A misfolded but active dimer of bovine seminal ribonuclease

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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 M×I, where × 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]BS-RNase and [H119D]BS-RNase) prepared by site-directed mutagenesis. This activity came from a dimer (M×I) 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 M×I, respectively. Adventitious oxidation can thus lead to the formation of a misfolded but active enzyme (M×I).

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 of RNA to form a 2′,3′-cyclic phosphodiester intermediate and the hydrolysis of this cyclic intermediate to form a 3′-phosphomonoester (Thompson et al., 1994). 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. 1. 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, intrasubumit disulfide bonds form, and the oxidized protein folds to pro-

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Abbreviations. BS-RNase, bovine seminal ribonuclease; CM-Cys, carboxymethyl cysteine; Nbs$_2$, 5,5′-dithiobis(2-nitrobenzoic acid); RNase A, bovine pancreatic ribonuclease A; S15, residues 1−15 of RNase A; S-peptide, residues 1−20 of RNase A; S-protein, residues 21−124 of RNase A.
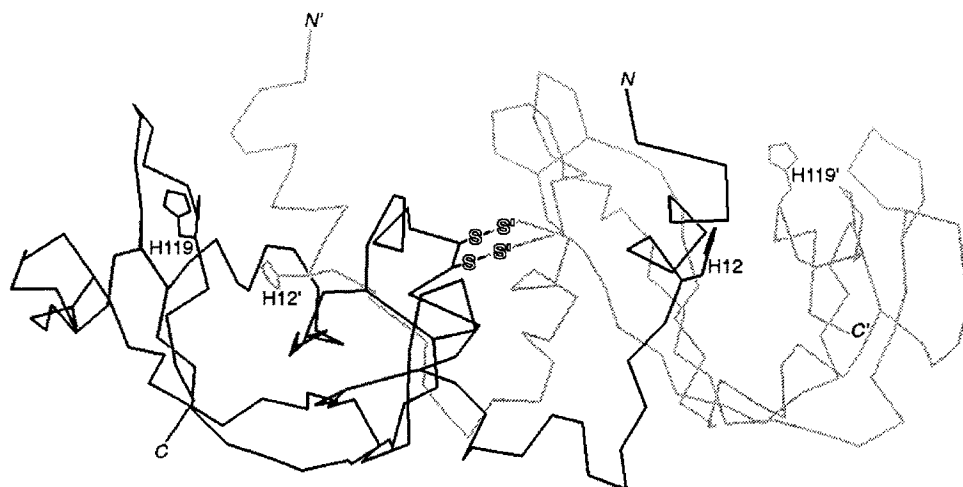
Enzyme. Ribonuclease (EC 3.1.27.5).

duce native monomer, designated as M. Disulfide bonds then form between two monomers to yield M = M. Finally, M = M equilibrates slowly (that is, in days) with M×M. At equilibrium, the ratio of M×M to M = M is approximately 2:1. The distinct conformational transitions of BS-RNase makes this protein an intriguing system with which to study the formation of quaternary structure.

BS-RNase 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 (Dostál and Matoušek, 1973; Souček et al., 1986; Tamburrini et al., 1990; D'Alessio et al., 1991; Laccetti et al., 1992). Form M, like the two dimers, is a potent ribonuclease. Yet, many of the unusual enzymic and biological properties of dimeric BS-RNase (D'Alessio et al., 1991) are absent from the monomeric form and are present to different extents in M×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* (Kim and Raines, 1993a). 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. Here, we report on the characterization of this third form, and on the relationship between the three forms and one additional inactive form.

Fig. 1. Structure of the crystalline M×M form of BS-RNase (Mazzarella et al., 1987). The chains (α-carbons only) are drawn in black or gray ('). The active-site histidine residues, intersubunit disulfide bonds, and N-termini and C-termini are indicated.

EXPERIMENTAL PROCEDURES

Materials

Expression vector pET22b and *E. coli* strain BL21(DE3)/pLysS were from Novagen. Reagents for DNA synthesis were from Applied Biosystems except for acetonitrile, which was from Baxter Healthcare. Restriction endonucleases and T4 DNA ligase were from Promega. T7 DNA polymerase was from New England Biolabs. Bacto tryptone and Bacto yeast extract were from Difco. Ampicillin (sodium salt) was from International Biotechnologies. 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). Poly(cytidylic acid), [poly(C)], was from Midland Reagent. RNase A (type III-A) and ribonuclease S-protein (residues 21−124 of RNase A) were from Sigma Chemical. Ribonuclease S15 (residues 1−15 of RNase A) was synthesized by Operon Technologies. All other chemicals and reagents were of commercial reagent grade or better, and were used without further purification.

