Dissociation of Bovine Seminal Ribonuclease into Catalytically Active Monomers by Selective Reduction and Alkylation of the Intersubunit Disulfide Bridges†

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ABSTRACT: The hypothesis previously advanced that interchain disulfide bridges link the two identical subunits of bovine seminal ribonuclease BS-1 has been confirmed. The sedimentation rate and the electrophoretic mobility of the protein are not affected by denaturing agents unless thiol reagents are present in the denaturation mixtures. Reduction under controlled conditions results in the immediate cleavage of only 2 disulfide bonds out of 10 present in the dimeric protein. Under these conditions, and the results do not change when partial reduction is followed by S-alkylation, 30% of the protein dissociates, while the remaining is found to consist of a dimeric species easily dissociable by denaturing agents without addition of thiol reagents. This indicates that the dimeric structure of seminal ribonuclease is maintained not only by disulfide bridges, but also by noncovalent forces. The protein derivative prepared by selective reduction and alkylation has been identified as monomeric bis-*S*-carboxymethylcysteine-31,32-ribonuclease BS-1. This is on the basis of the characterization of the ^{14}C-labeled S-carboxymethylated peptides isolated from a thermolytic hydrolysate of the derivative prepared with iodo-2-[^{14}C]acetic acid. Monomeric, selectively alkylated ribonuclease BS-1 is stable and catalytically active. The importance of such a derivative is discussed both in the light of the recent studies on the biological actions of seminal ribonuclease and as the fourth component of an experimental system of ribonucleases consisting of two homologous dimers (bovine seminal ribonuclease BS-1 and dimerized bovine pancreatic ribonuclease A) and two homologous monomers (ribonuclease A and the monomeric derivative of ribonuclease BS-1).

Seminal ribonuclease (RNase)[1] BS-1 and pancreatic RNase A may be regarded as composing an interesting system for the study of the relationships between structure and function in enzymic proteins. While pancreatic ribonuclease is a single-chain protein with a molecular weight of 13,700 (see Richards and Wyckoff, 1971, for a review) the seminal ribonuclease has a dimeric structure and a molecular weight of 29,000 (D'Alessio et al., 1972a,b). However, the amino acid sequence of the subunit of RNase BS-1 is strictly homologous to the sequence of pancreatic RNase A (Leone et al., 1972, and manuscript in preparation). This structural relation between the two enzymes is reflected in their catalytic properties, which are apparently identical, except for a lower k_{cat} value of RNase BS-1 (Floridi et al., 1972), and for the ability of RNase BS-1 to degrade also double-stranded RNA and DNA–RNA hybrids (Libonati and Floridi, 1969; Taniguchi and Libonati, 1974).

The most relevant difference between the primary structures of the two proteins has been detected in two amino acid replacements at sequence positions 31 and 32, where a lysine and a serine of RNase A have been substituted in the sequence of RNase BS-1 subunit by two adjacent half-cystine residues. Half-cystine residues 31 and 32 are not linked to any of the other eight half-cystines present in the RNase BS-1 subunit, which are located in the subunit chain at sequence positions identical with those of the eight half-cystine residues of RNase A and which form disulfide pairs identical with those of RNase A (A. Di Donato and G. D'Alessio, manuscript in preparation). Recently, a dimeric peptide structure made up of two identical peptides linked by two disulfide bonds has been isolated from an enzymic hydrolysate of RNase BS-1. The half-cystine residues involved in these bridges have been identified as the adjacent half-cystine residues 31 and 32 (Di Donato and D'Alessio, 1973).

These results and the independent observation that the disulfide bonds formed by half-cystine residues 31 and 32 are extremely reactive to reductive cleavage by thiol reagents (Malorni et al., 1972) led us to propose for RNase BS-1 a quaternary structure where the two adjacent half-cystine residues 31 and 32 form interchain disulfide bridges with the corresponding half-cystine residues of the other subunit, thus being responsible for the dimeric structure of the protein (Malorni et al., 1972; Di Donato and D'Alessio, 1973).

