-9, bottom, lane 4) contained several truncated polypeptides along with full-length fusion protein. A similar ensemble of polypeptides was likely co-purified by Ni^{++} chelation chromatography. Unfortunately, polypeptides cannot be detected by zymogram electrophoresis if they have lost their D14N S15 tag. Thus, only the full-length fusion protein was observed by zymogram electrophoresis after Ni^{++} chelation chromatography (Fig. II-9, bottom, lane 5). No significant amount of truncated polypeptides was observed by zymogram electrophoresis after purification by S-protein affinity chromatography followed by Ni^{++} chelation chromatography (Fig. II-9, bottom, lane 6).

Discussion

Target proteins fused to a carrier polypeptide can be detected, immobilized, or purified based on the interaction of the carrier with a specific ligand. For example, the interaction of a carrier with an appropriate antibody can be used to detect as few as 10^{-13} mol of a fusion protein. Detection methods that rely on the catalytic activity of an enzymic carrier can be even more sensitive. Unfortunately, enzymes are relatively large, and are therefore more likely than simple peptides to perturb a

target protein or to be immunogenic. S-Peptide is an unusual carrier because it combines a small size with a high sensitivity of detection.

Sensitive methods exist for assaying ribonuclease activity in solution or in a gel matrix. We have demonstrated that these methods are also applicable to fusion proteins having an S-peptide carrier. For example, as few as 10^{-16} mol of intact RNase A can be detected after its electrophoresis in a zymogram, which is a polyacrylamide gel containing a polymeric substrate for RNase A (Thomas and Hodes, 1981; Blank *et al.*, 1982; Ribó *et al.*, 1991). Similarly, 10^{-16} mol of a fusion protein having an S-peptide carrier can be detected after zymogram electrophoresis and activation with S-protein. This assay is most sensitive at low temperature, which is known to minimize the dissociation constant of RNase S (Schreier and Baldwin, 1977). The sensitivity is also enhanced by the presence of bovine serum albumin, which probably serves to limit the nonspecific interaction of S-protein with the gel matrix. Zymogram electrophoresis is useful not only because of its high sensitivity, but also because it reports the molecular weight of the fusion protein and reveals the presence of truncated forms that co-purify with the intact fusion protein. Finally, it may be possible to increase the sensitivity of this method even further by using cascades that amplify the signal generated by RNA cleavage (Schreier and Baldwin, 1977).

The spacer in our fusion protein was designed to allow the intact target to be liberated from the remainder of the fusion protein. Yet, the native fusion protein, which contains in its spacer the Ile–Glu–Gly–Arg sequence recognized by blood coagulation factor X_a protease, is not cleaved

by factor X_a. This absence of cleavage may result from an inability of factor X_a to access the spacer sequence. Similar results have been observed with another fusion protein that also contains an Ile–Glu–Gly–Arg spacer sequence preceeding a β-galactosidase target protein (Maina *et al.*, 1988). There as here, denatured fusion protein is digested by factor X_a, but several products are produced. This nonspecific digestion is likely due to the ability of factor X_a to catalyze cleavage at sequences other than Ile–Glu–Gly–Arg (Nagai and Thϕgersen, 1987).

The native fusion protein is cleaved by trypsin. The predominant product of trypsin digestion (Fig. II-7, lane 10) has an apparent molecular weight of 116 kD and an *N*-terminal sequence that indicates that cleavage occurred primarily after Arg10 of the S15 carrier. No cleavage is detected after the Arg residue of the spacer. Thus trypsin, like factor X_a, may be unable to access the Arg residue of the spacer in the native fusion protein. Unfortunately, no method currently exists for the precise liberation of an intact target protein from its carrier (Ford *et al.*, 1991; Nilsson *et al.*, 1992).

In addition to those described above, two other properties of S-peptide increase its versatility as a carrier in fusion proteins. First, the mature domains of exported proteins bear information necessary for the effective secretion of a protein (Wickner *et al.*, 1991). RNase A is secreted effectively from exocrine cells of the cow pancreas and is readily translocated in both bacteria and yeast (S. B. delCardayré & R. T. Raines, unpublished results). An S-peptide is therefore unlikely to inhibit the efficient translocation of a fusion protein.

Secondly, S-peptide associates with S-protein such that both the *N*- and *C*-termini of S-peptide are accessible to solvent (Fig. II-1). This topology suggests that binding of S-peptide to S-protein would not suffer if a protein were fused to either termini of S-peptide. Indeed, hybrid biopolymers in which DNA is attached to the *N*-terminus of S-peptide have been described, and these constructs allow for the formation of a hybrid RNase S that is an active ribonuclease (Zuckermann and Schultz, 1988). The accessibility of both termini suggests that a protein fused to either (or both) termini of S-peptide would not prevent binding to S-protein.

Much is known about the biochemistry and biophysics of the intermolecular interaction that produces RNase S. Detailed studies on the binding of S-peptide to S-protein have indicated that three residues (Phe8, His12, and Met13) seem to be essential for the formation of a catalytically active RNase S, and four others (Glu2, Lys7, Arg10, Gln11, and Asp14) contribute to the stability and activity of the complex (Beintema, 1987). Variation in these seven residues creates a range of binding energies, some suitable for immobilization and others for purification of a fusion protein having an S-peptide carrier. Conversely, the residues in S-protein that interact with these seven residues in S-peptide can be varied by applying recombinant DNA technology to RNase A. Generating affinity resins from mutant S-proteins would enable a fusion protein with a single S-peptide carrier to be either immobilized or purified. This and other desirable properties make S-peptide a tag useful for detecting, immobilizing, or

purifying proteins *in vitro* as well as for exploring the behavior of proteins *in vivo*.

Karpeisky *et al.* (1994) have also developed a fusion system based on the ribonuclease S-peptide: S-protein interaction, in which human epidermal growth factor fusion protein was expressed and quantified in the presence of S-protein by ribonuclease activity assay.

Fig. II-1. Structure of crystalline complex formed by S15 and S-protein (Taylor *et al.,* 1981). This complex is a truncated but fully active RNase S. The side chains of the two active-site histidine residues (His12 and His 119) are shown.

(figure on the following page 29)

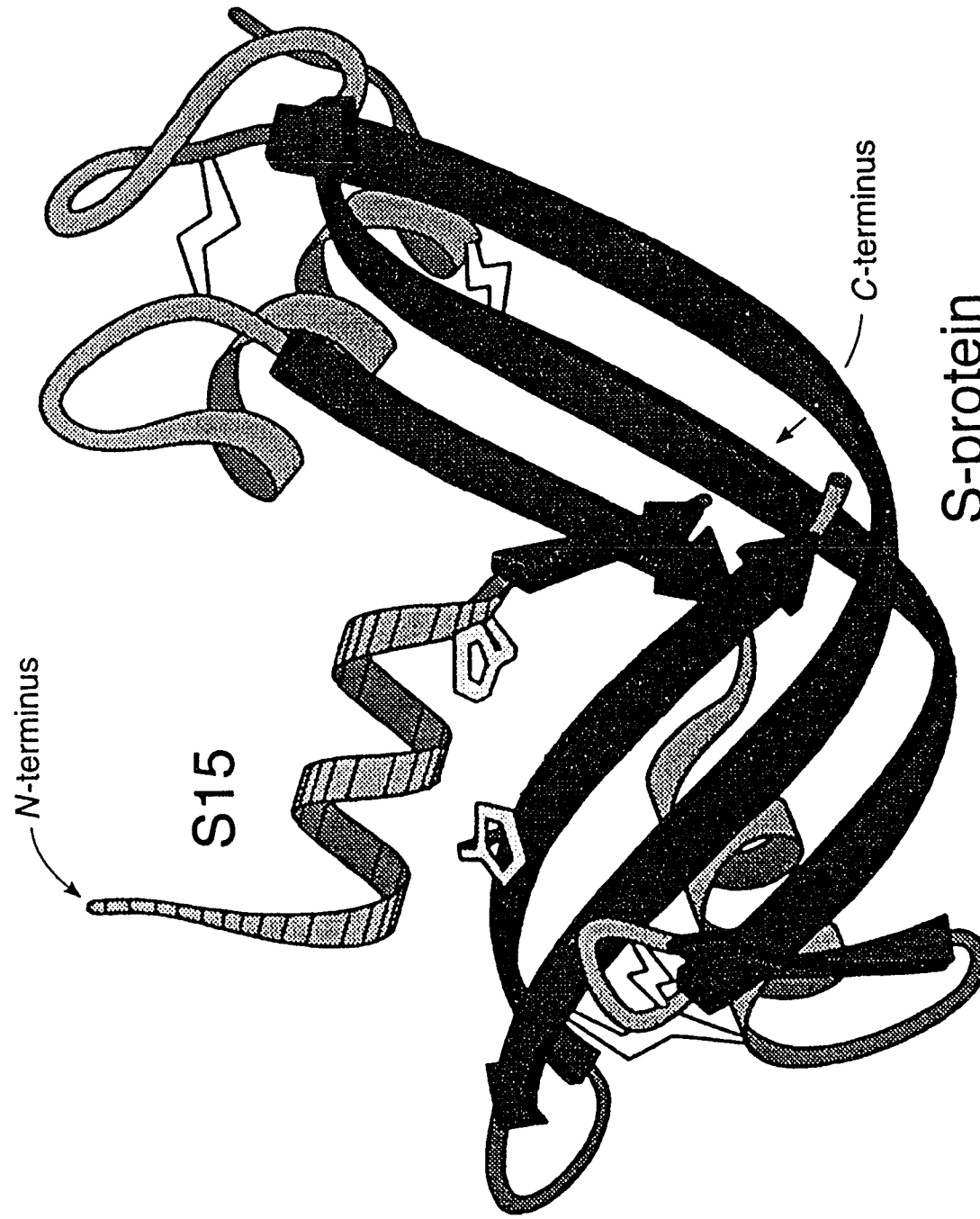


Fig. II-2. Map of plasmid pRS501, which directs the production of proteins fused to the *C*-terminus of an S15 carrier. In this work, the gene that codes for the target protein, β-galactosidase, was inserted into the *Stu*I and *Pst*I sites of pRS501 to yield plasmid pSG601, which directs the production of S15~Pro_3–Ile–Glu–Gly–Arg~β-galactosidase. In addition, the codon for Asp14 of S15 pSG601 was mutated to that for Asn to yield plasmid pSG919, which directs the production of D14N S15~Pro_3–Ile–Glu–Gly–Arg~β-galactosidase.

(figure on the following page 31)

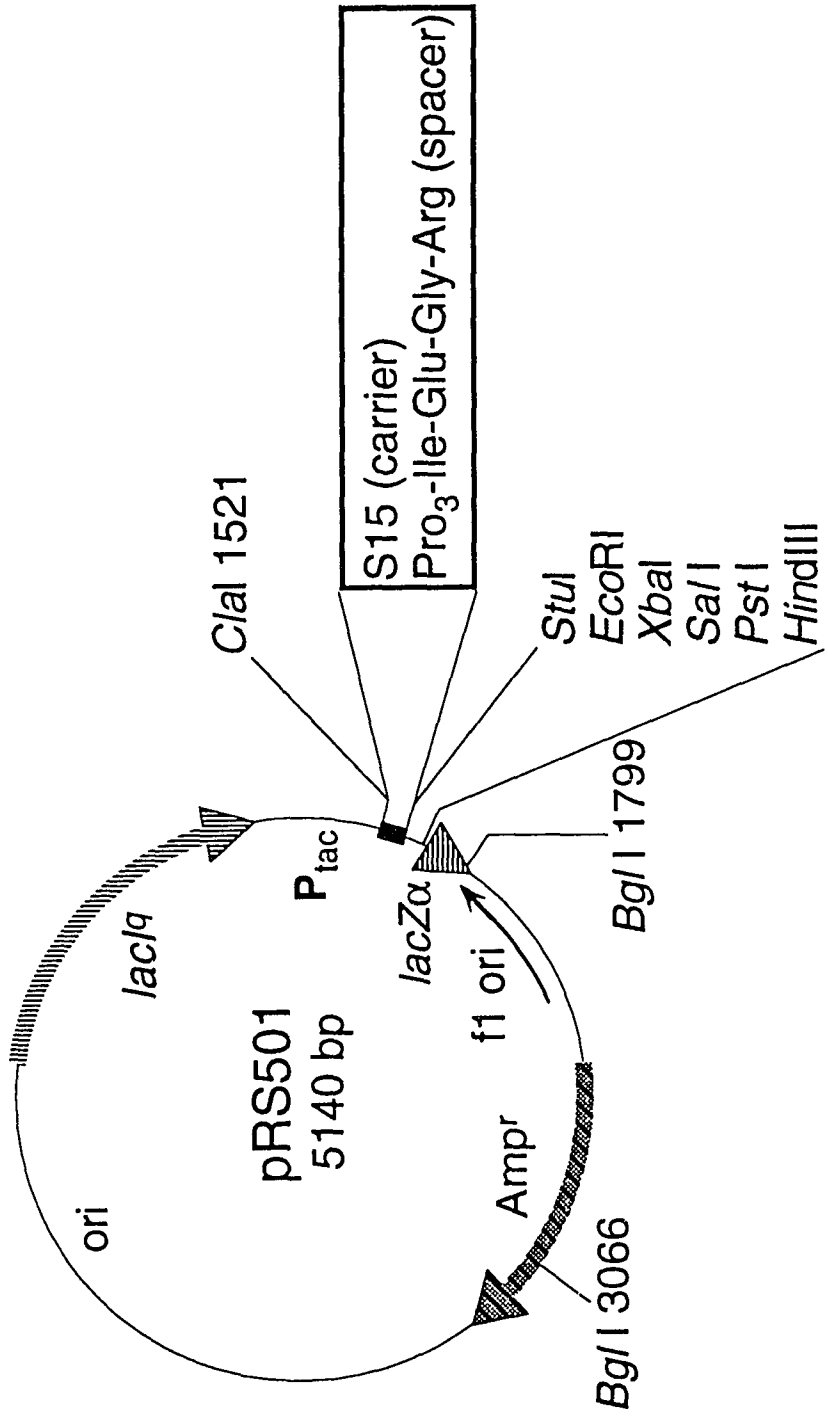
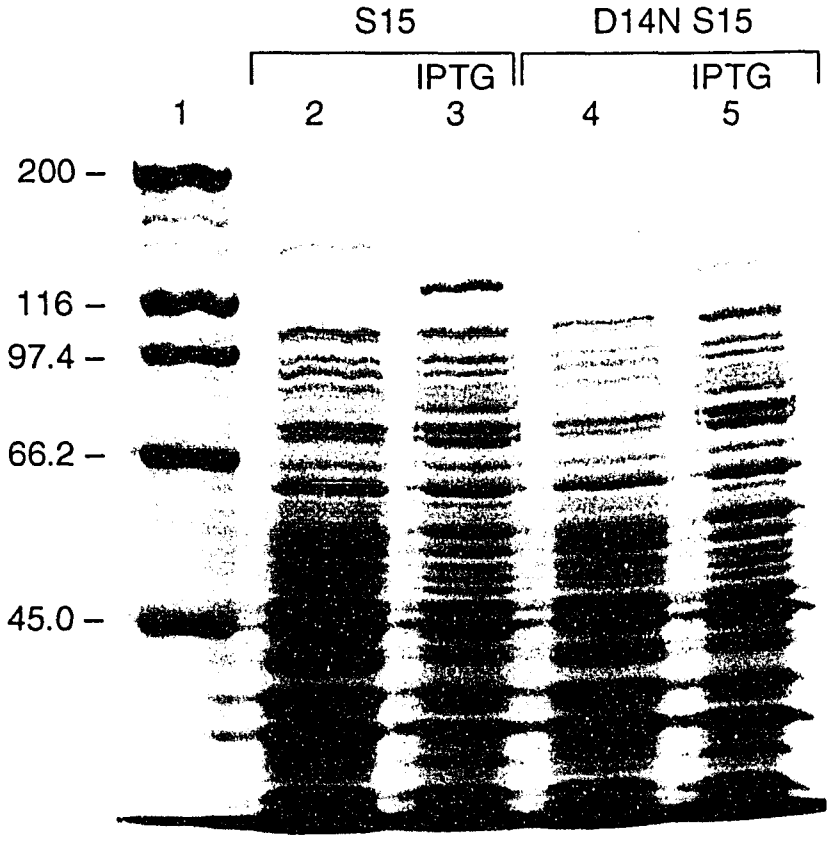


Fig. II-3. Production of fusion proteins with S15 or D14N S15 as carrier. Fusion protein with S15 as carrier was produced from CSH27/pSG601 (lanes 2 and 3). Fusion protein with D14N S15 as carrier was produced from CSH27/pSG919 (lanes 4 and 5). Results were analyzed by staining with Coomassie Brilliant Blue after SDS–PAGE. Lane 1, molecular weight markers (kDa); lanes 2 and 4, total protein from uninduced cells CSH27/pSG601; lanes 3 and 5, total protein from IPTG-induced cells.

(figure on the following page 33)



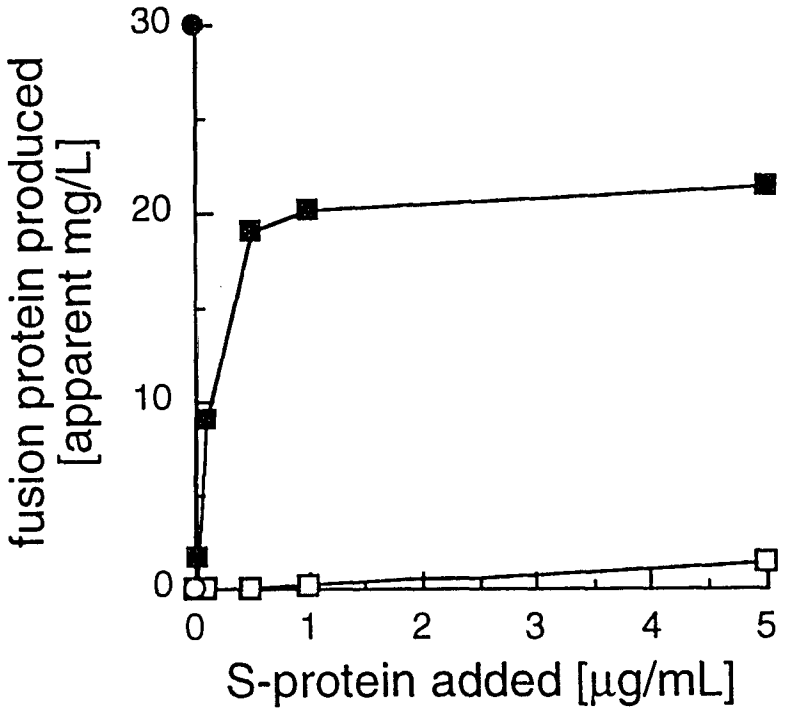


Fig. II-4. Production of fusion protein with S15 as carrier. Apparent concentrations were determined by measuring β-galactosidase activity (O, ●) or ribonuclease activity after activation with different amounts of S-protein (□, ■). Cells were uninduced (open symbols) or IPTG-induced (closed symbols).

Fig. II-5. Affinity purification of fusion protein under nondenaturing conditions. Fusion protein with D14N S15 as carrier was produced from CSH27/pSG919. Results were analyzed by staining with Coomassie Brilliant Blue after SDS–PAGE (top) or by zymogram electrophoresis (bottom). The bottom gel contains ten-fold less of each sample than the top gel. Lane 1, molecular weight markers (kDa); lane 2, soluble protein from IPTG-induced cells; lane 3, flow-through at 4 °C; lanes 4–6, buffer washes at 4 °C; lanes 7–9, buffer eluates at 37 °C.

(figure on the following page 36)

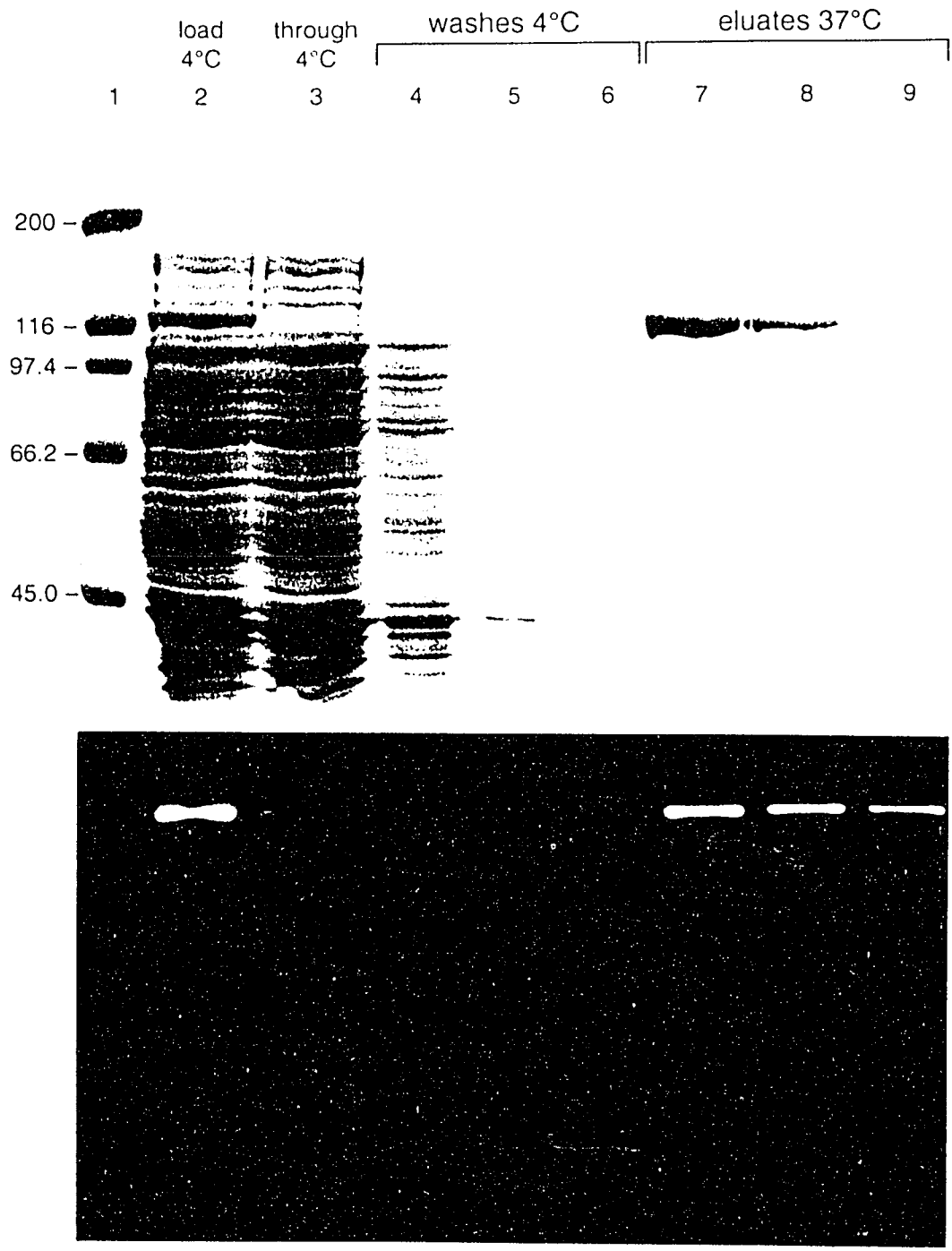


Fig. II-6. Sensitivity of detection of fusion protein after zymogram electrophoresis. The sample was identical to that in Fig. II-5, lane 7. Lane 1, molecular weight markers (kDa); lane 2, 1 ng (10^{-14} mol); lane 3, 100 pg (10^{-15} mol); lane 4, 10 pg (10^{-16} mol).