Expression and purification of BS-RNase

The synthetic gene for BS-RNase was expressed in *E. coli* as described (Kim and Raines, 1993a). 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 (Kim and Raines, 1993a), or in the presence of glutathione (3.0 mM oxidized, 0.6 mM reduced) or EDTA (1.0 mM). The resulting protein solutions were concentrated by ultrafiltration on an Amicon YM10 membrane, and the concentrate was loaded onto an FPLC HiLoad™26/60 Superdex™75 gel-filtration column that had been equilibrated with 50 mM sodium acetate, pH 5.0, containing 0.1 M NaCl. The column was eluted with the same buffer, and the fractions 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

with a GENECLEAN II kit from BIO101 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. Site-directed mutagenesis was performed by the method of Kunkel (1987). Oligonucleotide HD2.33 (AGAGTCCATGTCTTGTCTTTCAAACTTAGC-AGC) was used to change the CAC codon for His12 to the GAC codon for Asp. Oligonucleotide HD9.46 (ATCTGCA-GAATTGATTAAACAGAAGCGTCGAAGTCAACTGG-AACGG) was used to change the CAC codon for His119 to the GAC codon for 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 5,5'-dithiobis(2-nitrobenzoic acid)

(Nbs₂) in a solution containing 6 M guanidinium hydrochloride (Creighton, 1989).

Other methods

Ribonuclease activity was assayed by the method of Kunitz (1946). Protein concentration was determined as described (Kim and Raines, 1993a). DNA oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer by using the β-cyanoethyl phosphoramidite method (Sinha et al., 1984). Other manipulations of DNA were performed as described (Ausubel et al., 1989). Ultraviolet and visible absorbance measurements were made on a Cary 3 spectrophotometer equipped with a Cary temperature controller. PAGE was performed in the presence of SDS (0.1%, mass/vol.) according to Ausubel et al. (1989). Gels were fixed and stained by washing with aqueous methanol (40%, by vol.), containing acetic acid (10%, by vol.) and Coomassie brilliant blue (0.1%, mass/vol.). Molecular-mass standards for SDS/PAGE were from Bio-Rad: phosphorylase B (97.4 kDa unstained, 106 kDa prestained), serum albumin (66.2 kDa, 80.0 kDa), ovalbumin (45.0 kDa, 49.5 kDa), carbonic anhydrase (31.0 kDa, 32.5 kDa), trypsin inhibitor (21.5 kDa, 27.5 kDa), and lysozyme (14.4 kDa, 18.5 kDa). Zymogram electrophoresis was performed as described (Kim and Raines, 1993b, 1994), except that ribonuclease S-protein was omitted.

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. 2). 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 M×I, 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 by Kim and Raines (1993a). Their designation here as M×I, M = M and M is based on data given below. Another form, M×M, was purified with M = M. The rate of interconverion between M = M and M×M is slow, and M×M represented less than 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 M×I and I migrated more slowly and diffusely than did M = M and M, respectively, during SDS/PAGE analysis (Fig. 2). 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).