(figure on the following page 38)

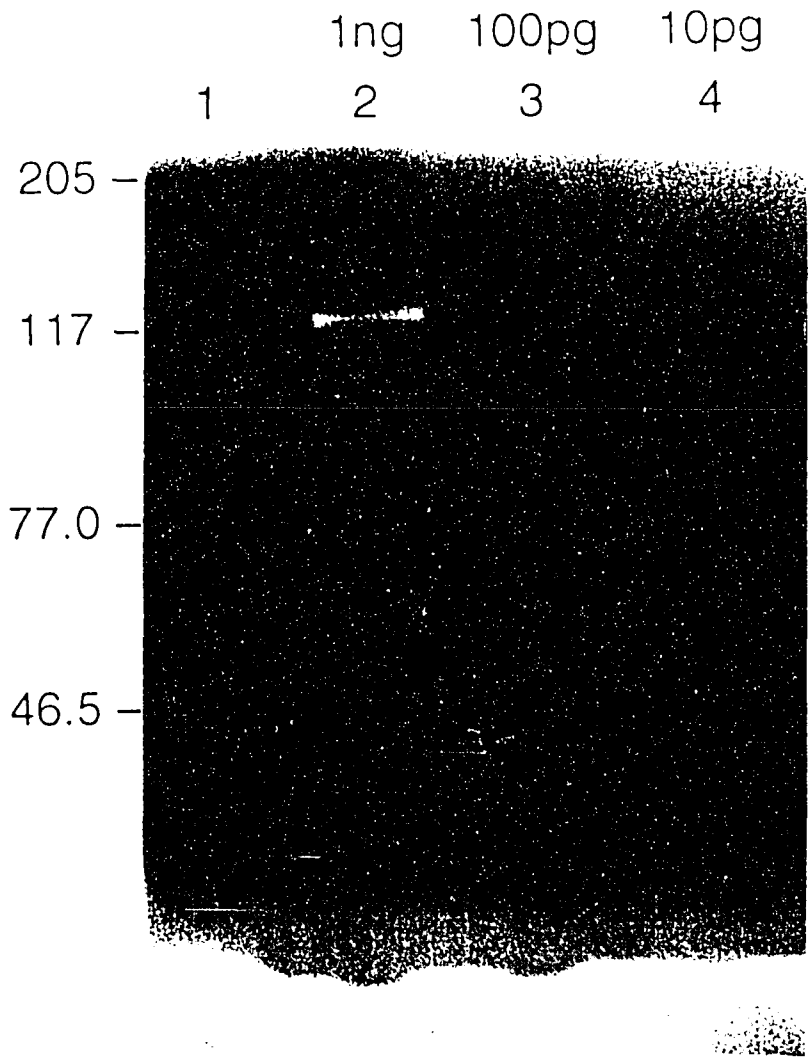


Fig. II-7. Liberation of target protein by digestion of fusion protein with trypsin. Fusion protein was produced from CSH27/pSG601, purified, and digested with trypsin for various times. The results were analyzed by zymogram electrophoresis (left) or by staining with silver after SDS–PAGE (right). Lanes 1 and 8, molecular weight markers; lane 2, authentic S15; lanes 3 and 9, before digestion with trypsin; lane 4-6, after digestion for 1 h, 4 h, or 8 h, respectively; lanes 7 and 10, after digestion for 17 h.

(figure on the following page 40)

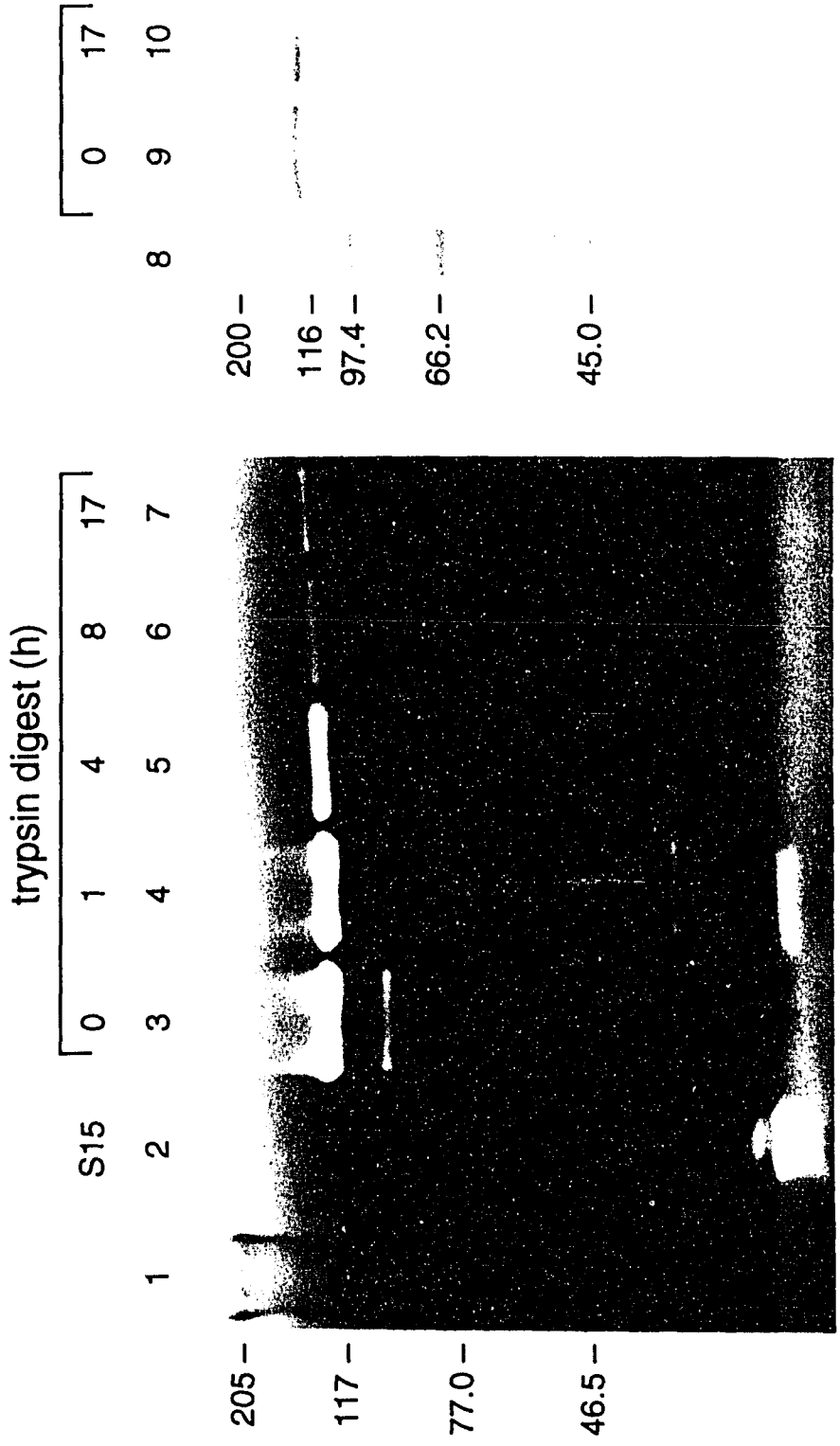


Fig. II-8. Fusion protein with a peptide tag at each terminus, and the dual affinities used in its purification. In this work, the target protein was β-galactosidase.

(figure on the following page 42)

Fig. II-9. Analysis of the purity of D14N S15~β-galactosidase~His_6. Proteins from *E. coli* BL21(DE3)/pSGT12 were analyzed by staining with Coomassie Brilliant Blue after SDS–PAGE (top) or by zymogram electrophoresis (bottom). Lane 1, molecular weight markers (kDa); lane 2, total protein from uninduced cells; lane 3, soluble protein from IPTG-induced cells; lane 4, protein purified by S-protein affinity chromatography; lane 5, protein purified by Ni^{++} chelation chromatography; lane 6, protein purified by both S-protein affinity chromatography and Ni^{++} chelation chromatography.

(figure on the following page 44)

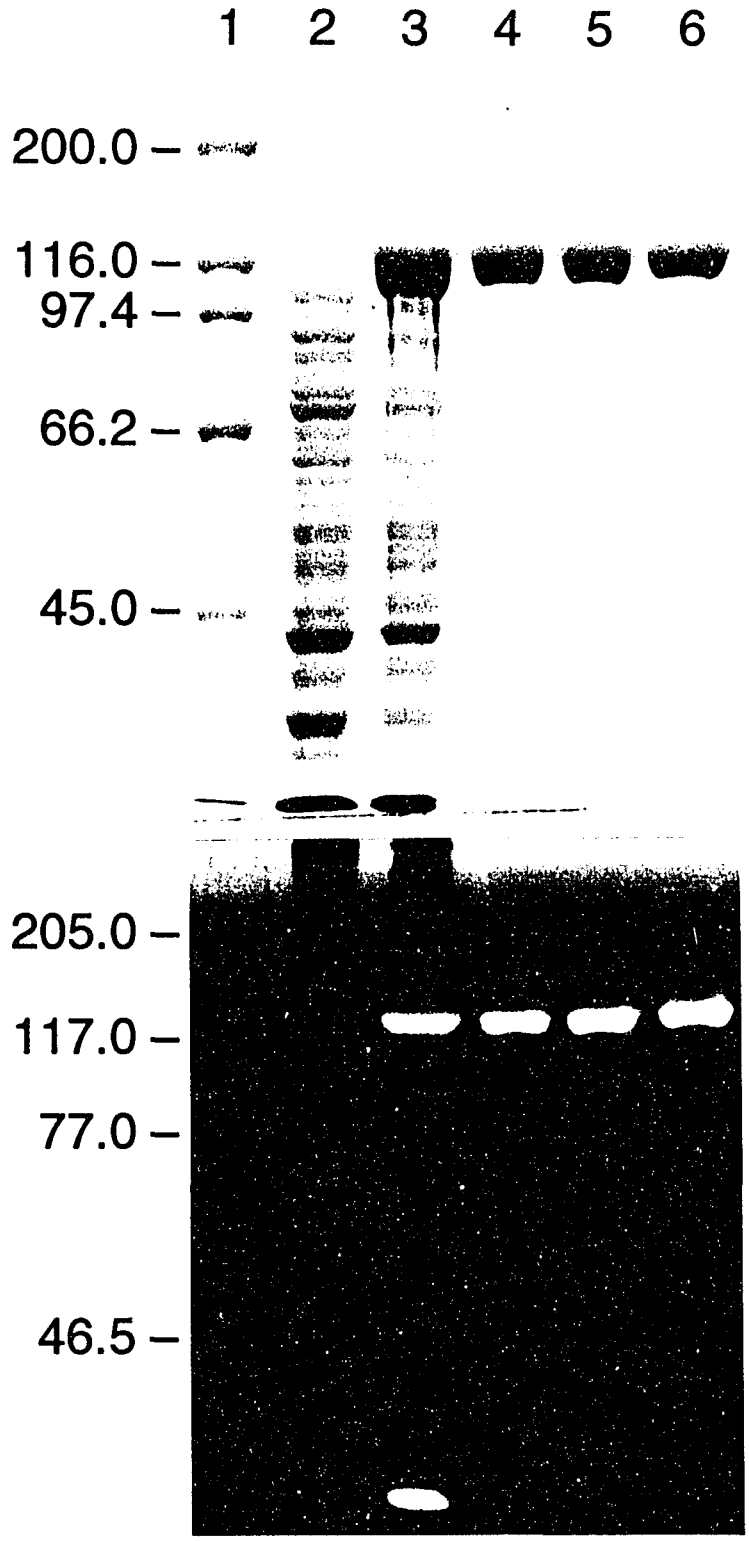


Table II-1. Purification of D14N S15~β-galactosidase from a 0.1 L Culture of *E. coli* strain CSH27/pSG919.

	total catalytic activity (units)[a]	yield (%)	specific catalytic activity (units/mg)[a]	purification factor	total protein (mg)
crude lysate	3.8×10^5	100	5.9×10^3	1	64
S-protein affinity chromatography	2.8×10^5	74	1.9×10^5	31	1.5

[a] Units refer to β-galactosidase units (Miller, 1972).

Table II-2. Purification of D14N S15~β-galactosidase~His_6 protein from 50-mL culture of *E. coli* strain BL21(DE3)/pSGT12.

purification step	total catalytic activity (10^6 units)[a]	total protein (mg)	specific catalytic activity (10^5 units/mg)[a]	purification factor	yield (%)
crude extract	2.1	37	0.56	1.0	100
S-protein affinity	1.5	6.8	2.2	3.9	71
Ni^{++} chelation	1.1	5.5	2.0	3.6	52
S-protein affinity + Ni^{++} chelation	1.1	5.0	2.2	3.9	52

[a] Units refer to β-galactosidase units.

CHAPTER III

Production and Purification of Bovine Seminal Ribonuclease

Originally published as

Kim, J.-S. and Raines, R. T. (1993) Bovine seminal ribonuclease produced from a synthetic gene. *J. Biol. Chem.* **268**, 17392-17396.

Summary

Bovine seminal ribonuclease (BS-RNase), a homolog of bovine pancreatic ribonuclease A (RNase A), is isolated as a dimer in which the subunits are crosslinked by two disulfide bonds. In addition to this anamolous quaternary structure, the enzyme also has extraordinary biological properties, such as antispermatogenic, antitumor, and immunosuppressive activities. The molecular bases for these properties are well-suited for exploration with the techniques of recombinant DNA. Accordingly, a gene encoding BS-RNase was designed based on criteria expected to maximize the translational efficiency of its mRNA in *Escherichia coli*. This gene was constructed from twelve synthetic oligonucleotides, and expressed with the phage T7 system. The protein thus produced was insoluble, and accumulated under optimal conditions to 15% of total cellular protein or 200 mg per liter of culture. Ribonuclease activity was generated by air-oxidation of the reduced and denatured protein. Three forms of active BS-RNase were isolated by gel filtration chromatography: the well-characterized dimer and monomer, and a previously uncharacterized form. The ribonuclease activities of all three forms were equivalent to or higher than that of dimeric BS-RNase isolated from bull seminal plasma.

Introduction

Bovine seminal ribonuclease (BS-RNase; EC 3.1.27.5) is an unusual member of the superfamily of secretory ribonucleases (Beintema, 1987). Although the amino acid sequence of BS-RNase is 81% identical to that of bovine pancreatic ribonuclease (RNase A), the quaternary structure and biological properties of BS-RNase have diverged greatly from those of RNase A and other homologous ribonucleases. For example, in contrast to its homologs, BS-RNase is isolated as a dimer in which the subunits are cross-linked by two disulfide bonds. The structure of crystalline dimeric BS-RNase shows that each active site can be composed of residues from different polypeptide chains (Capasso *et al.*, 1983; Mazzarella *et al.*, 1987).

Bovine seminal ribonuclease, like other ribonucleases, catalyzes RNA transphosphorylation and hydrolysis. Yet, BS-RNase has several unusual enzymatic properties. For example, the enzyme catalyzes the efficient cleavage of both double-stranded and single-stranded RNA (Sorrentino *et al.*, 1980). Also, BS-RNase is allosteric during catalysis of the hydrolysis of nucleoside 2',3'-cyclic monophosphates (Piccoli *et al.*, 1988).

Bulls are the only mammals known to produce a seminal ribonuclease. The concentration of BS-RNase in bull seminal plasma has been reported to be as high as 1.5 mg/mL, which corresponds to three percent of total seminal protein (D'Alessio *et al.*, 1991). Although its physiological role is not known, BS-RNase has been shown to have antispermatogenic, antitumor, and immunosuppressive activities (Dostal

and Motousek, 1973; Soucek *et al.*, 1986; Tamburrini *et al.*, 1990; Laccetti *et al.*, 1992).

As a small protein (2 x 13.6 kDa) with unusual properties and a known three-dimensional structure, BS-RNase is an exquisite object for studies that use the techniques of recombinant DNA (Piccoli *et al.*, 1991). To begin to illuminate structure–function relationships within BS-RNase and in comparison to RNase A, a gene that codes for BS-RNase has been designed on criteria based largely on the secondary structure predicted for the corresponding mRNA and on codon usage. This gene has been synthesized and expressed, and the resulting protein has been characterized. This chapter describes the first heterologous production of active BS-RNase, and the isolation of three active forms of this enzyme.

Results

Design and construction of the synthetic BS-RNase gene

A gene was designed to code for BS-RNase, and to maximize translational initiation and protein elongation in *E. coli*. To facilitate the initiation of translation, the nucleotide sequence of the gene was adjusted to expose the start codon and Shine/Dalgarno sequence of its transcript in the vector pSR1. The structure of lowest free energy predicted for this transcript by the program FOLD ($\Delta G°$ = –45.9 kcal/mol) is shown in Fig. III-1. In this structure, as well as in the lowest free energy structure predicted by the program MFOLD ($\Delta G°$ = –38.3 kcal/mol), the start codon and two

residues of the Shine/Dalgarno sequence are free of base pairing. To facilitate protein elongation, the codons used were those found in highly expressed genes of *E. coli.* To make future mutagenesis more convenient, seven unique restriction endonuclease recognition sites were incorporated by considering codon redundancy. The synthetic gene was constructed from twelve overlapping oligonucleotides, as shown in Fig. III-2. (The cDNA sequence of the natural precursor protein of BS-RNase was reported by Preuß *et al.* (1990)) The nucleotide sequence of the synthetic gene was found to be as expected.

Expression and purification

E. coli strain BL21(DE3)/pLysS containing pSR1 was grown to late log phase. Expression of the BS-RNase gene was then induced by the addition of IPTG. The amount of BS-RNase produced by induced cells was not dependent on the precise time of induction during late log phase. BS-RNase thus produced constituted about 15% of the total *E. coli* protein or about 100 mg of BS-RNase from the 0.5 L culture. BS-RNase appeared to form inclusion bodies, which facilitated its purification. The insoluble fraction was dissolved in 6 M guanidine-HCl (GdnCl) and reduced with dithiothreitol (DTT). The resulting solution of denatured and reduced protein was dialyzed versus dilute acid to remove GdnCl and DTT while inhibiting the formation of disulfide bonds. BS-RNase remained soluble during dialysis, but most other proteins precipitated and were hence easily removed by centrifugation.

Generation of ribonuclease activity by air-oxidation

Ribonuclease activity was generated by air-oxidation. The catalytic activity of ribonuclease (Kunitz units/mg) generated after 14 h of air-oxidation was independent of protein concentration, if the protein concentration was less than 0.7 mg/mL. Less active ribonuclease was generated at protein concentrations greater than 0.7 mg/mL, presumably due to protein aggregation. The concentration of active multimeric forms of BS-RNase increased with time, as shown in Fig. III-3. Free sulfhydryl groups, determined by DTNB assay, disappeared faster than activity was generated (data not shown), as has been observed with RNase A (Creighton, 1977). After incubation for 24 h at a protein concentration of 0.7 mg/mL, active dimeric BS-RNase had accumulated to about 40% of total protein. Sequence analysis indicated that dimeric BS-RNase had a methionine residue at its *N*-terminus.

Purification of active BS-RNase

Four forms of BS-RNase were isolated by gel filtration chromatography. The migrations of these forms are shown in Fig. III-4. The catalytic activity of the peak II protein (21 units/mg protein) was similar to that of native BS-RNase (22 units/mg) purified from seminal plasma and contained a protein that comigrated during nonreducing SDS–PAGE with dimeric BS-RNase from seminal plasma, as shown in Fig. III-5. Although the peak II fraction showed the same gel filtration behavior two weeks after isolation, this fraction contained an inactive form of BS-RNase that comigrated during SDS–PAGE with monomeric BS-RNase (Fig. III-5,

lane 6) as well as a covalent dimer. The peak III protein was the most active catalyst (53 units/mg), which is consistent with a previous report that artificially monomerized BS-RNase was more active than the native dimer (Tamburrini *et al.*, 1989), and comigrated during SDS–PAGE with monomeric BS-RNase from seminal plasma (data not shown). The peak I protein was as active a catalyst (26 units/mg) as was the dimeric enzyme from seminal plasma, but showed a lower mobility during nonreducing SDS–PAGE. Under reducing conditions, the proteins from peaks I, II, and III comigrated with monomeric BS-RNase from bull seminal plasma, as expected (data not shown). Native PAGE performed at pH 3.8 revealed that these three fractions were homogeneous, and that only the peak II protein comigrated with the dimeric BS-RNase from bull seminal plasma (data not shown).

Discussion

The T7 expression system has been used to produce many proteins in *E. coli* (Studier *et al.*, 1990). The high selectivity of T7 RNA polymerase for the T7 promoter, coupled with the high chain elongation rate of this polymerase, results in the accumulation of a large number of transcripts. Yet, an abundance of transcripts does not necessarily translate into an abundance of protein, because of either poor translation initiation or protein elongation (Gold, 1990). The secondary structure of mRNA has been postulated to play an important role in the initiation of translation

(Iserentant and Fiers, 1980). In particular, the accessibility of the start codon and Shine/Dalgarno sequence is believed to be critical for maximal expression (Gheysen *et al.*, 1982; Li *et al.*, 1991). The secondary structure of the mRNA transcribed from the synthetic BS-RNase gene was designed to allow for such access. The codons of the synthetic gene were also chosen for efficient protein elongation by considering the codons common in highly expressed genes of *E. coli*.

BS-RNase produced in *E. coli* by the T7 RNA polymerase expression system appears to form inclusion bodies, which here has several advantages. For example, the heterologous production of active ribonucleases is thought to be toxic to *E. coli* (Nambiar *et al.*, 1987; Hartley, 1989). The BS-RNase in inclusion bodies is likely to be inactive. Another advantage is that isolation of inclusion bodies after cell lysis results in a substantial purification of BS-RNase (Fig. III-5). This purification is of particular utility in separating endogenous *E. coli* ribonucleases, such as RNase I and RNase II (Cannistraro and Kennell, 1991), from BS-RNase. Fortunately, these *E. coli* enzymes remain soluble, and are therefore easily separable from insoluble inclusion bodies containing BS-RNase.

The air-oxidation of reduced and denatured protein was followed by zymogram electrophoresis (Fig. III-3), which monitors simultaneously the catalytic activity and molecular weight of ribonucleases. At least five minor bands appear between monomer and dimer (Fig. III-3, lanes 3 and 4). These bands are not visible in a polyacrylamide gel stained with Coomassie brilliant blue (data not shown). We suspect that these bands (<5% of total protein) are mixed disulfides between peptides and

monomeric BS-RNase. (Zymogram electrophoresis is performed in a denaturing but nonreducing gel.) Multimers seen above the dimer region (Fig. III-3, lanes 3 and 4) may also appear during gel filtration chromatography (Fig. III-4, shoulder on peak I), but were not further characterized.

Bovine seminal ribonuclease isolated from seminal plasma is a dimer (Fig. III-5, lane 8). Nevertheless, different multimeric forms have been produced by refolding and reoxidizing BS-RNase that had been reduced and denatured. For example, Smith *et al.* (1978), were unable to regenerate a significant amount of dimer from oxidation by glutathionine. In contrast, Parente & D'Alessio (1985) reported that a substantial amount (40%) of dimer was regenerated by air-oxidation of the enzyme from seminal plasma. We have generated a similar fraction of dimer by air-oxidation of the enzyme from *E. coli*. Further, despite the presence of an *N*-terminal methionine residue, the dimer produced by *E. coli* is as active a catalyst as that isolated from seminal plasma. The structure of crystalline BS-RNase indicates that the *N*-terminus of each subunit is on the surface of the protein, far removed from the active site (Capasso *et al.*, 1983). The presence of an *N*-terminal methionine residue therefore should not (and apparently does not) affect its ability to cleave single-stranded RNA.

We have also observed that two forms of BS-RNase, in addition to the monomer and dimer reported by previous workers, are generated by air-oxidation. One is inactive (based on zymogram analysis; data not shown) and copurified with the dimer. The other is as active as the dimer and isolated as peak III. The molecular weight of this active protein is

estimated by gel filtration chromatography to be 40 kDa, which is consistent with it being a BS-RNase trimer. The properties of this apparent trimer and its relationship with other forms of BS-RNase are discussed in the following chapter.

Fig. III-1. Predicted secondary structure of mRNA transcribed from pSR1. The secondary structure of lowest free energy (ΔG° = –45.9 kcal/mol) was predicted by the program FOLD (Zuker & Stiegler, 1981) and drawn by the program LOOPVIEWER (Don Gilbert, Indiana University, Bloomington, IN). The Shine/Dalgarno sequence is sidelined, and the start codon is boxed.

(figure on the following page 58)

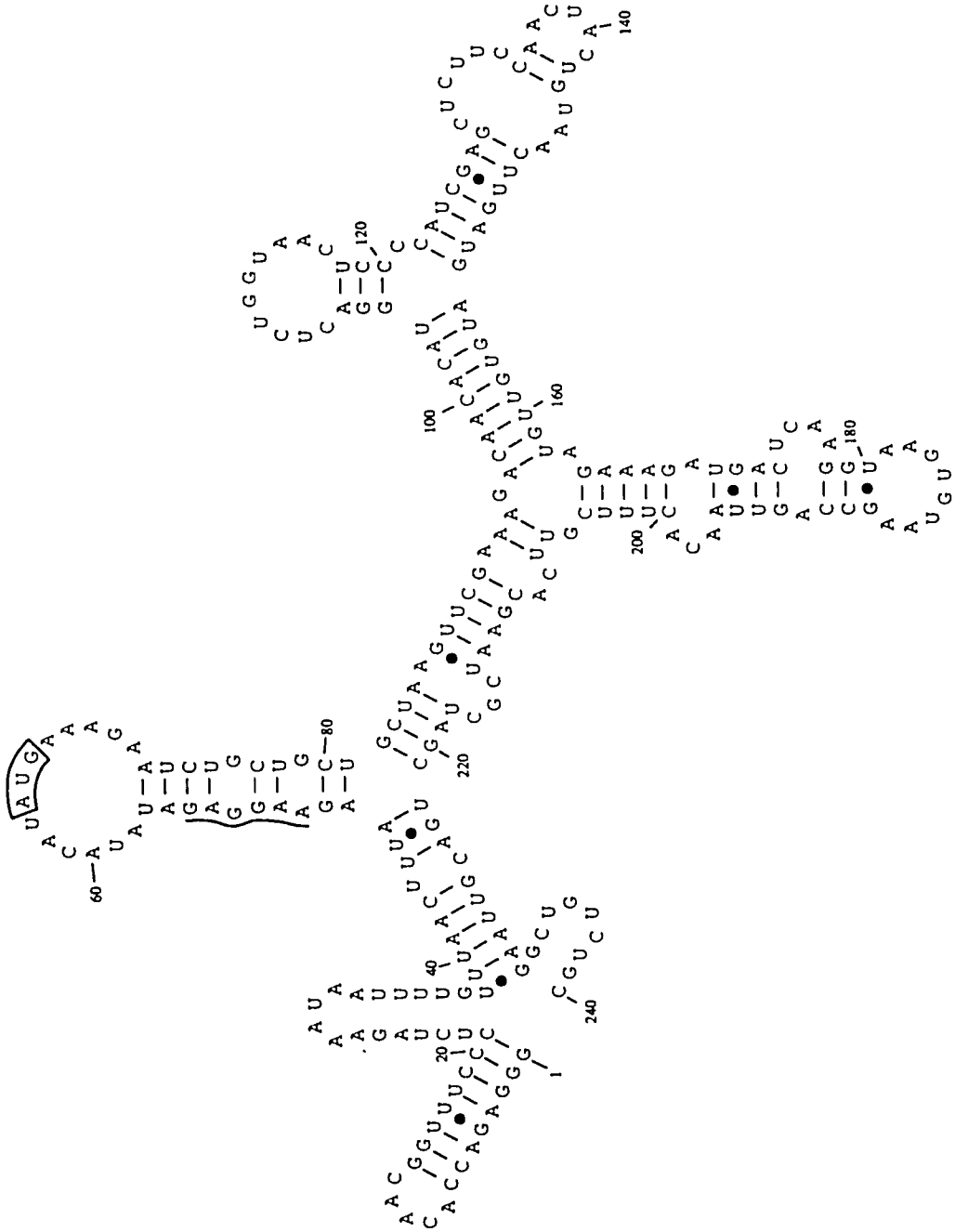


Fig. III-2. DNA sequence of synthetic BS-RNase gene. The individual oligonucleotides used to construct the synthetic gene are enclosed by contiguous white or black backgrounds. Restriction enzyme recognition sites not found in expression vector pET17b are indicated.

(figure on the following page 60)

Fig. III-3. Zymogram showing timecourse of generation of the activity during oxidation of BS-RNase from *E. coli*. BS-RNase activity was generated by air-oxidation of reduced and denatured protein. Protein samples (100 ng) after various times of oxidation were separated by electrophoresis in a nonreducing SDS–polyacrylamide gel containing poly(C). Ribonuclease activity left a clear band after staining with Toluidine blue O. Lane 1, 0 h of air-oxidation; lane 2, 4 h; lane 3, 17 h; lane 4, 40 h.

(figure on the following page 62)

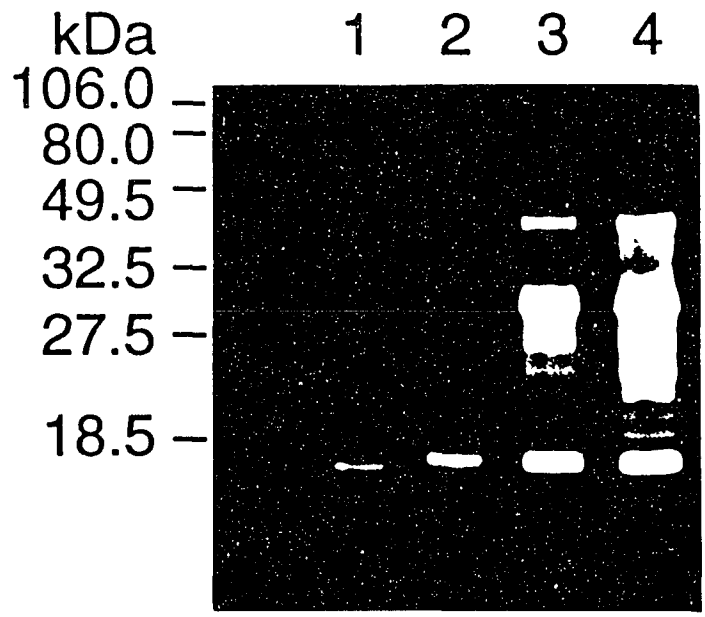


Fig. III-4. Gel filtration of oxidized BS-RNase from *E. coli*. BS-RNase was regenerated by air-oxidation and subjected to gel filtration on Sephadex G-75 resin. Absorbance (□) and ribonuclease activity (O) of fractions are shown. The column void volume (V_O), three peak fractions (I, II and III), and the predicted migration for a monomer (m), dimer (d) and trimer (t) of BS-RNase are indicated.

(figure on the following page 64)

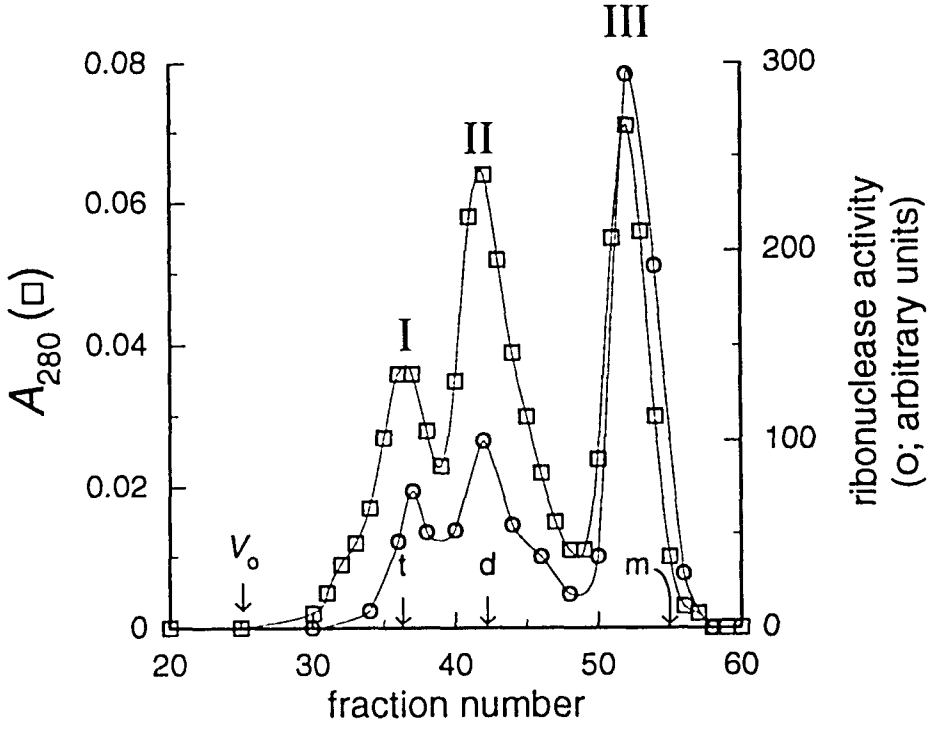


Fig. III-5. SDS–polyacrylamide gel showing purification of BS-RNase from *E. coli*. Recombinant BS-RNase was purified from *E. coli* strain BL21 (DE3)/pLysS/pSR1 and compared to native BS-RNase purified from bull seminal plasma (lane 8) and RNase A (lane 9). Proteins were separated by electrophoresis in an SDS–polyacrylamide gel under reducing conditions (lanes 1-4) or nonreducing conditions (lanes 5-9). Lane 1, total cellular protein from BL21(DE3)/pLysS/pSR1 before induction; lane 2, total cellular protein after induction by IPTG; lane 3, insoluble fraction collected by centrifugation of cell lysate; lane 4, reduced and denatured protein after dialysis in 20 mM acetic acid; lane 5, peak I fraction from chromatography on Sephadex G-75; lane 6, peak II fraction; lane 7, peak III fraction; lane 8, BS-RNase from seminal plasma; lane 9, RNase A.

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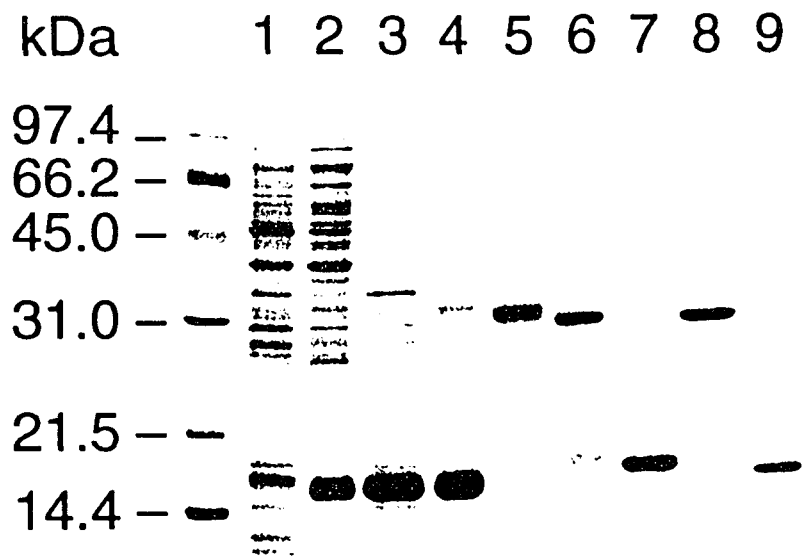

CHAPTER IV

A Misfolded but Active Dimer of Bovine Seminal Ribonuclease

Originally published as

Kim, J.-S. and Raines, R. T. (1994) A misfolded but active dimer of bovine seminal ribonuclease. *Eur. J. Biochem.* (in press)

Summary

Bovine seminal ribonuclease (BS-RNase) is an unusual homolog of RNase A. Isolated from bulls as a dimer, BS-RNase has special biological properties including anitspermatogenic, antitumor, and immunosuppressive activities. The structural bases for these properties are unknown. Four forms of BS-RNase were isolated after folding and air oxidation of the denatured and reduced protein produced in *Escherichia coli*: two dimers (**M=M** and **MxI**, where **x** signifies an active site composed of residues from both subunits) and two monomers (**M** and **I**). Considerable ribonuclease activity was generated by air oxidation of an equimolar mixture of two inactive mutant proteins (H12D and H119D) prepared by site-directed mutagenesis. This activity came from a dimer (**MxI**) with a composite active site. ^{1}H–NMR spectroscopy revealed that this dimer contained one correctly folded subunit (**M**), and one incorrectly folded subunit (**I**). Form **I**, which is a poor catalyst, was activated by ribonuclease S-protein, suggesting that the C-terminal portion of **I** was not folded properly. Electrospray ionization–mass spectrometry and sulfhydryl group titration indicated that **I** contains a single oxidized sulfhydryl group, which cannot participate in a disulfide bond. These results show that quaternary structure in BS-RNase is attained by the initial formation of two monomers, **M** and **I**, which then combine with another **M** to form **M=M** and **MxI**, respectively. Adventitious oxidation can thus lead to the formation of a misfolded but active enzyme (**MxI**).

Introduction

Bovine seminal ribonuclease (BS-RNase) is homologous to bovine pancreatic ribonuclease A (RNase A) (D'Alessio *et al.*, 1991). Like RNase A, BS-RNase catalyzes the transphosphorylation and hydrolysis of RNA, as shown in Fig. IV-1. The imidazole groups in His12 and His119 are likely to serve as a general acid and general base during catalysis by these enzymes (Thompson and Raines, 1994). Unlike RNase A, BS-RNase is isolated as a dimer having two distinct quaternary forms, designated as **M×M** and **M=M** (Piccoli *et al.*, 1992). Forms **M×M** and **M=M** each contain two intersubunit disulfide bonds, in addition to the four disulfide bonds within each subunit. In **M×M**, the *N*-terminal tail of one subunit (residues 1–17) stretches out from the body and fills a cleft in the body of the other subunit, as shown in Fig. IV-2. Thus in **M×M**, the key catalytic residues (His12 and His119) of each active site are contributed by different polypeptide chains. In the other form, **M=M**, such exchange has not occurred.

Refolding studies have revealed that **M×M** is formed by a sequence of conformational transitions. First, intrasubunit disulfide bonds form, and the oxidized protein folds to produce native monomer, designated as **M**. Then, disulfide bonds form between two monomers to yield **M=M**. Finally, **M=M** equilibrates slowly (that is, in days) with **M×M**. At equilibrium, the relative amount of **M×M** is 60–80%. The distinct conformational transitions of BS-RNase make this protein an intriguing system with which to study the formation of quaternary structure.

Bovine seminal ribonuclease constitutes 3% of the protein in bull seminal plasma. The physiological role of BS-RNase is unknown, but the protein does display surprising antispermatogenic, antitumor, and immunosuppressive activities (Dostal and Motousek, 1973; Soucek *et al.*, 1986; Tamburrini *et al.*, 1990; Laccetti *et al.*, 1992). Form **M**, like the two dimers, is a potent ribonuclease. Yet, many of the unusual enzymatic and biological properties of dimeric BS-RNase are absent from the monomeric form (D'Alessio *et al.*, 1991) and are present to different extents in **M**x**M** and **M**=**M** (Piccoli *et al.*, 1993). The structural basis for these functional differences is unclear.

We are interested in exploring structure–function relationships within BS-RNase and in comparison to RNase A. Accordingly, we have expressed a synthetic gene for BS-RNase in *Escherichia coli* (CHAPTER III). The BS-RNase protein produced is insoluble, and requires folding and oxidation to generate ribonuclease activity. After folding/air oxidation, three forms of active BS-RNase can be isolated by gel filtration chromatography: dimer (**M**=**M**), monomer (**M**), and a previously uncharacterized form that migrates as a trimer. In this chapter, we describe the characterization of this third form, and on the relationship between the three forms and one additional inactive form.

Results

Purification of Four Forms of BS-RNase

Four forms of BS-RNase were isolated by gel filtration chromatography after folding/air oxidation of the reduced and denatured protein (Fig. IV-3, top). This gel filtration pattern is similar to that from BS-RNase isolated from bull semen and refolded *in vitro* (Parente and D'Alessio, 1985). Three of these forms, designated as **MxI**, **M=M**, and **M**, showed ribonuclease activity equivalent to or higher than that of dimeric BS-RNase isolated from bull seminal plasma. (These three forms were labeled as peak I, peak II, and peak III, respectively, in CHAPTER III. Their designation here as **MxI**, **M=M**, and **M** is based on data given below. Another form, **MxM**, was co-purified with **M=M**. The rate of interconverion between **M=M** and **MxM** is slow, and **MxM** represented <5% of this mixture as judged by gel filtration after the selective reduction of intersubunit disulfide bonds.) The fourth form, designated as **I**, appeared as a shoulder of the **M=M** peak. Form **I** was a poor catalyst, having less than 5% of the specific activity of **M**. Forms **MxI** and **I** migrated more slowly and diffusely than did **M=M** and **M**, respectively, during SDS–PAGE analysis (Fig. IV-3, bottom). The relative amount of **I** formed during folding/oxidation was not changed significantly if a chelating agent, EDTA, was used to complex metal ions during air oxidation or if glutathione rather than air was the oxidizing agent (data not shown). Amino acid analysis showed that all of the four forms of BS-RNase contained identical amino acid compositions.

Site-Directed Mutagenesis

BS-RNase has two histidine residues in its active site (Fig. IV-2). These two residues were changed to aspartate by site-directed mutagenesis. Each mutation resulted in a greater than 10^3-fold loss of ribonuclease activity, based on zymogram electrophoresis and catalytic activity assay (Fig. IV-4). Unlike the wild-type enzyme, which formed fully active monomer and dimer after 20 h of folding/air oxidation (Fig. IV-4, lane 8), the two mutant enzymes showed no enzymatic activity after 20 h of folding/air oxidation (Fig. IV-4, lanes 2 and 4). Considerable activity was detected, however, in an equimolar mixture of the two BS-RNase mutants, and zymogram electrophoresis showed that this activity was from a covalent dimer (Fig. IV-4, lane 6). As shown in Fig. IV-3, the dimer region on a SDS–polyacrylamide gel contains two forms of active BS-RNase: **M**x**I** and **M**=**M**. Gel filtration and PAGE (both native and denaturing) analyses (data not shown) revealed that the ribonuclease activity of the mixture of mutant proteins was from **MxI**.

NMR Analysis

BS-RNase and RNase A each have 4 histidine residues. The resonance of the hydrogen atom on the $C^{\varepsilon 1}$ atom of histidine residues is usually well-resolved, and often provides useful information on protein conformation. This approach has been used previously to study the conformation of RNase A (Markley, 1975) and BS-RNase (Andini *et al.*, 1983).

Each form of BS-RNase described above was purified and used for ^{1}H–NMR studies (Fig. IV-5). The spectrum of **M** contained 5 major resonances in the region expected for hydrogen atoms on the $C^{\varepsilon 1}$ atom of histidine residues. The one additional resonance is probably due to the splitting of the resonance from His48 (Andini *et al.*, 1983). In contrast, the spectrum of **I** in this region showed only 3 major resonances. The difference in the spectra of **M** and **I** indicated that the conformations of **M** and **I** are different, which is consistent with the results of gel filtration chromatography and catalytic activity assays. Further, the NMR spectrum of **MxI** was the sum of the spectra of **M** and **I**, suggesting that **MxI** is a dimer consisting of **M** and **I**.

Sulfhydryl Group Content

The extended conformation of **I**, inferred from its slow migration during gel filtration chromatography and SDS–PAGE, suggested that **I** lacks one or more disulfide bonds. To determine sulfhydryl group content, BS-RNase was treated with iodoacetic acid after folding/air oxidation, and the resulting dimeric forms were purified to homogeneity. The content of carboxymethyl cysteine (CM-Cys) residues was determined by amino acid analysis. Native BS-RNase dimer has 10 cysteine residues per subunit: 8 of which form 4 intrasubunit disulfide bonds and 2 of which form intersubunit disulfide bonds. As expected, the **M=M** dimer was found to contain no CM-Cys residues. In contrast, the **MxI** dimer contained 1.2 CM-Cys residues. Titration of iodoacetate-treated proteins with DTNB in a

solution containing denaturant revealed no free sulfhydryl groups, indicating that carboxymethylation had been complete.

Interconversion between the Forms of BS-RNase

Interconversion between M, I, M=M, and MxI was studied as follows. Reduced and denatured BS-RNase was air-oxidized and the four forms of BS-RNase were isolated without carboxymethylation of the sulfhydryl groups. The four forms were then isolated by repetitive gel filtration chromatography, which was performed at low pH to minimize further oxidation of sulfhydryl groups. Each isolate was concentrated to 1 mg/mL, and the concentrates were buffered by addition of 1/10 volume of 1.0 M Tris-acetic acid buffer, pH 8.5. The resulting solutions were incubated at room temperature for 24 h, and then treated with iodoacetate. Gel filtration chromatography of the resulting proteins indicated that more than 60% of M had been converted to M=M. On the other hand, no interconversions between M, I, and MxI were observed. Finally, M and I purified without carboxymethylation were denatured and reduced. The subsequent folding/air oxidation of M resulted in a regain of ribonuclease activity. In contrast, the subsequent folding/air oxidation of I did not produce any activity, indicating that neither M nor M=M was produced from denatured and reduced I.

Activation of I with Ribonuclease S-Protein

S-Peptide (residues 1–20) and S-protein (residues 21–124) are the enzymatically inactive products of the limited digestion of RNase A by

subtilisin. S-Peptide binds S-protein with high affinity to form RNase S, which has full enzymatic activity (Richards, 1955). The truncated RNase S formed from S-protein and the first 15 residues of S-peptide (S15) has the same enzymatic activity and dissociation constant as does RNase S (Potts *et al.*, 1963). Although the I form of BS-RNase has little enzymatic activity, it could in theory be activated by forming a complex with either S-protein (if its S-peptide portion is folded properly) or with S15 (if its S-protein portion is folded properly). As shown in Table IV-1, substantial enzymatic activity was generated by activation with S-protein but not with S15.

Mass Spectrometry

The molecular masses M and I, determined by electrospray ionization–mass spectrometry, were 13,733 Da and 13,749 Da, respectively, suggesting that I had suffered a covalent modification that increased its molecular mass by 16 Da. The calculated molecular mass of monomeric recombinant BS-RNase that possesses an additional methionine residue at *N*-terminus is 13,734 Da. This value is calculated for monomeric protein with 4 intramolecular disulfide bonds and all sidechains uncharged.

Discussion

Bovine seminal ribonuclease is known to fold first into a monomer (M) and then into a dimer (M=M) (Parente and D'Alessio, 1985). This dimer converts slowly to another dimer (MxM) in which the *N*-terminal

tail (residues 1–17) from the two subunits are exchanged (Piccoli *et al.*, 1992). The different quaternary forms of BS-RNase appear to have distinct enzymatic and biological activities (Piccoli *et al.*, 1993). To monitor the quaternary structure of BS-RNase, we made mutant proteins in which each of the active-site histidine residues was changed to aspartate. These mutants have little ribonuclease activity (Fig. IV-4), as expected from the alteration of a residue that is critical for catalysis (Thompson and Raines, 1994). Likewise, **M=M** prepared from a mixture of H12D BS-RNase and H119D BS-RNase should have little ribonuclease activity, since each active site would lack either His12 or His119. Yet, a substantial amount of ribonuclease activity was produced in a mixture of the H12D and H119D BS-RNases. This activity could have resulted only from a dimer that has a composite active site. Thus, a previously unrecognized pathway must exist for the formation of quaternary structure in BS-RNase. We demonstrated the existence of a form of BS-RNase that migrates as a trimer during gel filtration (CHAPTER III). This form was referred to as the peak I protein. We now find that this form is responsible for the catalytic activity observed in the mixture of BS–RNase mutants.