Site-directed mutagenesis

BS-RNase has two histidine residues in its active site (Fig. 1). 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. 3).
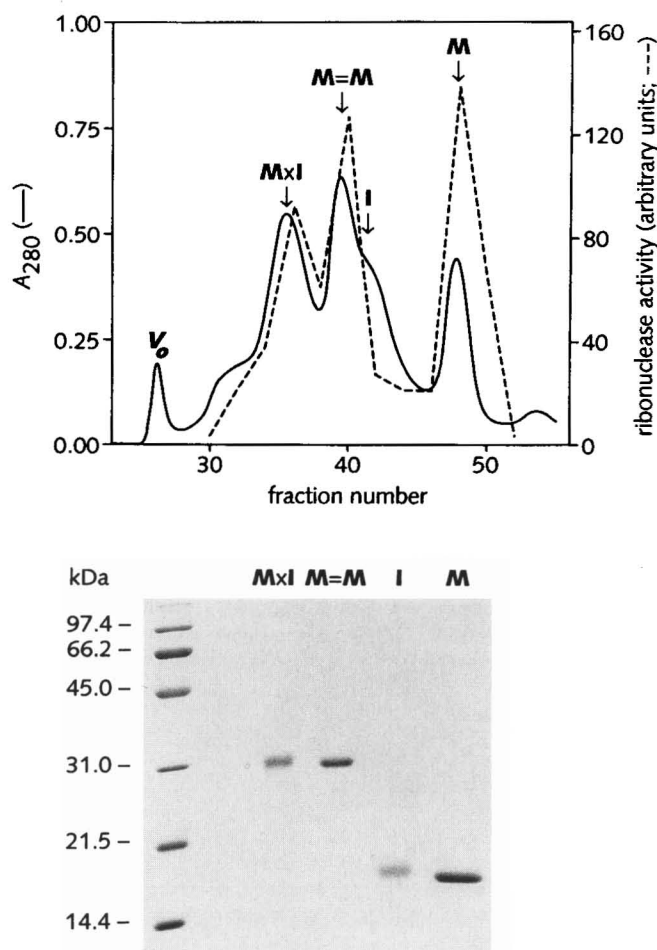

Fig. 2. **Purification and analysis of different forms of BS-RNase produced in *E. coli*.** Denatured and reduced BS-RNase from *E. coli* was folded and air oxidized. BS-RNase forms were purified by gel filtration through Superdex™75 (above). Ribonuclease activity is in units of $(\Delta A_{300}/\text{min})$ (-1.0×10^4). Purified BS-RNase forms were analyzed by SDS/PAGE (below).

Unlike the wild-type enzyme, which formed fully active monomer and dimer after 20 h of folding/air oxidation (Fig. 3), the two mutant enzymes showed no enzyme activity after 20 h of folding/air oxidation (Fig. 3). 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. 3). The 45-kDa protein (Fig. 3) corresponds to a BS-RNase multimer observed previously (Fig. 3 in Kim and Raines, 1993a). As shown in Fig. 2, the dimer region on a SDS/polyacrylamide gel contains two forms of active BS-RNase: M×I and M = M. Gel filtration and SDS/PAGE analyses (data not shown) revealed that the ribonuclease activity of the mixture of mutant proteins was from M×I.

NMR analysis

BS-RNase and bovine pancreatic ribonuclease (RNase A) each have four 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).
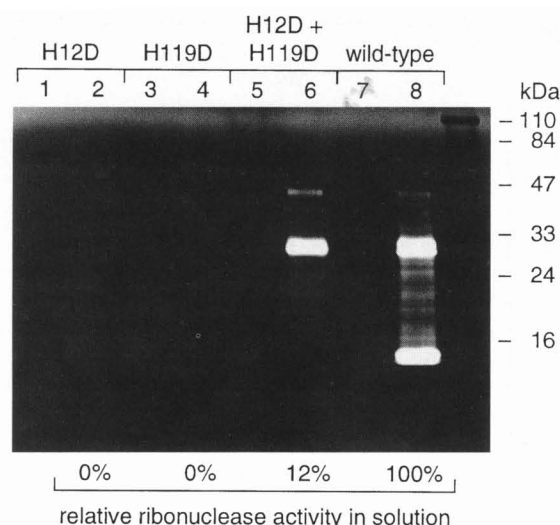
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Fig. 3. Zymogram analysis of wild-type and mutant BS-RNases. Folding/air oxidation was performed with [H12D]BS-RNase (lanes 1 and 2), [H119D]BS-RNase (lanes 3 and 4), an equimolar mixture of [H12D]BS-RNase and [H119D]BS-RNases (lanes 5 and 6), and 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 non-reducing SDS/poly-acrylamide gel containing poly(C). Ribonuclease activity is indicated by a clear band after staining with Toluidine Blue O. Relative ribonuclease activities assayed in solution before SDS/PAGE are also shown.
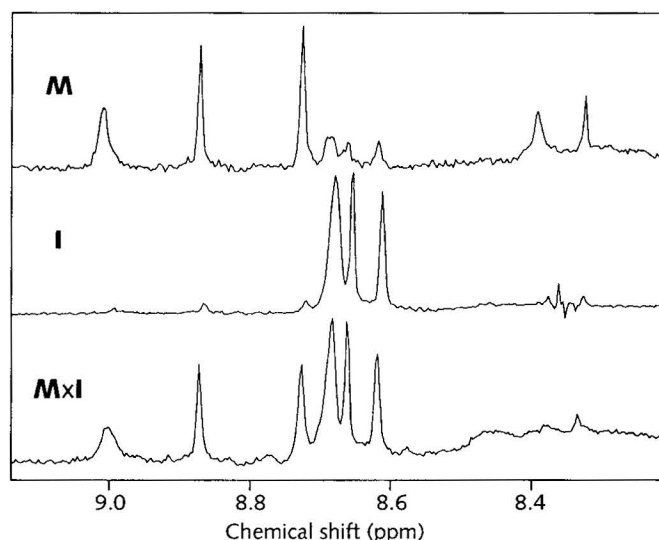