The structures of the various forms of BS-RNase were compared by using ^{1}H–NMR spectroscopy. The results of these studies (Fig. IV-5) show that the peak I protein is a dimer in which one subunit (**M**) is folded correctly and the other subunit (I) is not. This form has been designated as **MxI**, where the **x** signifies that it has an active site composed of residues from both subunits.

The I form of BS-RNase cannot be converted to other forms. Folding and air oxidation of denatured and reduced I produces I, but not M. Based on these findings, we suggest the pathway in Fig. IV-6 for the production of quaternary structure in BS-RNase. In this pathway, the reduced and denatured BS-RNase (U) folds in the presence of an oxidant into a monomeric state, M or I, each of which can combine with M to form M=M or MxI, respectively.

The inability of I to be converted to M suggests that I has suffered a covalent modification. The rapid elution of I during gel filtration chromatography indicates that I is in a relatively extended conformation. The fast accumulation of MxI relative to MxM (Fig. IV-6) can also be ascribed to the extended conformation of I. Such a conformation could result from the covalent modification of the sulfhydryl group of a cysteine residue, which would then be unable to form a disulfide bond. The sulfhydryl group of a cysteine residue can be oxidized by molecular oxygen to a sulfenic acid (–SOH), a sulfinic acid (–S(O)OH), or a sulfonic acid (–$S(O)_2OH$) in processes catalyzed by certain metal ions (Hayward *et al.*, 1987; Scopes, 1987). Sulfenic acids, sulfinic acids, and sulfonic acids cannot participate in disulfide bonds. The molecular masses of M and I differ by 16 Da, which is the molecular mass of the most prevalent isotope of oxygen. Apparently, the oxidation of one sulfhydryl group to a sulfenic acid in BS-RNase, results in incomplete disulfide bond formation and the formation of I. The relative amount of I formed during folding/oxidation was not changed significantly either when air oxidation was accomplished with

glutathione or when a chelating agent, EDTA, was used to complex metal ions.

The *C*-terminal S-protein portion of the I form contains all ten cysteine residues found in the protein. The I form itself is a poor catalyst and was not activated by addition of the *N*-terminal S15 (Table IV-1). This result suggests that the C-terminal S-protein portion of I was not folded properly, and is consistent with the presence of a covalent modification in the C-terminal portion of the I form. In contrast, the I form could be activated by the addition of S-protein, indicating that the *N*-terminal residues that are critical to catalysis or interaction with S-protein have not been covalently modified.

Proteins produced heterologously in *E. coli* often form inclusion bodies (Schein, 1991; Wilkinson and Harrison, 1991). Fortunately, these insoluble aggregates can be separated readily from soluble cellular components. Still, solubilization of inclusion bodies with a strong denaturant and subsequent refolding are required to obtain active proteins. If disulfide bonds are present in the native protein, the denatured protein must be exposed to oxidizing agents during refolding. Often, the folding/oxidation of a denatured and reduced protein results in a low yield of correctly folded/oxidized protein. This low yield can result from the oxidation of a methionine residue to a sulfoxide (Brot and Weissbach, 1991). Our results indicate that this low yield can also be due to the adventitious oxidation of the sulfhydryl group of a cysteine residue. Further, this oxidation may occur despite preventative measures, such as the presence of metal chelating agents or the use of alternative oxidants.

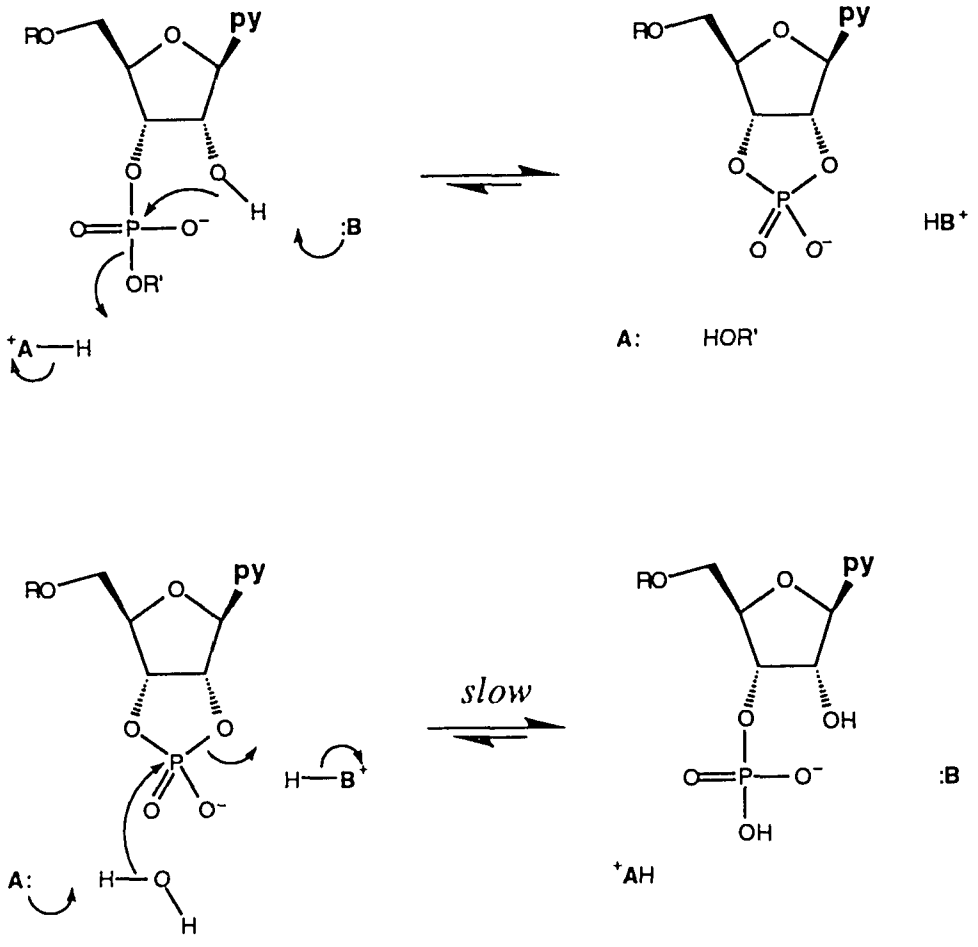

Fig. IV-1. Mechanism of the transphosphorylation (top) and hydrolysis (bottom) reactions catalyzed by BS-RNase. **B** is His12, **A** is His119, and **py** is a pyrimidine base.

Fig. IV-2. Structure of the crystalline **MxM** form of BS-RNase (Mazzarella *et al.*, 1987). Only α-carbons are shown. The chains are drawn in black or gray ('). The active-site histidine residues, intersubunit disulfide bonds, and *N*- and *C*-termini are indicated.

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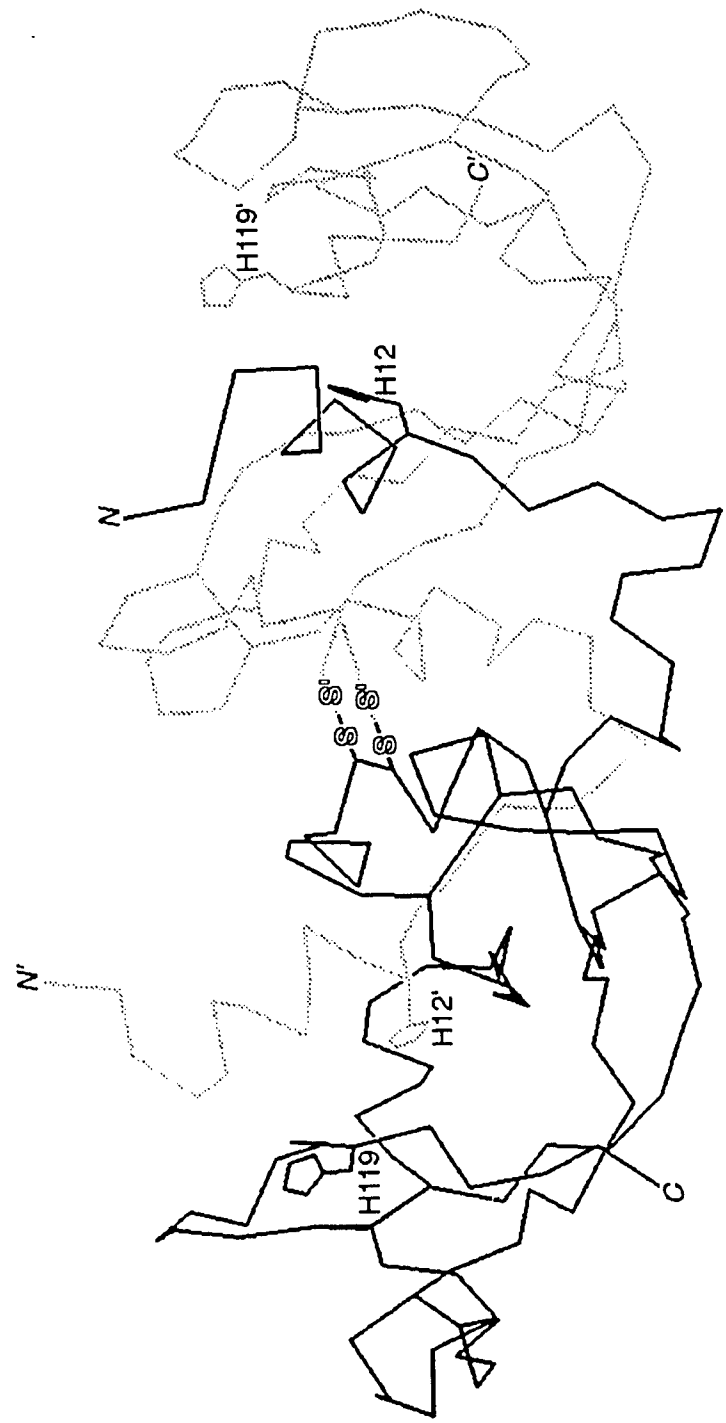
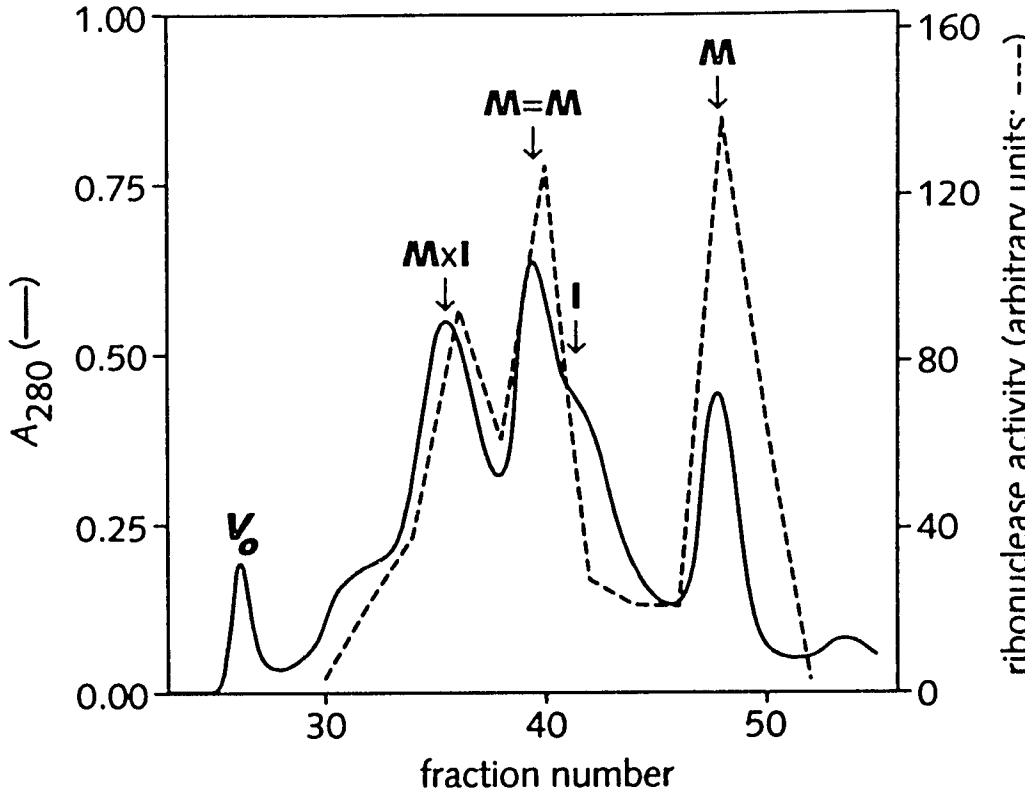


Fig. IV-3. Purification and analysis of different forms of BS-RNase produced from *E. coli*. Denatured and reduced BS-RNase from *E. coli* was folded and air oxidized. BS-RNase forms were purified by gel filtration through Superdex^{TM}75 (top). Purified BS-RNase forms were analyzed by SDS–PAGE (bottom) under nondenaturing conditions.

(figure on the following page 83)



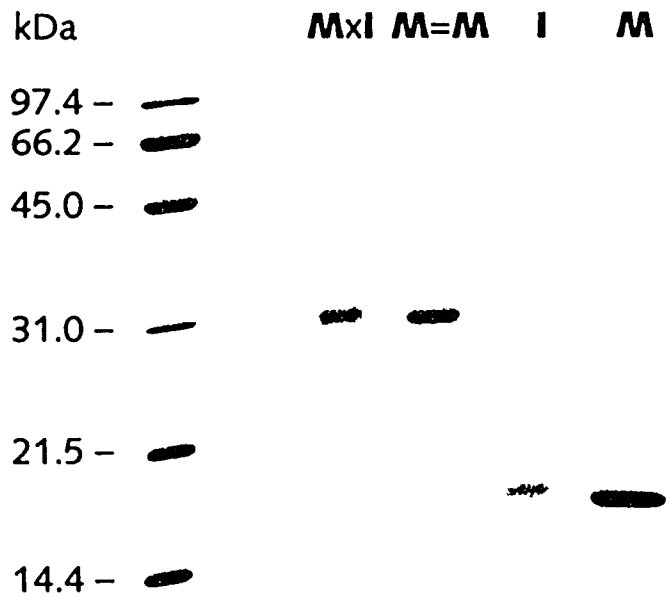


Fig. IV-4. Zymogram analysis of wild-type and mutant BS-RNases. Folding/air oxidation was performed with H12D BS-RNase (lanes 1 and 2), H119D BS-RNase (lanes 3 and 4), an equimolar mixture of H12D and H119D BS-RNases (lanes 5 and 6), and wild-type BS-RNase (lanes 7 and 8). Protein samples (100 ng) at 0 h (lanes 1, 3, 5, and 7) and 20 h (lanes 2, 4, 6, and 8) of incubation were separated by electrophoresis in a nonreducing SDS–polyacrylamide gel containing poly(C). Ribonuclease activity is evidenced by a clear band after staining with Toluidine Blue O. Relative ribonuclease activities assayed in solution before SDS–PAGE are also shown.

(figure on the following page 85)

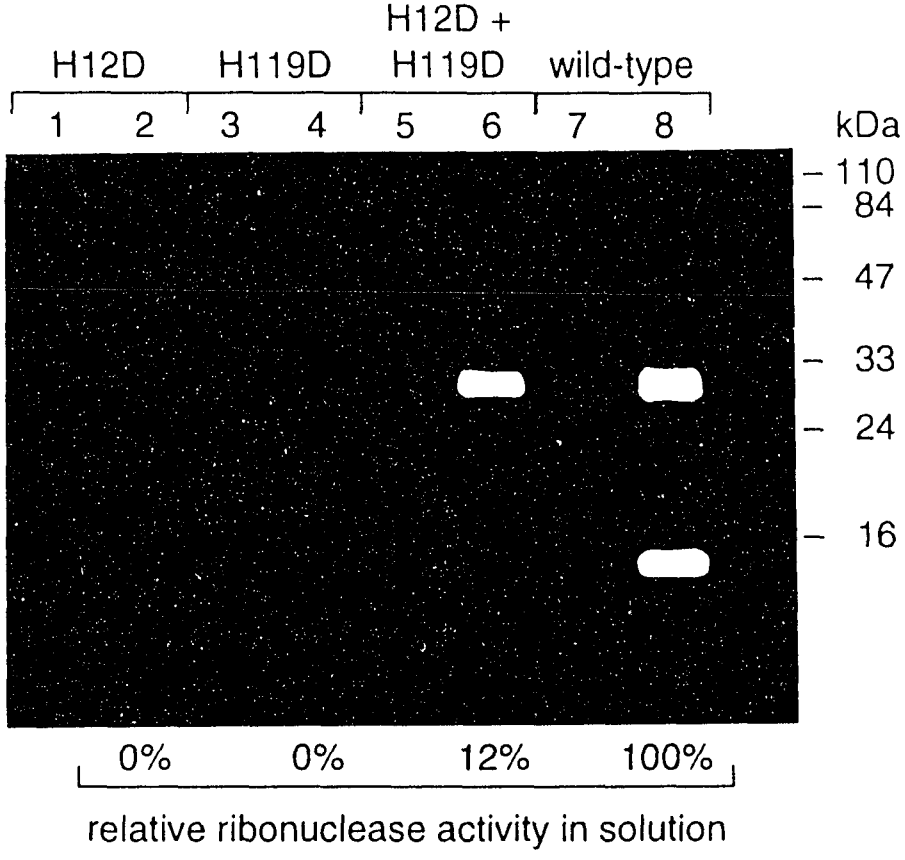
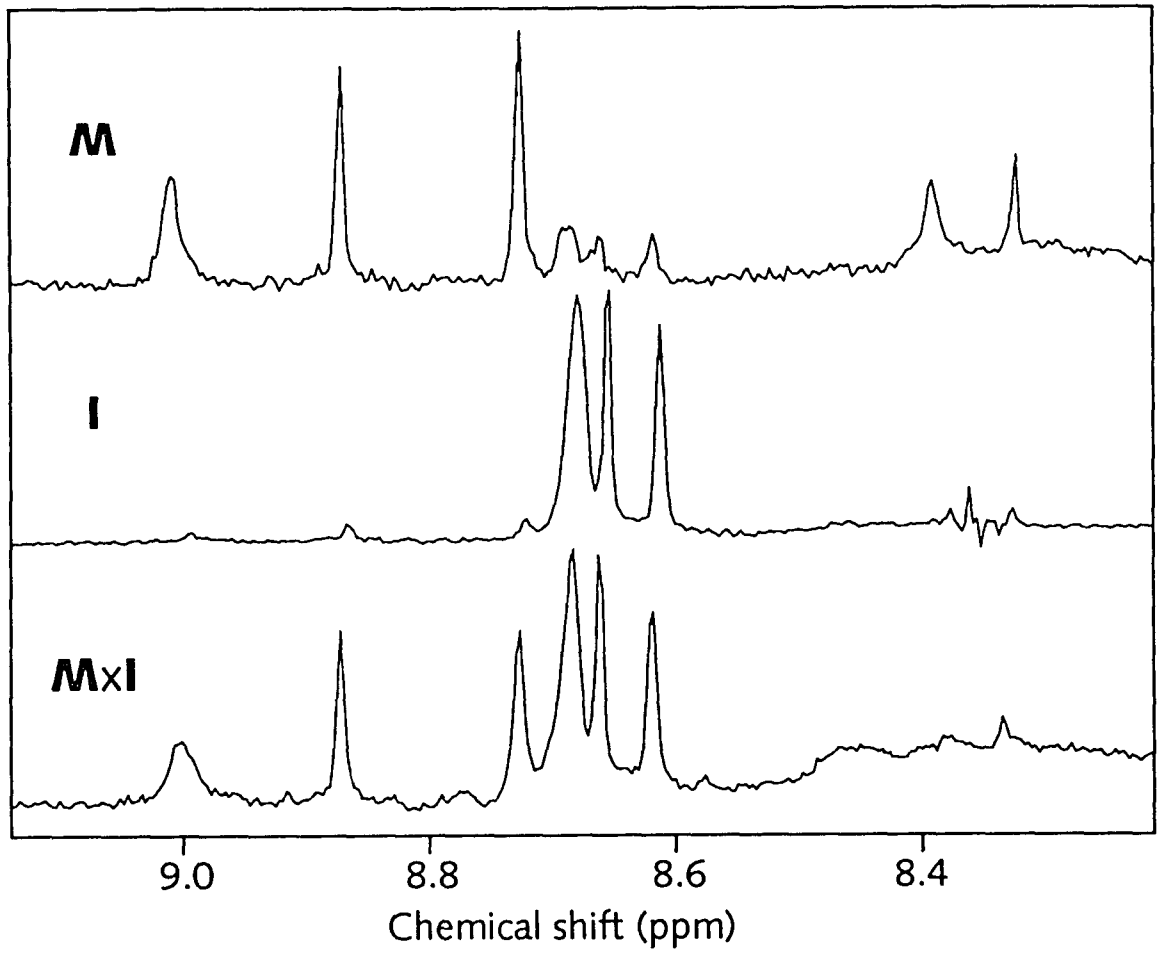


Fig. IV-5. Portion of ^{1}H–NMR spectrum of three forms of BS-RNase. Downfield region is shown for **M** (top), **I** (middle), and **MxI** (bottom).

(figure on the following page 87)



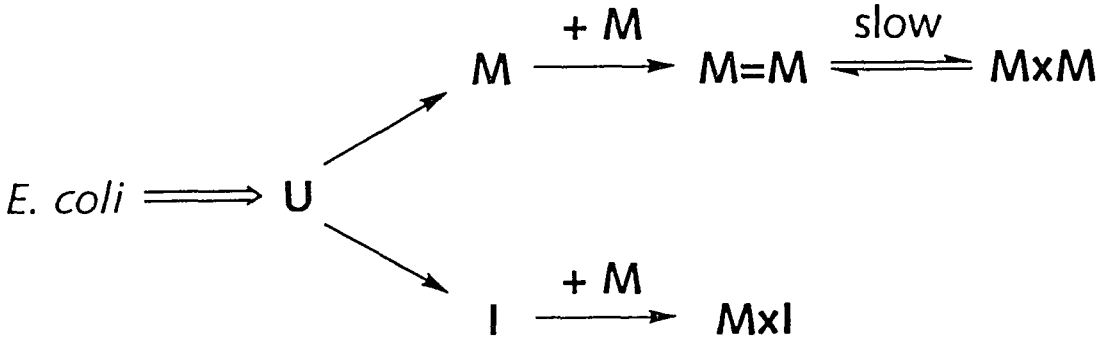


Fig. IV-6. Pathway for the production of quaternary structure in BS-RNase. The reduced and denatured BS-RNase (U) folds into M or I, each of which can combine with M to form M=M or MxI, respectively.

Table IV-1. Activation of I with S15 or S-Protein.

protein(s)	specific activity[a] (relative)
I	1.0
S15	0.0
S-protein	0.0
I + S15[b]	2.2
I + S-protein[c]	72
S-protein + S15[b]	72
RNase A	100

[a] Ribonuclease activity was assayed by the method of Kunitz (1946).

[b] Ribonuclease S15 was added in 10-fold molar excess.

[c] Ribonuclease S-protein was added in 2-fold molar excess.

CHAPTER V

Structural Basis for the Biological Activities of Bovine Seminal Ribonuclease: Role of Intersubunit Disulfide Bonds

Assays for biological activity were performed in collaboration with Dr. J. Soucek (Institute of Hematology and Blood Transfusion, Prague, Czech Republic) and Dr. J. Matousek (Institute of Animal Physiology and Genetics, Libechov, Czech Republic).

Summary

Bovine seminal ribonuclease (BS-RNase) is a homolog of bovine pancreatic ribonuclease (RNase A) with special biological properties that include antitumor, aspermatogenic, and immunosuppressive activity. In contrast to RNase A, BS-RNase is a dimer crosslinked by two disulfide bonds between Cys31 of one subunit and Cys32 of the other. At equilibrium, this dimer is an approximately equimolar mixture of two distinct quaternary forms, **M=M** and **MxM**. The conversion of **M=M** to **MxM** entails the exchange of the *N*-terminal α-helix between subunits. Extensive interconversion of **M=M** and **MxM** occurs during the time-course of assays for antitumor, aspermatogenic, and immunosuppressive activity, impeding biological analysis of the independent forms. Nevertheless, the activity of purified **MxM** was somewhat greater than that of purified **M=M**. To probe further the relationship between quaternary structure and biological activity, mutants of BS-RNase were created in which Cys31 or Cys32 was changed to a serine residue. In these enzymes, which were fully active ribonucleases, the fraction of **MxM** at equilibrium was reduced markedly from that of wild-type BS-RNase. The mutant BS-RNases also displayed much less antitumor, aspermatogenic, and immunosuppressive activity. These results suggest that the **MxM** form of BS-RNase is responsible for its special biological properties.

Introduction

Bovine seminal ribonuclease (BS-RNase) is a close homolog of bovine pancreatic ribonuclease A (RNase A). Eighty one percent of the amino acid sequence of the two proteins are identical (Suzuki *et al.*, 1987). Unlike RNase A, BS-RNase has antitumor, antispermatogenic, and immunosuppressive activities (Dostal and Motousek, 1973; Soucek *et al.*, 1986; Tamburrini *et al.*, 1990; Laccetti *et al.*, 1992). These special biological properties correlate with the oligomerization state assumed by BS-RNase, which is isolated from bull seminal fluid 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). In contrast, artificial monomers of BS-RNase lack these activities (Tamburrini *et al.*, 1990).

In dimers of BS-RNase, the subunits are crosslinked by two disulfide bonds between Cys31 of one subunit and Cys32 of the other subunit. These crosslinked 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 *N*-terminal tail (residues 1–17) of one subunit stretches out from the *C*-terminal body of the same subunit, and interacts with the body of the other subunit. In the minor form, M=M, this exchange does not occur. The two quaternary forms may differ in their enzymatic and biological properties (Piccoli *et al.*, 1993). 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 MxM:M=M has been reported to be 2:1 (Piccoli *et al.*, 1992).

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; J. J. Beintema, personal communication). The DNA sequence of each of these genes indicates that the residue corresponding to Cys31 in the encoded protein is replaced by a phenylalanine. This mutation eliminates one of the two intersubunit disulfide bonds.

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 one of the two 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 MxM dimer? and (3) What are the consequences of these mutations on the antitumor, aspermatogenic, and immunosuppressive activities of BS-RNase?

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 *Escherichia coli* as described (de Nigris *et al.*, 1993).

Interconversion of **M=M** *and* **MxM**

The **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 folded/oxidized monomer was treated with a 5-fold molar excess of dithiothreitol and then dialyzed at pH 8.5 to allow for air-oxidation. After dialysis for 24 h, >70% of each BS-RNase was converted to the **M=M** (>90%) or **MxM** (<10%) dimer. The ability of dimers of the C31S and C32S enzymes to catalyze the degradation of RNA was identical (± 5%) to that of wild-type BS-RNase.

In dimers of wild-type BS-RNase, the 2 intersubunit disulfide bonds have a lower reduction potential than do the 8 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 **MxM** remain associated as a noncovalent dimer (**NCD**) due to 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.

Selective reduction followed by gel filtration chromatography thus allowed us to distinguish **M=M** from **MxM**, as shown in Fig. V-1. We used this method to follow the time course of the interconverion of the **M=M** and **MxM** forms of wild-type, C31S, and C32S BS-RNase. The **M=M** form of wild-type BS-RNase equilibrated with the **MxM** form over several days, as shown in Fig. V-2. Numerical analysis of the data in Fig. V-2 gave the kinetic and thermodynamic parameters in Table V-1. At equilibrium, 57% of the wild-type dimer was present as the **MxM** form, indicating that the **MxM** 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 **MxM** 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 **MxM** form, indicating that the **M=M** form is more stable than is the **MxM** form in the C31S and C32S enzymes.

A comparison of the amino acid sequences of BS-RNase and RNase A revealed that the hinge region connecting the *N*-terminal tail and C-terminal body of BS-RNase varies from the analogous region in RNase A, as shown in Fig. V-3 (Suzuki *et al.*, 1987). This region corresponds to the short loop sequence (residues 15–20) that can be removed in RNase S without affecting either the enzymatic activity of RNase S or the affinity between S-peptide and S-protein (Potts *et al.*, 1963). A residue of note in the hinge region is Pro19. In RNase A, Pro19 is replaced by alanine. The interconversion of the *cis* and *trans* isomers of peptide bonds that include the nitrogen of proline residues can give rise to a slow kinetic phase during protein folding (Brandts *et al.*, 1975). The Ser18–Pro19 peptide bond

has been proposed to isomerize during the formation of **M×M** (Parente and D'Alessio, 1985).

We probed the role of Pro19 in the interconversion of **M=M** and **M×M** by two methods. First, a hybrid protein, SRA-I, was constructed in which the hinge region of BS-RNase was replaced with the corresponding residues of RNase A (Fig. V-3). The conversion of **M=M** to **M×M** in SRA-I was assayed by selective reduction and gel filtration chromatography. The rate and extent of formation of **M×M** did not differ significantly from that of wild-type BS-RNase (data not shown). Secondly, wild-type **M×M** was preincubated for 1 h with or without cyclophilin, which catalyzes the *cis/trans* isomerization of prolyl peptide bonds (Fischer *et al.*, 1984; Mücke and Schmid, 1992). The quaternary structure of BS-RNase was then assessed by selective reduction and gel filtration chromatography. The presence of cyclophilin made no significant difference in the amount of **M=M** (<5%) formed from **M×M**.

Antitumor activity

Wild-type BS-RNase inhibits the growth of tumor cells but not that of normal cells (Vescia *et al.*, 1980). We determined the ability of various forms of BS-RNase (monomeric BS-RNase, C31S BS-RNase, C32S BS-RNase, **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 growth of human leukemic cell lines K-562 and ML-1. The 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. V-4A.

The C31S BS-RNase had a modest cytotoxic effect. Monomeric BS-RNase and RNase A showed no measurable effect on the growth of K-562 cells.

All ribonucleases had a weaker effect on the growth of ML-1 cells than on that of K-562 cells, as shown in Fig. V-4B. The weaker response of the ML-1 cell line allowed us to better compare the efficacy 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 < **MxM**. Again, monomeric BS-RNase and RNase A showed no measurable effect on cell growth.

Aspermatogenic activity

Wild-type BS-RNase induces reversible infertility in rodents (Dostal and Motousek, 1973). We determined the aspermatogenic activity of various forms of BS-RNase and of RNase A by injecting the ribonucleases into mice testes 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 is reported in Fig. V-5 relative to that of an non-injected testis of the same mouse. Wild-type BS-RNase and the **MxM** 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 little activity.

Immunosuppressive activity

Wild-type BS-RNase inhibits the growth of lymphocytes in culture (Soucek *et al.*, 1986; Tamburrini *et al.*, 1990). We determined the ability of various forms of BS-RNase and of RNase A to inhibit the growth of human lymphocytes. C32S BS-RNase, **M=M** BS-RNase, wild-type BS-RNase, and **MxM** BS-RNase were highly toxic to lymphocytes, as shown in Fig. V-6. The C31S BS-RNase had a modest cytotoxic effect. Monomeric BS-RNase and RNase A showed little effect on cell growth.

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 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, a mutant of triosephosphate isomerase was created that loses only 10^3-fold in activity upon dimer dissociation (Borchert *et al.*, 1994).

BS-RNase differs in several ways from HIV-1 protease, triosephosphate isomerase, and all other multimeric enzymes with

composite active sites. First, monomers of BS-RNase have more catalytic activity than does **MxM**, the form of BS-RNase having a composite active site (Tamburrini *et al.*, 1989). Secondly, BS-RNase exists in two distinct quaternary forms. The interconversion of these two forms requires regional unfolding and movement of the *N*-terminal α-helix of each monomer. Under severe conditions (such as lyophilization from a solution of 50% acetic acid), RNase A forms a dimer in which the active sites are composite and thus similar to the active sites in the **MxM** form of BS-RNase (Crestfield *et al.*, 1962). In contrast, BS-RNase undergoes this conversion in physiological conditions. We have illuminated the molecular basis and biological consequences of this conversion.

We speculated that the two intersubunit disulfide bonds and the hinge region might be related to the conversion of **M=M** to **MxM**. When the hinge region of BS-RNase was replaced by the analogous amino acid residues of RNase A, no significant difference in the rate or extent of conversion was observed (data not shown). We also suspected that the interconversion of **M = M** to **M x M** might have physiological consequences, and thus might be catalyzed by an enzyme. It has been suggested that the *cis/trans* isomerization of the Ser18–Pro19 peptide bond could play a role in the formation of **MxM** (Parente and D'Alessio, 1985). The *cis/trans* isomerization of a proline peptide bond can be catalyzed by cyclophilin (Mücke and Schmid, 1992). We found that cyclophilin was not able to affect significantly the rate of conversion of **MxM** to **M=M**. These two results argue that the hinge peptide in general and the isomerization

of the Ser18–Pro19 peptide bond in particular do not play important roles in the interconversion of the two dimeric forms of BS-RNase.

Mutants of BS-RNase that lack one of the two intersubunit disulfide bonds were still able to form a dimer, M=M, but the extent of the conversion to MxM was reduced significantly. The cysteine to serine mutations each made the conversion of M=M to MxM approximately twofold slower and the conversion of MxM to M=M approximately twofold faster (Table V-1). The subunits of wild-type BS-RNase are covalently linked by two intersubunit disulfide bonds. Eliminating one of these crosslinks increases the conformational entropy of the dimer. Since the MxM form has less conformational entropy than does the M=M form, the MxM form is disfavored by eliminating an intersubunit disulfide bond.

The results of the biological activities suggest that the MxM form of BS-RNase has significantly more antitumor and aspermatogenic activity than does the M=M form. The order of these biological activities increased as M=M form < wild-type BS-RNase < MxM form (Fig. V-4 and 5). Still, these results are difficult to interpret. The problem is that the M=M and MxM 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 aspermatogenesis assay takes 10 days, and the immunosuppression assays takes 6 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 problem. The M=M preparations of the C31S and C32S enzymes contain far less MxM than does that of the wild-type BS-RNase at equilibrium. In the

biological assays, these enzymes are thus better representatives of the **M=M** form. The biological activities of the mutant enzymes increased as C31S BS-RNase < C32S BS-RNase < wild-type BS-RNase (Fig. V-4, 5, and 6). This same order described the fraction of the enzymes that existed at equilibrium as the **MxM** form (Fig. V-2 and Table V-1). The diminished biological effect of C31S and C32S BS-RNases seems therefore to arise from a decreased fraction of the more potent **MxM** form.

What is the molecular basis for the different biological efficacy of the two quaternary forms of BS-RNase? The **MxM** and **M=M** forms contain the same amino acids crosslinked by 10 disulfide bonds. Each form has a pI = 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, suggesting that the solvated molecules have the same Stokes radius. Thus the overall physical properties of the **MxM** and **M=M** forms are indistinguishable. The existence of a cellular receptor for BS-RNase is unlikely (Mancheno *et al.*, 1994). We suggest that the difference in the biological efficacy of the two forms derives from their different fate inside the cell. The basis of our separation of **MxM** and **M=M** is the ability of **MxM** but not **M=M** to remain a dimer in the presence of a reducing agent. The reduction potential of the cytosol of mammalian cells will quickly reduce the intersubunit but not the intrasubunit disulfide bonds of BS-RNase. In this environment, **MxM** but not **M=M** will remain a dimer. The **MxM** form of BS-RNase, with its composite active site, seems therefore to have evolved to maintain the enzyme as a dimer *in vivo*.

The dimeric form of BS-RNase has two distinct attributes that may be critical for its biological activity. First, placental ribonuclease inhibitor binds tightly to monomers but not dimers of BS-RNase (Murthy and Sirdeshmukh, 1992). Second, double-stranded RNA substrates are cleaved rapidly by dimers but not monomers of RNase (Libonati and Floridi, 1969; Sorrentino *et al.*, 1980). We therefore propose that the ability of BS-RNase to maintain a dimeric form *in vivo* leads to its special biological properties either because only the dimer is resistant to cytosolic ribonuclease inhibitor, or because only the dimer catalyzes the cleavage of double-stranded RNA, or both.

Our results make predictions about the structure and function of the mammalian seminal ribonucleases from deer, giraffe, and sheep (Breukelman *et al.*, 1993; J. J. Beintema, personal communication). Although these enzymes have yet to be isolated, they are likely to exist as dimers crosslinked 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.

Fig. V-1. 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 **MxM** form. At various times, aliquots were withdrawn and the intersubunit disulfide bonds were selectively reduced, thereby converting the **M=M** form to monomer (**M**) and any **MxM** form to noncovalent dimer (**NCD**). These forms were then separated by gel filtration chromatography, and quantified by A_{280}.

(figure on the following page 104)

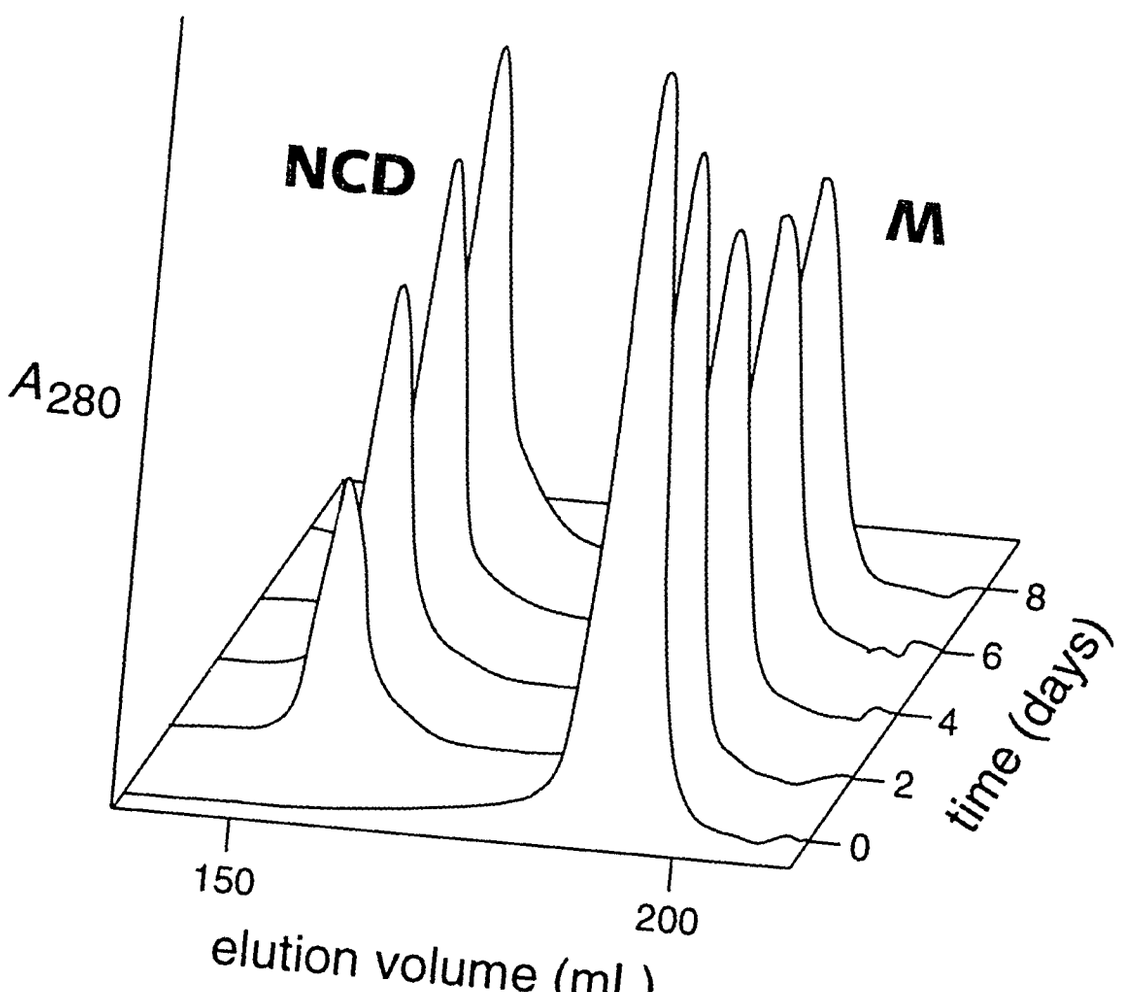


Fig. V-2. Time course for the equilibration of the M=M and MxM forms of wild-type (□), C31S (Δ), and C32S (▼) BS-RNase at 37 °C. The two forms were separated by selective reduction of the intersubunit disulfide bond(s) and gel filtration chromatography. The gel filtration profiles for the wild-type enzyme at 0, 2, 4, 6, and 8 days are shown in Fig. V-1.

(figure on the following page 106)

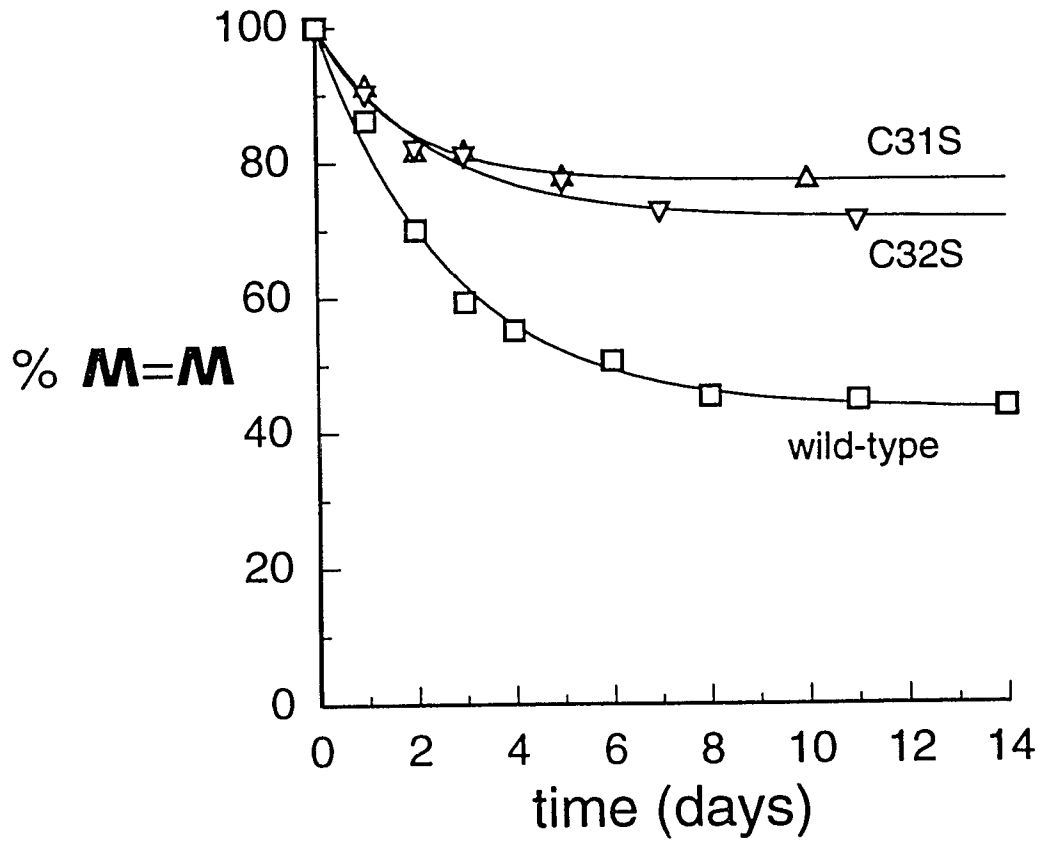


Fig. V-3. Sequence of the *N*-terminus of BS-RNase, RNase A, and the hybrid SRA-I. Differences from BS-RNase are underlined.

	1									10										20				
BS-RNase	K	E	S	A	A	A	K	F	E	R	Q	H	M	D	S	G	N	S	P	S	S	S	S	N..
RNase A	K	E	T	A	A	A	K	F	E	R	Q	H	M	D	S	S	T	S	A	A	S	S	S	N..
SRA-I	K	E	S	A	A	A	K	F	E	R	Q	H	M	D	S	S	T	S	A	A	S	S	S	N..

Fig. V-4. Effect of various forms of BS-RNase and of RNase A on the growth in culture of human tumor cell lines K-562 (A) and ML-1 (B). Values were determined by the incorporation of (^{3}H)thymidine into DNA and are reported as a % of the control, which was buffer containing no ribonuclease. Data were recorded 3 days after addition of ribonuclease to the culture.

(figure on the following page 109)

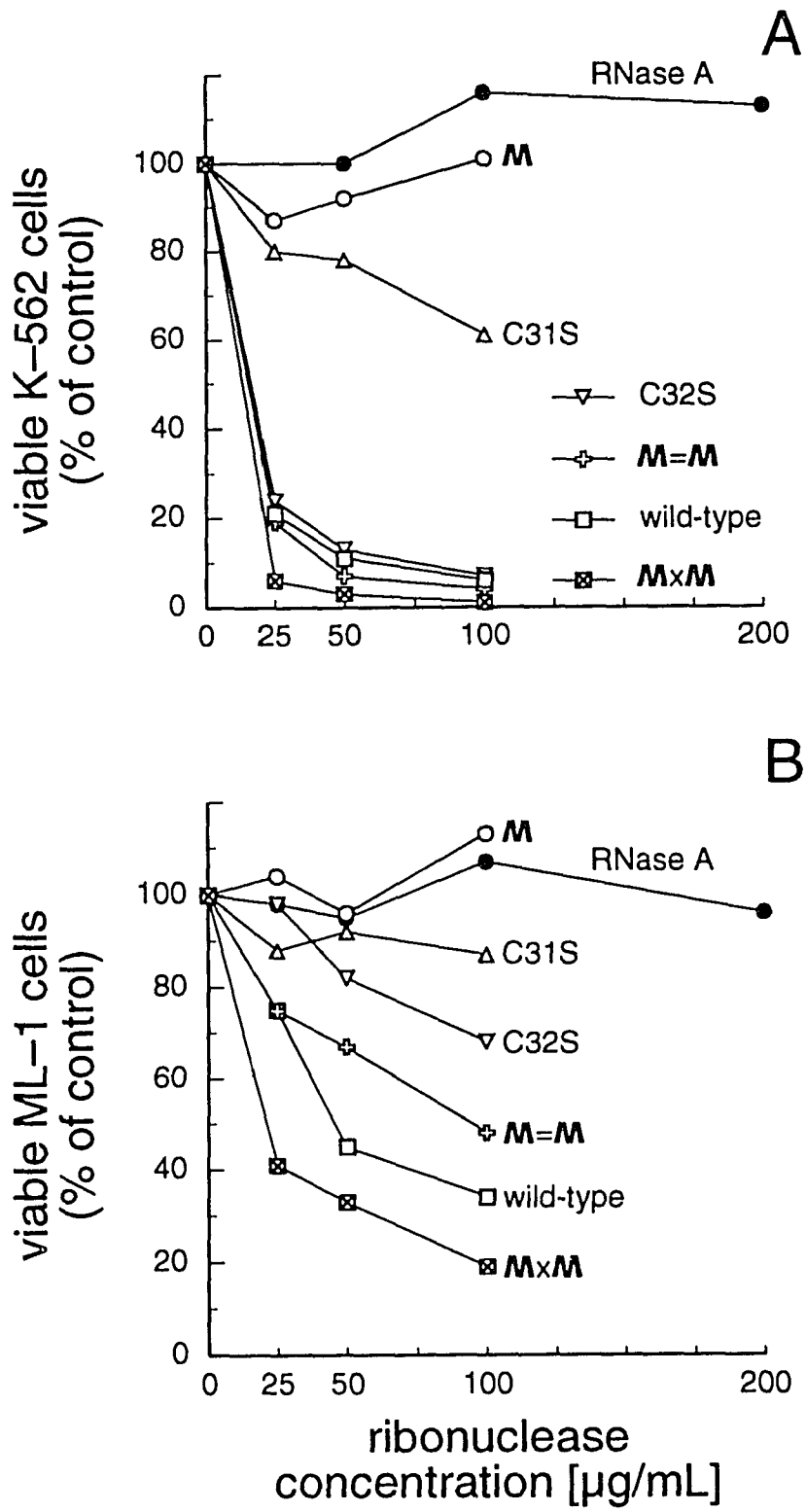


Fig. V-5. Effect of various forms of BS-RNase and of RNase A on mouse spermatogenesis. Each value is an average from 4 injected testes and is reported as a % of the control, which is the value from the non-injected testes of the same 4 mice. Data were recorded 10 days after injection.