Fig. 4. Portion of ^{1}H-NMR spectrum of three forms of BS-RNase. Downfield region is shown for M (top), I (middle), and M×I (bottom).

Each form of BS-RNase described above was purified and used for ^{1}H-NMR studies (Fig. 4). The spectrum of M contained five 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 three 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 M×I was the sum of the spectra of M and I, suggesting that M×I 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/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 M×I dimer contained 1.16 CM-Cys residues. Titration of iodoacetate-treated proteins with Nbs$_2$ 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 M×I 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 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, pH 8.5. The resulting solutions were incubated at room temperature for 24 h, then treated with iodoacetate. Gel-filtration chromatography of the resulting proteins indicated that more than 60% of M had been converted to M = M. However, no interconversions between M, I and M×I 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 enzymically 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 enzymic activity (Richards, 1955; Kim and Raines, 1993b). The truncated RNase S formed from S-protein and the first 15 residues of S-peptide (S15) has the same enzymic activity and dissociation constant as does RNase S (Potts et al., 1963; Kim and Raines, 1993b). Although the I form of BS-RNase has little enzymic 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 1, substantial enzymic activity was generated by activation with S-protein but not with S15.

Table 1. Activation of I with S15 or S-Protein. Ribonuclease activity was assayed by the method of Kunitz (1946).

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

[a] Ribonuclease S15 was added in 10-fold molar excess.
[b] Ribonuclease S-protein was added in twofold molar excess.

Mass spectrometry

The molecular masses M and I, determined by electro-spray-ionization mass spectrometry, were 13733 Da and 13749 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 13734 Da. This value is calculated for monomeric protein with four intramolecular disulfide bonds and all sidechains uncharged.

DISCUSSION

BS-RNase is known to fold first into a monomer (M) then into a dimer (M = M; Parente and D'Alessio, 1985). This dimer converts slowly to another dimer (M×M) 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 enzymic 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. 3), as expected from the alteration of a residue that is critical for catalysis (Fig. 1; 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]BS-RNase and [H119D]BS-RNase. 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. Previous work in our laboratory demonstrated the existence of a form of BS-RNase that migrates as a trimer during gel filtration (Kim and Raines, 1993a). 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. 4) 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 M×I, 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

Scheme 1. Proposed pathway for the production of quaternary structure in BS-RNase.
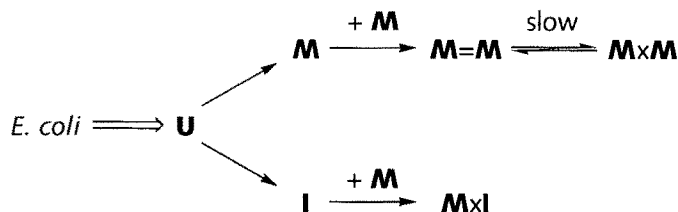
produces I, but not M. Based on these findings, we suggest the pathway in Scheme 1 for the production of quaternary structure in BS-RNase. In this pathway, the reduced and denatured BS-RNase (U) folds in the presence of an oxidant into a monomeric state, M or I, each of which can combine with M to form M = M or M×I, 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 M×I relative to M×M (Scheme 1) 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)$_2$OH], 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 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.

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