(figure on the following page 111)

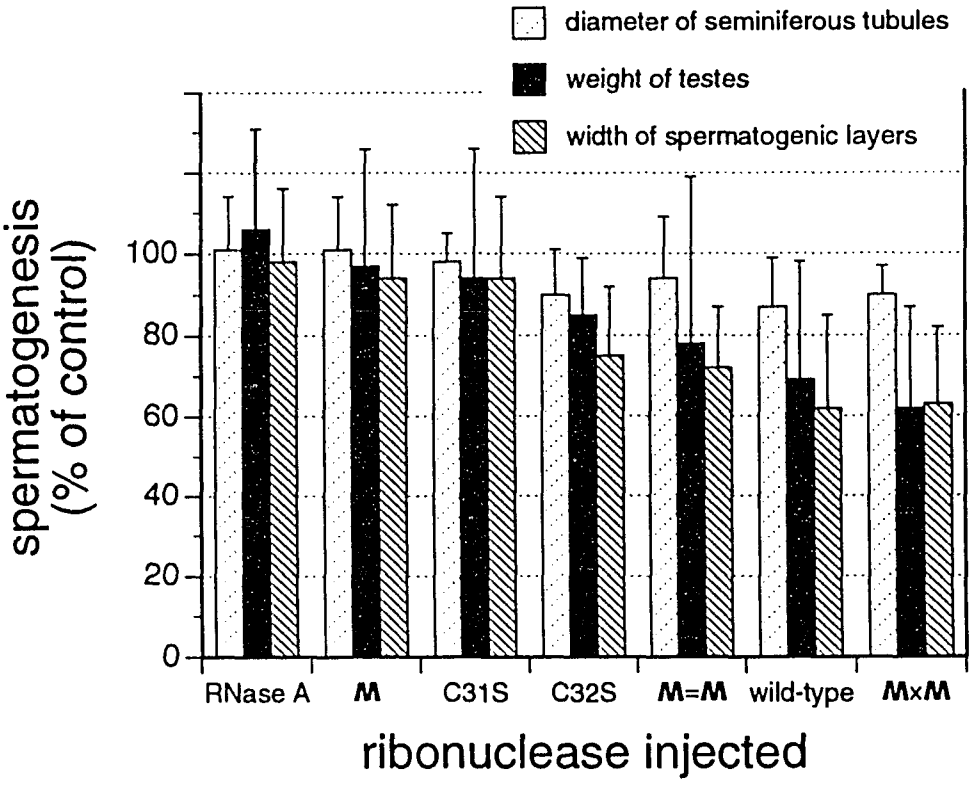


Fig. V-6. Effect of various forms of BS-RNase and of RNase A on the growth in culture of human lymphocytes. Values were determined by the incorporation of (^{3}H)thymidine into DNA and are reported as a % of the control, which was buffer containing no ribonuclease. Data were recorded 6 days after addition of ribonuclease to the culture.

(figure on the following page 113)

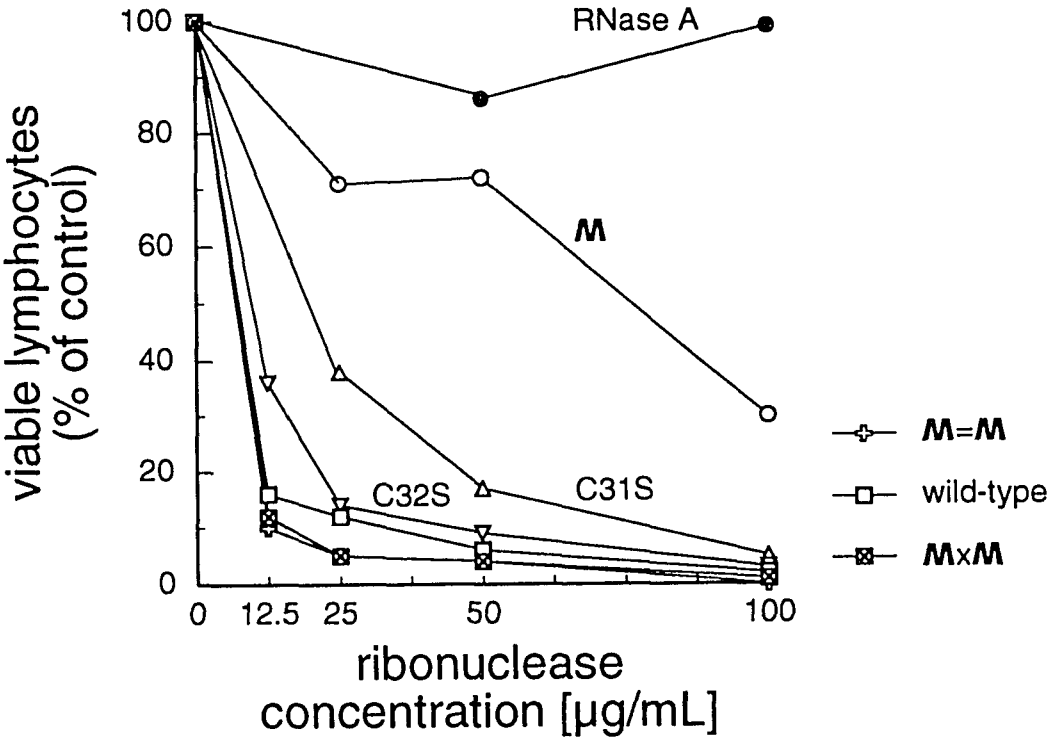


Table V-1. Kinetic and thermodynamic parameters for the $\mathbf{M} = \mathbf{M} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathbf{M}\times\mathbf{M}$ interconversion of wild-type and mutant BS-RNase.[a]

BS-RNase	k_1 (days^{-1})	k_{-1} (days^{-1})	K ($= k_1/k_{-1}$)	ΔG ° (kcal/mol)
wild-type	0.21	0.16	1.3	– 0.18
C31S	0.14	0.46	0.30	0.75
C32S	0.12	0.30	0.40	0.57

[a] Calculated by fitting the data in Fig. V-2 to the equation

$$(\%\ \mathbf{M} = \mathbf{M})_t = \frac{k_{-1} + k_1 e^{-(k_1 + k_{-1})t}}{k_1 + k_{-1}} \times 100.$$

The values of r^2 for this fit were 0.99, wild-type; 0.98, C31S; and 0.98, C32S.

CHAPTER VI

Structural Basis for the Biological Activities of Bovine Seminal Ribonuclease: Site-directed and Regional Mutagenesis

Assays for biological activity were performed in collaboration with Dr. J. Soucek (Institute of Hematology and Blood Transfusion, Prague, Czech Republic) and Dr. J. Matousek (Institute of Animal Physiology and Genetics, Libechov, Czech Republic).

Summary

Bovine seminal ribonuclease (BS-RNase) has unusual biological properties, including antispermatogenic, antitumor, and immunosuppressive activities. A closely related protein, bovine pancreatic ribonuclease (RNase A), lacks such activities. Differences in the primary structures of the two ribonucleases must give rise to the biological activities of BS-RNase. To identify the residues responsible for the biological activities, we constructed a series of hybrids between BS-RNase and RNase A in which divergent segments of RNase A replace analogous segments of BS-RNase, and we examined the biological activities of these hybrids. All the hybrids we tested displayed strong biological activities, comparable to those of wild-type BS-RNase. This result suggests that a receptor specific for BS-RNase may not exist. BS-RNase may enter cells by a nonspecific interaction with cell membranes (Mancheno *et al.*, 1994).

We also determined whether the ribonuclease activity of BS-RNase was necessary for its biological activities by preparing a mutant BS-RNase in which His119, a critical active-site residue, was changed to an aspartate. The mutation in H119D BS-RNase caused a 4,000-fold decrease in catalytic activity. The H119D BS-RNase was also inactive in our biological assays. This result indicates that the catalytic activity of BS-RNase is necessary for its biological activities.

Introduction

Bovine seminal ribonuclease (BS-RNase) displays extraordinary biological properties, such as antispermatogenic, antitumor, and immunosuppressive activities (Dostal and Motousek, 1973; Soucek *et al.*, 1986; Tamburrini *et al.*, 1990; Laccetti *et al.*, 1992). Among these biological properties, the immunosuppressive activity of BS-RNase is likely to be physiologically significant, since this activity may be required to suppress the female immune response against bull seminal components (James and Hargreave, 1984). The antitumor activity of BS-RNase has been of interest due to its therapeutic potential (Youle *et al.*, 1993).

These biological properties are closely related to the unique dimeric structure of BS-RNase. When monomerized, BS-RNase loses biological activities (Vescia *et al.*, 1980; Tamburrini *et al.*, 1990). In contrast, artificially dimerized RNase A shows an antitumor activity, although to a lesser extent (Bartholeyns and Baudhuin, 1976; Vescia *et al.*, 1980). Intact RNase A, which shares 81% amino acid sequence identity with BS-RNase, does not display any of the unusual biological activities. However, RNase A/BS-RNase hybrid proteins in which particular regions of primary structure in RNase A were replaced with the corresponding segments of BS-RNase showed various degrees of immunosuppressive activity (S. A. Benner, personal communication). This result implies that the differences in the primary structure as well as in the quaternary structure between the two ribonucleases may be responsible for the special biological effects of BS-RNase.

Regional mutagenesis by recombinant DNA techniques has been used to generate angiogenin/RNase A hybrid proteins, derivatives of angiogenin and RNase A in which particular regions of primary structure have been replaced with the corresponding segments of RNase A and angiogenin, respectively (Harper and Vallee, 1989; Allemann *et al.*, 1991). These studies allowed structural elements critical to angiogenesis to be identified. Similarly, the preparation of BS-RNase/RNase A hybrids and their characterization may allow us to identify structural components of BS-RNase necessary for the unusual biological properties.

Due to the unusual biological properties, BS-RNase belongs to a special group of ribonucleases termed RISBASEs (*RI*bonucleases with *S*pecial *B*iological *A*ctions) (D'Alessio *et al.*, 1991). This group includes ribonucleases with diverse biological properties from various sources. Human angiogenin is involved in blood vessel formation (Shapiro *et al.*, 1986). Onconase from frog, *Rana pipiens*, is a strong antitumor agent (Ardelt *et al.*, 1991). S-RNase from plants is responsible for self-incompatibility (McClure *et al.*, 1989; Taylor *et al.*, 1993). All these proteins have conserved active site residues and are able to cleave RNA. This catalytic activity of RISBASEs appears to be essential for their biological activities. For example, site-directed mutagenesis on a catalytic lysine residue in human angiogenin resulted in substantially reduced angiogenic effect (Shapiro *et al.*, 1989). Chemically modified onconase lost its cytotoxic effect on tumor cells (Ardelt *et al.*, 1991). Similarly, chemical modification of a catalytic histidine residue in BS-RNase resulted in the loss of its immunosuppressive activity (Tamburrini *et al.*, 1990) and antitumor

activity (Vescia *et al.*, 1980). Chemical modifications of proteins often lead to inconclusive results because the modification may be incomplete or it may cause structural and functional damage to the proteins (Knowles, 1987). For example, chemically-inactivated RNase A dimer was reported to show full antitumor effects (Bartholeyns and Baudhuin, 1976; Bartholeyns and Zenebergh, 1979).

We prepared a mutant BS-RNase in which a critical active-site residue has been changed. In this chapter, we report on the consequences of this active site mutation for the catalytic efficiency and biological activities of BS-RNase as well as on the characterization of BS-RNase/RNase A hybrids.

Results

Mutagenesis of BS-RNase

In order to construct BS-RNase/RNase A hybrids, first we compared the amino acid sequences of the two ribonucleases (Fig. VI-1). The amino acid sequence of BS-RNase is highly similar to that of RNase A, having only 23 substitutions in its 124 residues. Seventeen of the 23 substituted residues in BS-RNase are located in three main regions: residues 16–20, 28–39 and 102–115. One is in position 3, and the remaining five are located in the region between residue 55 and 80. Several of these substitutions appear to be particularly important in the acquisition of the dimeric structure of BS-RNase. The region of residues 28–39 contains two

substitutions that are essential for the dimeric structure of the protein: Cys31 and Cys32. The substitution of Leu28 of BS-RNase for a Gln residue in RNase A makes the region highly hydrophobic and thus facilitate the contact between the two subunits. Lys34 of BS-RNase was shown to be responsible for the high reactivity of Cys31 and Cys32 toward reducing agents. These residues that may be responsible for the dimeric structure of BS-RNase have not been changed in the hybrids we constructed.

Most of the other substitutions are located in surface loops as shown in Fig. VI-2. We divided these substitutions in five groups based on their location in the three-dimensional structure, and then prepared five corresponding hybrids between BS-RNase and RNase A: SRA-I to SRA-V (Table VI-1). The hybrid proteins were purified from *E. coli* to homogeneity. Wild-type BS-RNase was also purified from bull seminal plasma and from *E. coli*. All the hybrid proteins showed ribonuclease activity identical (± 5%) to that of wild-type BS-RNase.

In addition, H119D BS-RNase was prepared to investigate the consequences of the active site mutation for the catalytic efficiency and biological activities.

Kinetic studies of H119D BS-RNase

The kinetic parameters for wild-type and H119D BS-RNases were determined by using UpA as a substrate (Table VI-2). Wild-type BS-RNases from *E. coli* and from seminal plasma were indistinguishable in terms of catalytic efficiency (k_{cat}/K_m) and affinity for substrate (K_m). The active-site mutation in H119D BS-RNase, however, caused 4,000-fold decrease in the

catalytic efficiency without affecting the affinity for substrate. Similar results had been reported with RNase A mutants in which the two histidine residues were replaced by alanine, respectively (Thompson and Raines, 1994).

Biological assays

We examined the antitumor, immunosuppressive, and antiembryonic activities of wild-type BS-RNase from seminal plasma, wild-type BS-RNase from *E. coli* (designated as rBS-RNase in the following figures), BS-RNase/RNase A hybrids (SRA-I to SRA-V), and two monomeric ribonucleases (RNase A and monomers of BS-RNase). First, the antitumor activity was tested on human tumor cell line K-562 (Fig. VI-3). Wild-type BS-RNase from both seminal plasma and *E. coli* showed strong inhibitory effect on the growth of the tumor cells. All of the hybrids except SRA-V also displayed a similar antitumor effect. SRA-V had only a modest effect. H119D BS-RNase and the two monomeric ribonucleases (RNase A and BS-RNase monomer) showed no effect on the growth of the tumor cells. Next, we examined the ability of various forms of BS-RNase and of RNase A to inhibit the growth of normal human lymphocytes stimulated by mixed lymphocyte culture (MLC) as shown in Fig. VI-4. All the hybrids displayed strong immunosuppressive activity. The two monomers showed a modest effect at high concentration (100 μg/mL). H119D BS-RNase was ineffective. Finally, the antiembryonic effect of the ribonucleases was investigated. As shown in Fig. VI-5, all the hybrids and wild-type BS-RNase caused 100% mortality of bovine embryos at 24 after

injection. BS-RNase monomer and RNase A showed only a minimal effect. H119D BS-RNase was also ineffective.

Thus, the three biological assays we performed yielded consistent results: H119D BS-RNase and BS-RNase monomer were inactive, while all the hybrids were as active as wild-type BS-RNase.

Discussion

RISBASEs are ribonucleases with diverse biological properties from various sources (D'Alessio *et al.*, 1991). The mechanism by which RISBASEs display their biological properties has not been understood. Two hypotheses have been suggested on the mechanism of RISBASEs. The 'extracellular' hypothesis is based on the fact that all RISBASEs are secretory proteins. RISBASEs may display their biological properties by degrading 'communicator RNAs' between cells, which have yet to be isolated (Benner, 1988). In contrast, the 'intracellular' hypothesis assumes cell specific internalization of RISBASEs, which cleave RNA inside the cell (D'Alessio, 1993). This hypothesis predicts the existence of cell-surface receptors for RISBASEs. Several receptors for RISBASEs have been reported. Both hypotheses, however, have one thing in common: the ribonuclease activity of RISBASEs is proposed to be essential for their biological properties. We were able to confirm the proposal that the ribonuclease activity of BS-RNase is required for its biological effects by preparing an inactive BS-RNase mutant in which the active site residue

His119 has been replaced with aspartate. H119D BS-RNase showed minimal, if any, antitumor, immunosuppressive, and antiembryonic activities.

Unlike other ribonucleases in general and other RISBASEs in particular, BS-RNase is isolated as dimers. BS-RNase monomer formed by selective reduction displayed negligible biological activities in our experiments, confirming previous reports (Vescia *et al.*, 1980; Tamburrini *et al.*, 1990). It is possible that only dimers of BS-RNase are recognized by a specific receptor, which has not been isolated yet. The dimeric structure seems to be necessary but not sufficient for the biological activities of BS-RNase. Artificial dimers of RNase A showed only a modest antitumor effect (Vescia *et al.*, 1980). Thus, we assumed that the difference in the primary structure of RNase A and of BS-RNase is also related to the biological activities of BS-RNase.

"Homolog scanning" by regional mutagenesis is a useful method to identify the structural determinants that cause functional variation among homologous proteins (Cunningham *et al.*, 1989). In this method, the primary sequences of two homologs are compared. Only one of the homologs displays certain activities. Next, a series of hybrids between the two homologs are constructed by systematically substituting segments of sequences derived from the other homolog, and activities of the hybrids are examined. This strategy was used to identify the epitopes of human growth hormone (Cunningham *et al.*, 1989). Homolog scanning has also been applied to RNase A and angiogenin. The RNase A/angiogenin hybrid in which an external loop of RNase A was replaced with the

corresponding segment of angiogenin mimicked the characteristics of angiogenin (Allemann *et al.*, 1991). Similarly the reciprocal hybrid in which the loop of RNase A was inserted into angiogenin endowed RNase A-like catalytic properties to angiogenin (Harper and Vallee, 1989).

Encouraged by these previous successes, we adopted this strategy to identify the structural determinants of the biological activities of BS-RNase. We prepared and characterized BS-RNase/RNase A hybrids in which segments of RNase A sequences were inserted into the BS-RNase gene, replacing the corresponding segments of BS-RNase.

All the hybrids displayed strong biological activities in our assays. The 5 hybrids we tested contained substitutions for 16 of the 23 residues that differ between BS-RNase and RNase A. The 4 residues that are apparently responsible for the dimeric structure of BS-RNase (Leu28, Cys31, Cys32, and Lys34) were not changed in our hybrids (Capasso *et al.*, 1983; Parente *et al.*, 1985). The other 3 substitutions are located far from the 5 divergent clusters in the three-dimensional structure of BS-RNase (Fig. VI-2). Thus, it is not likely that these 3 substitutions affect significantly the biological activities of BS-RNase. Nevertheless, we are aware that particular combinations of all substitutions may be responsible for the biological properties.

Recently Mancheno *et al.* (1994) suggested that BS-RNase may permeate cells by altering the cell membrane. The membrane of tumor cells has a high net negative charge. BS-RNase, which is a basic protein with pI = 10.3, binds to the membrane bilayer and destabilizes it *in vitro*. Interestingly, monomeric BS-RNase has no effect on the membrane.

Monomeric BS-RNase showed no activities in our biological assays as well. It appears that our results with BS-RNase/RNase A hybrids support this modified intracellular model for the mechanism of BS-RNase action. Because all the hybrids displayed a similar extent of biological activities, it is not likely that a receptor specific for BS-RNase exists. This model also explains why artificial dimers of RNase A are less effective as antitumor agents. RNase A, with pI = 9.3, is less basic than BS-RNase. RNase A may be not able to interact as efficiently with negatively charged membrane as does BS-RNase. On entering cells, which is initiated by this ionic interaction, BS-RNase inhibits or kills the cells by cleaving RNA, perhaps double-stranded. It is not clear whether this ionic interaction model can be applied to other activities of BS-RNase.

We have discussed the molecular basis for the biological activities of BS-RNase in two aspects. In this chapter, we have addressed the recognition of BS-RNase by cells. Our data suggest that a receptor specific for BS-RNase is not likely to be involved in this process. BS-RNase may enter cells by destabilizing the negatively charged cell membrane. The positive charge density of dimeric BS-RNase appears to be important in this step, since the BS-RNase monomer does not interact with the lipid bilayer (Mancheno *et al.*, 1994). RNase A dimers, which have less net positive charge than do BS-RNase dimers, show much less antitumor effect possibly due to inefficient interaction with the membrane.

In CHAPTER V, we addressed the subsequent step of BS-RNase action. Once entering cells, the two dimeric forms of BS-RNase, **M=M** and **MxM**, meet with different fates. The high reduction potential of the

cytosol is likely to reduce the intersubunit but not intrasubunit disulfide bonds of BS-RNase (D'Alessio *et al.*, 1975; Hwang *et al.*, 1992). As a result, **M=M** dissociates, while **MxM** remains as a noncovalent dimer. Unlike monomers, the dimeric form of BS-RNase is resistant to ribonuclease inhibitor (Murthy and Sirdeshmukh, 1992) and able to cleave double-stranded RNA (Sorrentino *et al.*, 1980), which may lead to the biological activities of BS-RNase.

Thus, a ribonuclease must satisfy two requirements to be biologically active. First, it must be able to permeate the cell membrane: monomeric BS-RNase is not biologically active and dimeric RNase A is only slightly active because they are not able to permeate the membrane efficiently, possibly due to their lower net positive charge. Secondly, a biologically active ribonuclease must remain as a dimer inside the cell: the **M=M** form of BS-RNase may be ineffective because it dissociates inside the cell due to the reduction of intersubunit disulfide bonds. Monomeric BS-RNase cannot be biologically active even it permeates cell membrane according to this requirement.

This model for the structural basis of the biological activities of BS-RNase explains all the experimental data obtained by others and by us in a simple and consistent way. We suggest that the current model can be further tested in the following experiments. RNase A has 5 fewer basic residues than does BS-RNase. In the first experiment, several substitutions are made in RNase A by site-directed mutagenesis to construct a RNase A/BS-RNase hybrid in which the positively charged residues as well as

Cys31 and Cys32 are incorporated. The ionic interaction model predicts that the hybrid dimers will be as biologically active, as is BS-RNase.

Another experiment is designed to test the second half of the current model. A nonreducible covalent dimer of C31S BS-RNase can be made by using a crosslinking, thiol-specific fluorescent labeling agent, bBBr (Kosower and Kosower, 1987). This dimer is not likely to form **MxM** because the sulfhydryl groups of Cys31 in each subunit are separated by 5 atoms of the labeling agent. Furthermore, C31S BS-RNase itself has much reduced ability to form **MxM** (CHAPTER V). Our hypothesis assumes that the **MxM** form of BS-RNase is biologically active only because **MxM** remains as a dimer inside the cell despite the reducing environment. The crosslinked dimer of C31S BS-RNase is expected to be as biologically active as is the **MxM** form of wild-type BS-RNase, although the dimer is unlikely to form **MxM**. Since the crosslinking agent is also a fluorescent label, cells treated with this protein can be examined with a fluorescence microscope to assess internalization. A related agent, mBBr, can be used to label C31S BS-RNase to prepare monomeric fluorescent protein. The ionic interaction model predicts that the dimer permeates cells while the monomer does not, which can be examined by fluorescence microscopy. Alternately, the monomer may enter cells but be still inactive, perhaps because it is inhibited by ribonuclease inhibitor. In a related experiment, mutant monomeric BS-RNase whose net positive charge is increased by site-directed mutagenesis can be tested for its ability to permeate the cell membrane and to inhibit the growth of the cells. The interpretation of the results of these experiments, however, may not be straightforward.

Under extreme conditions, RNase A is able to form a dimer whose structure is thought to be similar to that of the **MxM** form of BS-RNase (Fruchter and Crestfield, 1965). Since such conditions cannot be met *in vivo*, Nature seems to have found another way to construct the **MxM** form of bovine ribonuclease by simply introducing several residues, especially Cys31 and Cys32, into the primary structure of the protein. As a result, the **MxM** form is endowed with different biochemical properties (such as stability against reducing environment and resistance to ribonuclease inhibitor), which lead to new biological properties.

This remarkable structural flexibility of bovine secretory ribonucleases was also successfully exploited in our protein fusion system as described in CHAPTER II. In conclusion, we have used the techniques of protein engineering to exploit an interesting structural property of bovine pancreatic ribonuclease and to explore the structural basis for the biological activities of bovine seminal ribonuclease.

Fig. VI-1. Comparison of the amino acid sequences of RNase A and BS-RNase. Identical matches are shown as dots (.) in the sequence of RNase A. The substitutions incorporated in the hybrids, SRA-I to SRA-V, are indicated by corresponding numbers.

(figure on the following page 130)

```
                          10        20        30        40        50
BS-RNase    KESAA AKFER QHMDS GNSPS SSSNY CNLMM CCRKM TQGKC KPVNT FVHES
RNase A     ..T.. ..... ..... ST.AA ..... ..Q.. KS.NL .KDR. ..... .....
SRA                           11 11                2  222

                          60        70        80        90        100
BS-RNase    LADVK AVCSQ KKVTC KNGQT NCYQS KSTMR ITDCR ETGSS KYPNC AYKTT
RNase A     ....Q ..... .N.A. ..... ..... Y...S ..... ..... ..... .....
SRA                     3 3                 4

                         110       120
BS-RNase    QVEKH IIVAC GGKPS VPVHF DASV
RNase A     .AN.. ..... E.N.Y ..... ....
SRA          44         5 5 5
```

Fig. VI-2. The three-dimensional location of the substitutions in the hybrids, SRA-I to SRA-V. Each cluster is shown in one subunit and color-coded: SRA-I (green), SRA-II (cyan), SRA-III (magenta), SRA-IV (red), and SRA-V (yellow). The 3 substitutions (residues 3, 55, and 76), located far from the clusters and thus not incorporated in the hybrids, are shown in gray. Only α-carbons are shown.

(figure on the following page 132: original in color)

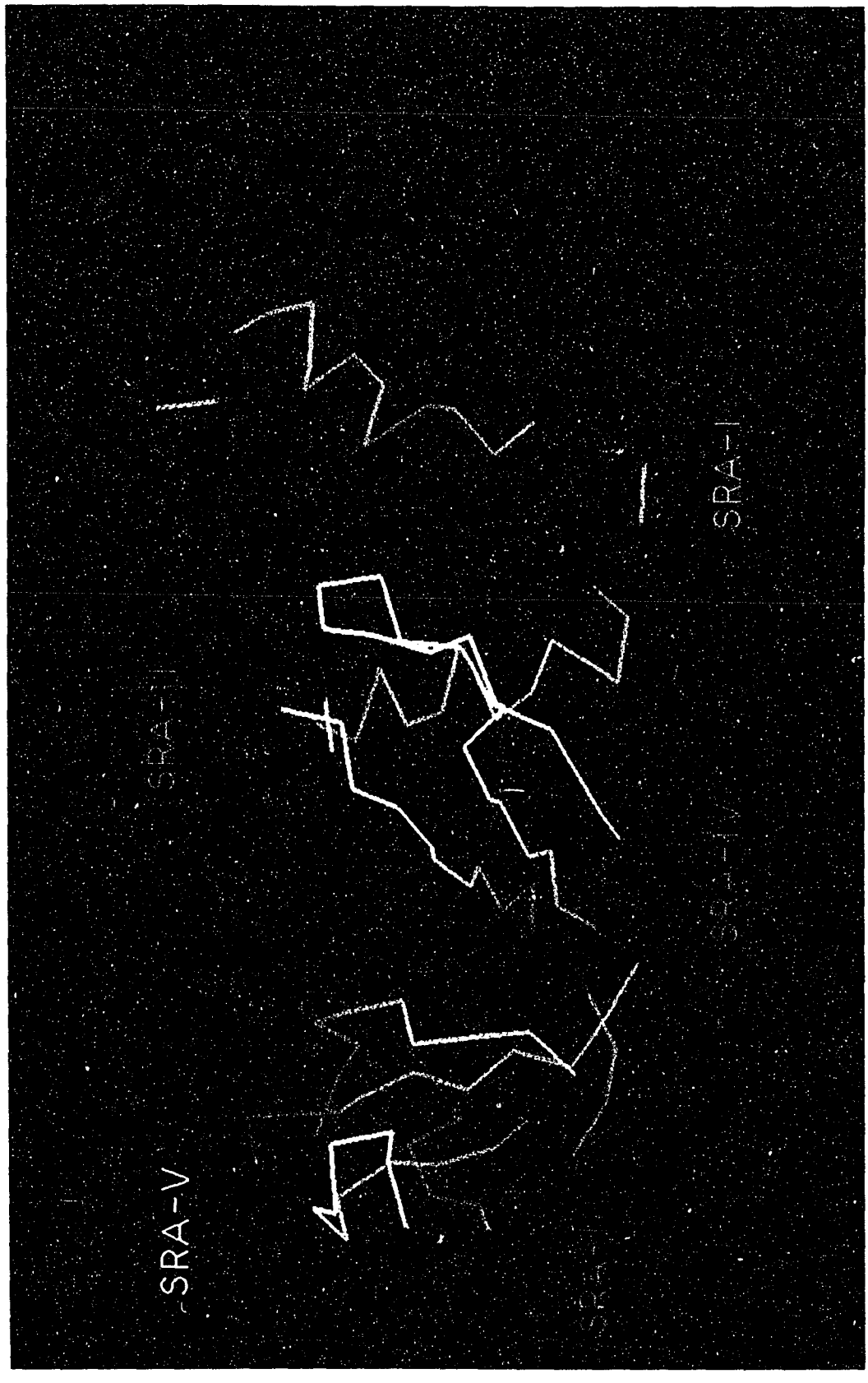


Fig. VI-3. Effect of various forms of ribonucleases on the growth in culture of human tumor cell line K-562. Values were determined by the incorporation of (^{3}H)thymidine into DNA and are reported as a % of the control, which was buffer containing no ribonuclease. Data were recorded 3 days after addition of ribonuclease to the culture.

(figure on the following page 134)

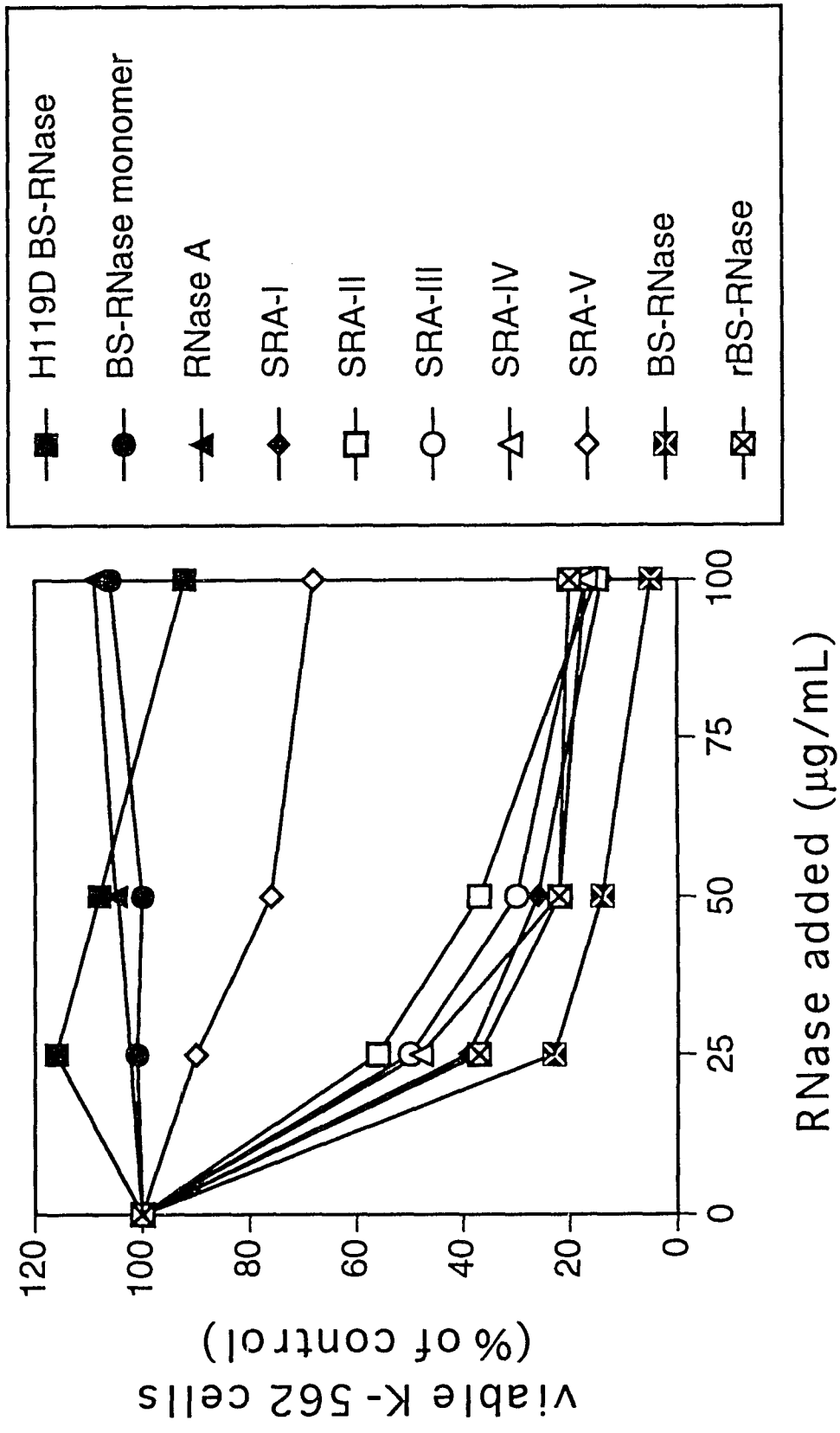


Fig. VI-4. Effect of various forms of ribonucleases on the growth in culture of human lymphocytes. Values were determined by the incorporation of (^{3}H)thymidine into DNA and are reported as a % of the control, which was buffer containing no ribonuclease. Data were recorded 6 days after addition of ribonuclease to the culture.

(figure on the following page 136)

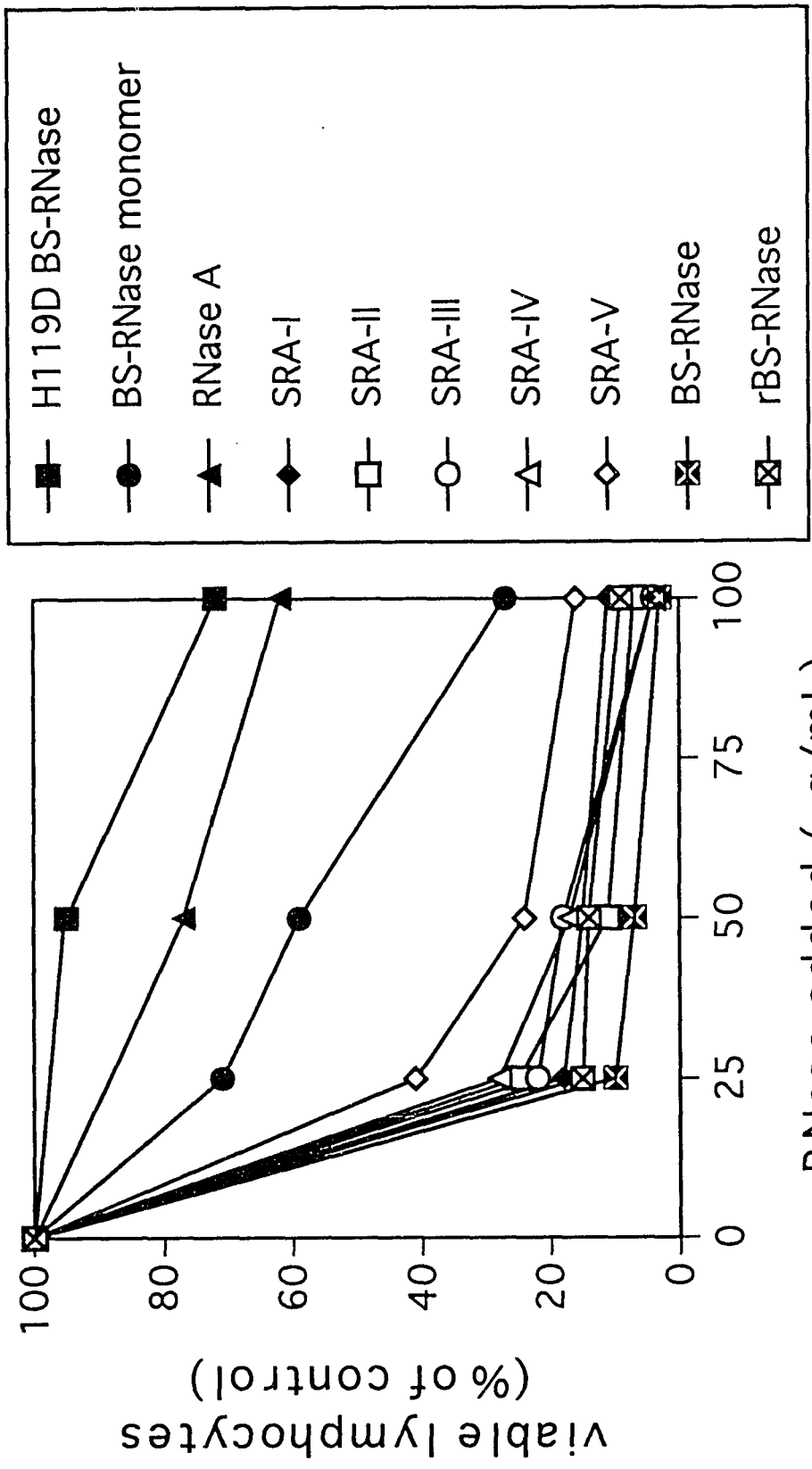


Fig. VI-5. Effect of various forms of ribonucleases on the growth of bovine embryos. The mortality of the embryos was assessed for 3 days after the addition of a ribonuclease (10 μg/mL). Only the effect of SRA-I is shown because all the hybrids, SRA-I to SRA-V, displayed almost identical effects. (figure on the following page 138)

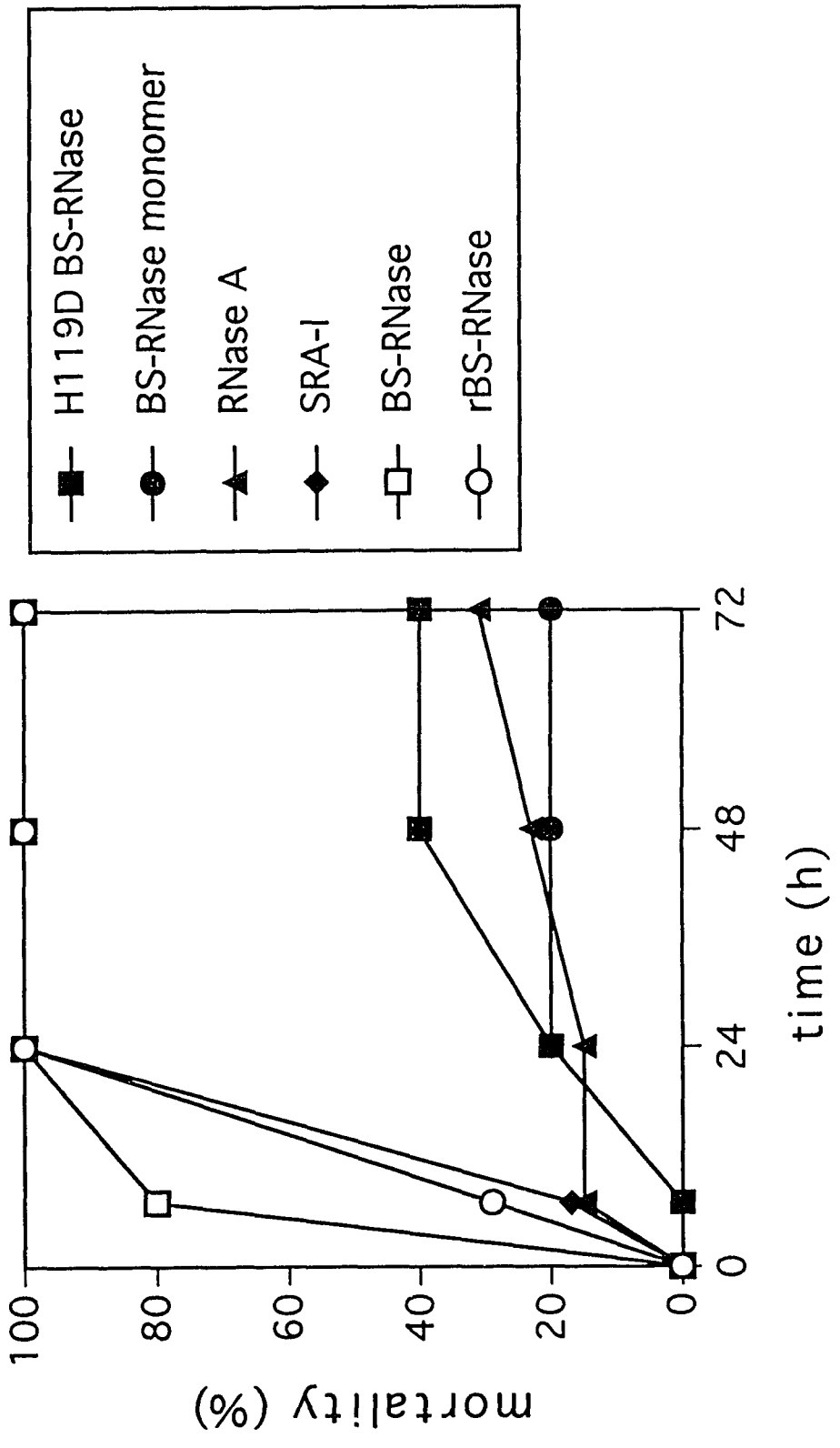


Table VI-1. Regional mutagenesis to construct the BS-RNase/RNase A hybrids.

hybrid proteins	substitutions	oligonucleotides used for mutagenesis[a]	plasmids
SRA-I	G16S, N17T, P19A, S20A	AA.45	pSRA10
SRA-II	M35L, Q37K, G38D, K39R	HC.55	pSRA20
SRA-III	K62N, T64A	SP.39	pSRA30
SRA-IV	R80S, V102A, E103N	VE.36 + RS.42	pSRA41
SRA-V	G111E, K113N, S115Y	SB.48	pSRA50

[a] The DNA sequences of the oligonucleotides are reported in CHAPTER VII, Experimental for CHAPTER VI.

Table VI-2. Steady-state kinetic parameters for cleavage of UpA by mutant and wild-type BS-RNases.

bovine seminal ribonuclease	k_{cat} (10^3 s^{-1})	K_m (mM)	k_{cat}/K_m (10^6 $M^{-1}s^{-1}$)
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

CHAPTER VII

Experimental

General

Materials

Plasmid pBluescript II SK(–) was from Stratagene (La Jolla, CA). Expression vector pET17b and pET22b were from Novagen (Madison, WI). *Escherichia coli* strain JM109 was from Promega (Madison, WI), and was used for transformation and plasmid isolation. *E. coli* strains BL21(DE3) and BL21(DE3)/pLysS were from Novagen (Madison, WI), and used for protein production. *E. coli* strain CJ236 was from Bio-Rad (Richmond, CA), and was used for site-directed mutagenesis. Helper phage M13K07, also from Bio-Rad, was used for site-directed mutagenesis.

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. T7 DNA polymerase was from New England Biolabs (Beverly, MA). Bacto-tryptone and bacto-yeast extract were from Difco (Detroit, MI). Ampicillin (sodium salt) was from International Biotechnologies (New Haven, CT). Kanamycin (monosulfate salt) and chloramphenicol were from Sigma Chemical (St. Louis, MO). IPTG was also from Sigma Chemical.

Luria-Bertani medium (LB) contained (in 1 L) bacto-trypton (10 g), bacto-yeast extract (5 g), and NaCl (10 g). Terrific Broth (TB) contained (in 1 L) bacto-tryptone (12 g), bacto-yeast extract (24 g), glycerol (4 mL), KH_2PO_4 (2.31 g), and K_2HPO_4 (12.54 g). Polycytidylic acid (poly(C)) was from Midland Reagent (Midland, TX).

RNase A (type III-A), S-protein, adenosine deaminase and UpA were from Sigma Chemical. Authentic S15 was synthesized by Operon Technologies (Alameda, CA). (α-^{35}S)Deoxyadenosine 5'-triphosphate was from Amersham (Arlington Heights, IL).

All other chemicals and reagents were of commercial reagent grade or better, and were used without further purification.

Methods

pH was measured with a Beckman pH meter fitted with a Corning electrode, calibrated at room temperature with standard buffers from Fisher (Chicago, IL). Ultraviolet and visible absorbance measurements were made on a Cary 3 spectrophotometer equipped with a Cary temperature controller. DNA oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer by using the β-cyanoethyl phosphoramidite method (Sinha *et al.*, 1984). DNA sequencing was performed with a Sequenase 2.0 kit from United States Biochemical. DNA fragments were isolated from agarose gels with a GENECLEAN II kit from Bio101 (La Jolla, CA). Site-directed mutagenesis was performed on single-stranded DNA isolated from *E. coli* strain CJ236 according to Kunkel *et al.* (1987). Other manipulations of DNA were performed as described by Ausubel *et al.* (1989).

Protein concentration was determined with the Protein Assay kit from Bio-Rad (Richmond, CA). The concentration of purified BS-RNase was determined by using an absorption coefficient of $\varepsilon^{1.0\%}{}_{1\ cm} = 4.65$ at 278 nm for the native enzyme, or $\varepsilon^{1.0\%}{}_{1\ cm} = 4.40$ at 274 nm for the reduced and

denatured protein (Parente and D'Alessio, 1985). The concentration of RNase A (Richards and Wyckoff, 1971) and S-protein was determined by using absorption coefficients of $\varepsilon^{1.0\%}_{1\ cm} = 7.09$ and of $\varepsilon^{1.0\%}_{1\ cm} = 8.40$ (= 7.09 x 13, 690 (molecular weight of RNase A) / 11,542 (molecular weight of S-protein)) at 278 nm, respectively. Ribonuclease activity was assayed by the method of Kunitz (1946). Free sulfhydryl groups were detected by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (Creighton, 1989).

Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl sulfate (SDS; 0.1% w/v) according to Ausubel *et al.* (1989). Gels were fixed and stained by washing with aqueous methanol (40% v/v), containing acetic acid (10% v/v) and Coomassie brilliant blue (0.1% w/v). The molecular weight standards were from Bio-Rad (Richmond, CA): phosphorylase B (97.4 kDa unstained; 106 kDa prestained), serum albumin (66.2; 80.0), ovalbumin (45.0; 49.5), carbonic anhydrase (31.0; 32.5), trypsin inhibitor (21.5; 27.5), and lysozyme (14.4; 18.5). Native PAGE was performed at pH 3.8 as described (Hames, 1990). Zymogram electrophoresis was performed as described (Blank *et al.*, 1982) with the following modification. After electrophoresis, the activity gel containing poly(C) (0.5 mg/ml) was washed with a solution of isopropanol (20% v/v) to remove the SDS and then with 10 mM Tris-HCl buffer, pH 7.5. The gel was incubated at room temperature for 10 min and then stained with a solution of toluidine blue O (0.2 % w/v), which stains only polymeric nucleic acid.

Protein to be sequenced was separated by electrophoresis in an SDS–polyacrylamide gel, and then transferred to a PVDF membrane (Bio-Rad;

Richmond, CA) by using a Mini Trans-Blot Cell (Bio-Rad). *N*-terminal sequence was determined by using an Applied Biosystems 470A protein sequencer at the Biotechnology Center, University of Wisconsin-Madison.

Experimental for CHAPTER II

Materials

E. coli strains CAG598 (*htpR165*-Tn*10*) and CAG629 (*lon*$^-$ *htpR165*-Tn*10*) were gifts from C. A. Gross. *E. coli* strain CSH27 (F$^-$ ara Δ(lac proAB) thi tet^S recA56 srl$^-$) was a gift of W. S. Reznikoff. Plasmid pMAL-c was from New England Biolabs. Plasmid pMC1871 was from Pharmacia (Piscataway, NJ). β-Galactosidase was from Sigma Chemical. The molecular weight standards from Bio-Rad were either unstainded: myosin (200 kDa), β-galactosidase (116), phosphorylase B (97.4), serum albumin (66.2), and ovalbumin (45.0); or prestained: myosin (205 kDa), β-galactosidase (117), bovine serum albumin (77.0), and ovalbumin (46.5).

Construction of plasmids

An f1 origin was inserted into plasmid pMAL-c as follows. Plasmid pMAL-c was digested with *Bgl*I, and the 5.0 kb fragment was isolated. Plasmid pBluescript II SK(–) was also digested with *Bgl*I and the 1.3 kb fragment was isolated. These two fragments were ligated to yield phagemid pML. A unique *Cla*I site was created in phagemid pML by site-

directed mutagenesis with oligonucleotide JS21 (CAAGGACCATCGATT ATGAAA) to yield phagemid pML305.

The sequence coding for S15–Pro_3 was inserted into phagemid pML305 as follows. Phagemid pML305 was digested with *Cla*I and *Eco*RI, and the 5.1 kb fragment was isolated. Oligonucleotide RS92 (CCAACAAGGACCATCGATT ATG AAG GAA ACC GCT GCC GCG AAA TTC GAA CGT CAG CAC ATG GAC TCC CCG CCG CCC ATC GAG GGT AGG CCT G) was annealed to oligonucleotide SR94 (AA TTC AGG CCT ACC CTC GAT GGG CGG CGG GGA GTC CAT GTG CTG ACG TTC GAA TTT CGC GGC AGC GGT TTC CTT CAT AATCGATGGTCCTTGTT). The resulting cassette was digested with *Cla*I, and then ligated to the 5.1 kb fragment to yield phagemid pRS501.

The sequence coding for β-galactosidase was inserted into phagemid pRS501 as follows. Phagemid pRS501 was digested with *Stu*I and *Pst*I, and the 5.1 kb fragment was isolated. pMC1871 was digested with *Sma*I and *Pst*I, and the 3.1 kb fragment was isolated. These two fragments were ligated to yield phagemid pSG601 (Fig. II-2). Phagemid pSG601 directs the production of S15~Pro_3–Ile–Glu–Gly–Arg fused to β-galactosidase under the control of P_{tac}, a promoter induced by IPTG.

Site-directed mutagenesis

The GAC codon for Asp14 of the S-peptide portion of pSG601 was converted to the AAT codon for asparagine by site-directed mutagenesis with oligonucleotide NE21 (T CAG CAC ATG AAT TCC CCG CC) to yield phagemid pSG919. This phagemid directs the production of D14N

S15~Pro_3–Ile–Glu–Gly–Arg fused to β-galactosidase under the control of promoter P_{tac}.

Production of fusion protein

E. coli strain CSH27/pSG601 or CSH27/pSG919 was grown at 37 °C in rich media containing ampicillin (100 μg/mL) until the absorbance at 600 nm was 0.5. IPTG was then added to a final concentration of 1 mM. The culture was further incubated for 2 hr at 37 °C. The cells were harvested by centifugation, resuspended in 1/10 volume of lysis buffer, which was 10 mM phosphate buffer, pH 7.0 containing NaCl (0.5 M), and lysed by ultrasonication. Insoluble debris was removed by centrifugation to yield crude extract.

Detection of fusion protein

β-Galactosidase activity in crude extract was measured as described (Miller, 1972). Ribonuclease activity in crude extract was measured as described (Ipata and Felicioli, 1968) after adding various amounts of S-protein and incubating the mixture for 30 min on ice. The apparent amount of fusion protein present was deduced from activity assays by using a standard curve generated with authentic β-galactosidase or RNase A.

The fusion protein was also detected in the crude extract by using a gel that allows for assay of ribonuclease activity. This gel was a Laemmli gel containing poly(C) (0.3 mg/mL), which is a substrate for RNase S. After electrophoresis, the activity gel was washed with aqueous isopropanol

(20% v/v). The fusion protein was then activated for cleavage of the poly(C) by incubating the gel at 4°C in 10 mM Tris-HCl buffer, pH 7.5, containing S-protein (1 μg/mL) and bovine serum albumin (1 mg/mL). After 10 min, the gel was washed with 10 mM Tris-HCl buffer, pH 7.5, to remove excess S-protein. The gel was then incubated at 4°C with 10 mM Tris-HCl buffer, pH 7.5. After 30 min, the gel was stained with a solution of toluidine blue O (0.2 % w/v).

Preparation of S-Protein~Sepharose

S-Protein was coupled to CNBr-activated Sepharose 4B as described (Kato and Anfinsen, 1969). In the coupling reaction, 2 mg of S-protein was used per 1 g of freeze-dried resin. Alternatively, RNase A was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. In the coupling reaction, 2 to 5 mg of RNase A was used per 1 g of freeze-dried resin. The resulting resin was treated with subtilisin and washed extensively with acetic acid (50% v/v) to elute S-peptide from the resin.

Purification of fusion protein

Crude extract containing the fusion protein was mixed with 1/10 volume of swollen S-protein~Sepharose gel. After incubation for 30 min on ice, the solution was removed by filtration. The beads were washed 3 times, each with 5 volumes of lysis buffer at 4 °C. Elution was performed at 37 °C with 20 mM Tris-HCl buffer, pH 7.4, containing sodium chloride (0.5 M) or sodium thiocyanate (3 M).

Liberation of target protein

β-Galactosidase was liberated from the fusion protein by treating the purified fusion protein (native or denatured) with factor X_a according to the manufacturer's instructions, or by treating the purified fusion protein (native) with trypsin (0.02% w/w) on ice.

Peptide tags for a dual affinity fusion system

The DNA fragment encoding D14N S15~β-galactosidase in plasmid pSG919 was amplified by PCR and inserted into plasmid pET29b+ (Novagen) to yield expression vector pSGT12. In this vector, the phage T7 RNA polymerase promoter controls the production of a fusion protein in which β-galactosidase is sandwiched between D14N S15 and His_6 (Fig. II-8). The D14N S15~β-galactosidase~His_6 fusion protein was purified from *E. coli* extract by column chromatography on S-protein~Sepharose resin (Novagen) or Ni^{++} chelation resin (Novagen) or both. About 2 mL of each resin was used to purifiy up to 7 mg of the fusion protein.

Experimental for CHATER III

Design of gene for BS-RNase

A gene that codes for BS-RNase was designed using criteria expected to maximize the production of BS-RNase in *E. coli*. Four computer programs from the University of Wisconsin–Genetics Computer Group (Madison, WI) were used to expedite the design process. First, the amino

acid sequence of BS-RNase (Suzuki *et al.*, 1987) was reverse translated into an mRNA sequence by using the BACKTRANSLATE program with the codon frequency file based on highly expressed *E. coli* proteins. Then, the fraction of A/T base pairs near the 5'-terminus of the coding region was maximized so as to minimize the stability of the mRNA secondary structure in this region. Next, the mRNA sequence was adjusted such that the most stable secondary structure predicted by the FOLD program (Zuker and Stiegler, 1981) had the AUG start codon positioned in a loop region and the Shine/Dalgarno sequence relatively exposed. The secondary structure of this sequence was also predicted and examined with the program MFOLD (Zuker, 1989). Finally, restriction endonuclease recognition sites not found in the expression vector pET17b were incorporated by using the MAP/SILENT program. These sites will facilitate future engineering of the gene.

Synthesis of gene for BS-RNase

A gene that codes for BS-RNase was constructed by a method analogous to that of Guillemette *et al.* (1991). Briefly, twelve overlapping oligonucleotides coding for both strands of the BS-RNase gene were synthesized chemically. The 5' hydroxyl groups of ten of the oligonucleotides were phosphorylated with the chemical phosphorylation reagent, (2-cyanoethoxy)-2-(2'-*O*-4,4'dimethoxytrityloxyethylsulphonyl) ethoxy-*N*,*N*-diisopropylaminophosphine (Horn and Urdea, 1986), from Glen Research (Sterling, VA). The oligonucleotides at the two 5' termini of the gene were not phosphorylated. The individual oligonucleotides

were purified by denaturing polyacrylamide gel electrophoresis. The twelve oligonucleotides were annealed, and then ligated *in situ* by using T4 ligase. The assembled DNA fragment was inserted into pBluescript II SK(–), which had been previously digested with *Eco*RI and *Bam*HI and gel purified, to yield pBSR.

Expression of synthetic gene in E. coli

Plasmid pBSR was digested with *Nde*I and *Eco*RI. The 460-bp fragment containing the BS-RNase gene was isolated and inserted into expression vector pET17b, which had been previously digested with *Nde*I and *Eco*RI, to yield plasmid pSR1. The plasmid pSR1 was used to transform *E. coli* strain BL21(DE3)/pLysS, the expression host.

To express the gene for BS-RNase, *E. coli* strain BL21(DE3) /pLysS/pSR1 was grown at 37 °C in TB (0.5 L), containing ampicillin (100 μg/mL), until the absorbance at 600 nm was 4.0 O.D. units. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.4 mM, and the cells were grown overnight at 37 °C.

Purification of BS-RNase from E. coli

Cells were harvested by centrifugation, and then resuspended in 20 mM sodium phosphate buffer, pH 7.0, containing EDTA (10 mM). The resuspended cells were lysed by passage twice through a French press. Insoluble material containing BS-RNase was collected by centrifugation at 12,000g for 30 min, and then washed with the same buffer. The pellet was solubilized by adding 5 mL of 10 mM Tris-HCl buffer, pH 8.0, containing

GdnCl (6 M) and EDTA (10 mM). Reduced DTT was then added to a final concentration of 0.1 M. After incubation at room temperature for 3 h, the solution was dialyzed exhaustively versus 20 mM acetic acid. The solution was then centrifuged to remove material that had precipitated during dialysis. Active BS-RNase was generated by diluting the dialyzed solution to various protein concentrations in 0.1 M Tris-HOAc buffer, pH 8 (Parente and D'Alessio, 1985). The regeneration solution was incubated in an open container at room temperature to allow for air-oxidation. After 24 h, iodoacetamide was added to a final concentration of 10 mM to alkylate any remaining sulfhydryl groups. The appearance of active BS-RNase was monitored by measuring ribonuclease activity and sulfhydryl content, and by zymogram electrophoresis.

Purification of multimeric forms of BS-RNase from E. coli

The solution of oxidized enzyme was concentrated by using a Centriprep-10 concentrator (Amicon; Beverly, MA). The resulting concentrate (10 mL) was loaded on to a column (100 cm x 5 cm^2) of Sephadex G-75 superfine resin that had been equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was eluted at a rate of 10 mL/h with the same buffer, and fractions (6.4 mL) were collected. The fractions were assayed for A_{280} and ribonuclease activity. The apparent molecular weight of BS-RNase was determined by comparing its elution volume with that of proteins of known molecular weight, including aggregates produced by lyophilization of RNase A from aqueous acetic acid (50% v/v) (Crestfield *et al.*, 1962). The molecular weight standards were

RNase A monomer (13.7 kDa), RNase A dimer (27.4), RNase A trimer (41.0), ovalbumin (43.0), RNase A tetramer (54.7), and bovine serum albumin (67.0). The molecular weight of each multimer of BS-RNase was calculated by assuming that the logarithm of its molecular weight was inversely proportional to its elution volume minus the column void volume.

Purification of native BS-RNase from bull seminal plasma

Bull seminal plasma was obtained from American Breeders Service (De Forest, WI). BS-RNase was purified by Mono-S cation exchange and Superdex 75 gel filtration FPLC (Pharmacia, Uppsala, Sweden) as described (Tamburrini *et al.*, 1986).

Experimental for CHAPTER IV

Expression and Purification of BS-RNase

The plasmid pSR1, which contains the synthetic gene for BS-RNase under the control of the phage T7 promoter, was used to produce the protein in *E. coli* strain BL21(DE3)/pLysS. Plasmids pLSR12 and pLSR119 were used to produce H12D BS-RNase and H119D BS-RNase, respectively. Folding/air oxidation was performed as described above, or in the presence of glutathione (3.0 mM oxidized, 0.6 mM reduced). The resulting protein solutions were concentrated by ultrafiltration on an Amicon YM10 membrane, and the concentrate was loaded onto an FPLC HiLoad^{TM}26/60

Superdex^{TM}75 gel filtration column that had been equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing NaCl (0.1 M). The column was eluted with the same buffer, and the fractions (4.0 mL) collected were assayed for A_{280} and ribonuclease activity.

Site-Directed Mutagenesis

Plasmid pSR1 was digested with *Xho*I and *Xba*I, and the 460-bp fragment containing the BS-RNase gene was isolated after electrophoresis in an agarose gel. This fragment was inserted into expression vector pET22b, which had been digested with *Xba*I and *Xho*I and isolated after electrophoresis in an agarose gel, to yield phagemid pLSR1. Oligonucleotide HD2.33 (AGAGTCCATGTCTTGTCTTTCAAACTTAGC AGC) was used to change the CAC codon of His12 to the GAC codon of Asp. Oligonucleotide HD9.46 (ATCTGCAGAATTGATTAAACAGAAGC GTCGAAGTCAACTGGAACGG) was used to change the CAC codon of His119 to the GAC codon of Asp. The resulting plasmids, pLSR12 and pLSR119, were used to produce H12D BS-RNase and H119D BS-RNase, respectively.

NMR Spectroscopy and Mass Spectrometry

Samples of BS-RNase were prepared and analyzed by NMR spectroscopy as described previously for RNase A (Markley, 1975). NMR spectroscopy was performed on a Bruker AM500 instrument at the National Magnetic Resonance Facility at Madison (NMRFAM) of the University of Wisconsin–Madison. Samples of BS-RNase prepared for

mass analysis were purified without iodoacetic acid treatment after folding/air oxidation, and the purified proteins were dialyzed exhaustively against 20 mM acetic acid. Electrospray ionization–mass spectrometry was performed at the Mass Spectrometry Facility (MSF) of the University of California, San Francisco.

Sulfhydryl Group Content

BS-RNase was folded and air oxidized by incubation of a 0.7 mg/mL solution for 20 h in an open container at room temperature. Iodoacetic acid was then added to a final concentration of 10 mM, and the resulting solution was incubated for 30 min. The two dimeric forms of BS-RNase were purified to homogeneity by repetitive gel filtration chromatography. To determine the fraction of cysteine residues that are carboxymethylated, these two forms were subjected to amino acid analysis at the Protein/Nucleic Acid Shared Facility of the Medical College of Wisconsin. The efficiency of carboxymethylation was determined by titration of carboxymethylated protein with DTNB in a solution containing guanidinium hydrochloride (6 M) (Creighton, 1989).

Experimental for CHAPTER V

Site-directed mutagenesis

The construction of phagemid expression vector pLSR1 was described above. Oligonucleotide C31S.41 (ACACTTACCTTGGGTCATC

TTTCTACAAGACATCATCAAGT) was used to change the TGT codon of Cys31 to the TCT codon of serine. Oligonucleotide C32S.39 (AC ACTTACCTTGGGTCATCTTTCTAGAACACATCATCAA) was used to change the TGT codon of 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. Plasmid pSRA10 was constructed by using oligonucleotide AA.45 (GTAGTTGGAAGAGCTAGCTGCGGAGGTACTA GAGTCCATGTGTTG) to produce hybrid ribonuclease SRA-I.

Production and purification of wild-type and mutant BS-RNases

Wild-type and mutant BS-RNases were produced in *E. coli* and partially purified as described above. Folding/oxidation was performed as described (de Nigris *et al.*, 1993) with minor modifications. Denatured and reduced protein was oxidized for 24 h at a concentration of 0.7 mg/mL in 0.1 M Tris-HOAc 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 an FPLC HiLoad^{TM}26/60 Superdex^{TM}75 gel filtration column that had been equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing NaCl (0.1 M). Fractions corresponding to monomeric BS-RNase were collected and concentrated. Glutathione was removed by selective reduction with a 5-fold molar excess of dithiothreitol, and the resulting protein was air-oxidized by dialysis for 24 h versus 0.1 M Tris-HCl buffer, pH 8.0. The dialyzed protein was concentrated and subjected to gel filtration chromatography as described above. Fractions corresponding

to dimeric BS-RNase were collected, and buffer was changed to 0.1 M Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) for the interconversion assay or to 10 mM sodium phosphate buffer, pH 7.0, containing NaCl (0.1 M) for the antitumor, aspermatogenic, and immunosuppressive activity assays. The concentration of all forms and mutants of BS-RNase was determined by using an absorption coefficient of $\varepsilon^{1.0\%}_{1\ cm} = 4.65$ at 278 nm (D'Alessio *et al.*, 1972).

Purification of **M=M** *and* **MxM**

Wild-type BS-RNase was purified to >95% homogeneity from bull seminal fluid by S-Sepharose cation exchange and gel filtration chromatography. The purified enzyme was selectively reduced, and then subjected to gel filtration chromatography to separate the noncovalent dimer (**NCD**) and monomer (**M**). The **MxM** form was prepared by air oxidation of **NCD**, and purified by gel filtration chromatography. Similarly, the **M=M** form was prepared by air oxidation of **M**, and purified by gel filtration chromatography. This method of purification yielded **MxM** and **M=M** that were >90% free of the other form, as judged by gel filtration chromatography. The purified **MxM** and **M=M** forms were stored at –70 °C, at which temperature their interconversion was undetectable.

In the biological assays, ribonucleases labeled "wild-type", "**MxM**", and "**M=M**" were prepared from bull seminal fluid, and ribonucleases labeled "C31S" and C32S" were **M=M** forms prepared from *E. coli*. In the conversion assays, the three **M=M** forms were from *E. coli* and the wild-

type **M**x**M** form was from bull seminal fluid. Wild-type BS-RNase prepared from bull seminal fluid and that from *E. coli* (which has an additional *N*-terminal methionine residue) had identical enzymatic activity (CHAPTER III) and antitumor activity (data not shown).

Conversion of **M=M** *and* **M**x**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 reduced dithiothreitol (56 μg; 360 nmol) such as to reduce only the intersubunit disulfide bonds (D'Alessio *et al.*, 1975). The fraction of **M=M** that had been converted to **M**x**M** was determined by selective oxidation and gel filtration chromatography as described above. The % **M=M** was determined by integration of the gel filtration profile obtained at 280 nm.

Effect of cyclophilin on conversion of **M**x**M** *to* **M=M**

The purified **M**x**M** form of wild-type BS-RNase (1.0 mg) was incubated at 37 °C with or without cyclophilin (0.1 mg) in 0.1 M Tris-HCl buffer, pH 8.0, containing EDTA (1 mM). After 1 h, the fraction of **M**x**M** that had been converted to **M=M** was determined by selective reduction and gel filtration chromatography as described above.

Antitumor activity assay

The effect of various ribonucleases on the growth of human leukemic cell lines K-562 and ML-1 was assessed as follows. Cultures (0.2

mL) were established in microtitration plates and cultivated at 37 °C in RPMI 1640 medium supplemented with inactivated human serum (20% v/v) under a humidified atmosphere containing CO_2 (5% v/v). A known concentration of RNase was added to each of 3 cultures. After 3 days, the ability of cells to proliferate was assessed by measuring the incorporation of (^{3}H)thymidine into newly synthesized DNA. Results were compared to untreated cells.

Aspermatogenic activity assay

The effect of various ribonucleases on the production of sperm in mice was assessed as follows. The left testes of CBA mice (5 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 x testes weight/body weight), and the width of spermatogenic layers. Results were recorded as the mean ± standard error of the mean (SEM), and compared to the untreated right testes of the same mice.

Immunosuppressive activity assay

The effect of various RNases on the growth of lymphocytes stimulated by MLC was assessed as follows. Lymphocytes were isolated with a Ficoll-pague solution gradient from the difibrinated blood of 2 unrelated humans. The 2 preparations were mixed 1:1, and the resulting

MLC was grown as described in the antitumor activity assay. A known concentration of RNase was added to each of 3 cultures. After 6 days, the ability of treated cells to proliferate was assessed by measuring the incorporation of (^{3}H)thymidine into newly synthesized DNA. Results were compared to untreated cells. The assay was also performed on human lymphocytes stimulated with either phytohemagglutinin or concanavalin A.

Experimental for CHAPTER VI

Oligonucleotides used for mutagenesis

The DNA sequences of the oligonucleotides used for mutagenesis to construct the hybrid proteins, SRA-I to SRA-V, are as follows (See Table VI-2): AA.45 (GTAGTTGGAAGAGCTAGCTGCGGAGGTACTAGAGTCC ATGTGTTG), HC.55 (GTGAACGAAAGTGTTTACTGGCTTACAACGAT CTTTAGTCAGCTTTCTACAACAC), SP.39 (GGTTTGACCGTTCTTGCAT GCAACATTCTTTTGGCTGCA), VE.36 (CAACAATGATGTGCTTGTTAG CTTGAGTGGTCTTGT), RS.42 (TCTACAGTCAGTGATGCTCATAGTAC TCTTAGATTGGTAACA), and SB.48 (AAGTGAACTGGAACGTATGGG TTACCTTCGCATGCAACAATGATGTGC).

Enzyme kinetic studies

The cleavage of UpA was monitored with an adenosine deaminase coupled assay (Ipata and Felicioli, 1968). The $\Delta\varepsilon$ for this reaction was -6000

$M^{-1}cm^{-1}$ at 265 nm. Assay conditions were as described previously (delCardayré and Raines, 1994). The values for k_{cat}, K_m, and k_{cat}/K_m were determined from initial velocity data with the program HYPERO (Cleland, 1979).

Biological assays

The antitumor and immunosuppressive acivities were assayed as described above. To assay for embryotoxicity, bovine embryos were grown at 37 °C in MEMD medium supplemented with fetal calf serum (20% v/v). The mortality of the embryos was assessed for 3 days after the addition of a ribonuclease (10 μg/mL).

Determination of protein concentration

The concentration of all hybrids except for SRA-V was determined by using an absorption coefficient of $\varepsilon^{1.0\%}{}_{1\ cm} = 4.65$ at 278 nm. The concentration of SRA-V, which has an additional Tyr residue, was determined by assuming that $\varepsilon^{1.0\%}{}_{1\ cm} = 5.67$ (= 4.65 + 1.02, the latter term was calculated by the method of Gill and von Hippel (1989).).

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A dissertation entitled

PROTEIN ENGINEERING TO EXPLOIT AND EXPLORE

BOVINE SECRETORY RIBONUCLEASES

submitted to the Graduate School of the
University of Wisconsin-Madison
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

by

JIN-SOO KIM

Degree to be awarded: December 19___ May 19___ August 19 94

Approved by Dissertation Readers:

Major Professor

July 28, 1994
Date of Examination

Dean, Graduate School