The results of the experiments reported in the present paper strongly support this hypothesis, as they show that a necessary and sufficient condition for promoting dissociation of RNase BS-1 is the selective reduction of the interchain disulfide bridges formed by half-cystine residues 31 and 32. Furthermore, a procedure is described for the preparation of a monomeric derivative of the protein, which is stable and catalytically active. The possibility is then discussed that the availability of such a derivative and, on the other hand, of dimeric derivatives of RNase A (Crestfield et al., 1962; Bartholeyns and Moore, 1974) can greatly increase the utility of the protein system proposed above.

Materials and Methods

Proteins. RNase BS-1 was prepared and checked for ho-

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[1] Abbreviations used are: RNase A, bovine pancreatic ribonuclease (EC 3.1.4.22); RNase BS-1, bovine seminal ribonuclease; Gdn · HCl, guanidine hydrochloride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); HSEtOH, 2-mercaptoethanol.

mogeneity as previously described (D'Alessio et al., 1972a). RNase A was Sigma type XII-A. Cytochrome *c* (from horse heart, type III), pepsin and α-chymotrypsinogen A were also obtained from Sigma. Thermolysin, a Calbiochem product, was treated according to Guy et al. (1971) before use.

Analytical Ultracentrifugation. Sedimentation analyses were performed in a Beckman Model E analytical ultracentrifuge equipped with electronic speed control. Samples were dissolved in and then dialyzed for 72 hr against 0.01 *M* sodium phosphate (pH 7), containing 0.1 *M* NaCl, 2 *M* Gdn · HCl (Mann Lab, Ultra Pure), with or without 0.01 *M* 1,4-dithiothreitol (Calbiochem). The centrifuge was operated with an An-H rotor at 59,780 rpm and 25°. Values of s_{obsd} were corrected to $s_{25,w}$ according to Schachman (1957). Viscosity and density factors for Gdn · HCl solutions were estimated from the data of Kawahara and Tanford (1966). The value of partial specific volume calculated for native RNase BS-1 (D'Alessio et al., 1972a) was used. Protein concentrations were 0.5%.

Gel Electrophoresis. Electrophoreses on polyacrylamide gel at pH 4.5 were carried out as described elsewhere (D'Alessio et al., 1972a).

Electrophoreses on polyacrylamide gel in sodium dodecyl sulfate were performed according to Weber and Osborn (1969). Denaturation of the proteins was carried out as described by Dunker and Ruekert (1969) with 1% sodium dodecyl sulfate (British Drug House, specially pure), 4 *M* urea (British Drug House, Aristar grade), and 1% HSEtOH (Fluka), where appropriate HSEtOH was omitted. Cytochrome *c* treated with sodium dodecyl sulfate was added to each sample before the electrophoresis was run and mobilities were calculated relative to the mobility of the cytochrome band. The molecular weight values for the standard proteins used for constructing the calibration curve were those listed by Weber and Osborn (1969), except for pepsin; in this case the value proposed by Mills and Tang (1967) was used.

Methods of Measuring Disulfide Cleavage. The extent of reaction of RNase BS-1 with 1,4-dithiothreitol was measured by two procedures.(a) Aliquots of 20 μl, containing 0.30 mg of protein, were withdrawn from the reduction mixture, incubated for 60 min with 0.2 ml of 0.6 m*M* iodoacetic acid, and then quenched in 2 ml of ice-cold 0.3% HCl in acetone (v/v). After a 30-min incubation in the cold, the precipitated protein was collected by centrifugation (15 min at 15,000*g*), washed twice with the acetone–HCl mixture, and then dissolved in water and quantitatively transferred to a hydrolysis vial. Carboxymethylcysteine content was estimated from duplicate amino acid analyses on the basis of the content of several amino acid residues in the protein. Iodoacetic acid solutions were prepared with recrystallized iodoacetic acid dissolved in water and neutralized.

(b) Aliquots (100 μl) of reduction mixture, containing 0.75 mg of protein, were incubated with $NaAsO_2$ for 5 min and then assayed with Nbs_2, following the procedure suggested by Zahler and Cleland (1968). Within 2 to 3 min from addition of Nbs_2 the curves of absorbance vs. time became linear and allowed accurate extrapolations.

Paper Electrophoresis and Chromatography. High-voltage paper electrophoresis was performed in a Gilson apparatus with pyridinium acetate buffers, at 3000 V for 50 min. Descending paper chromatography was carried out in 1-butanol–acetic acid–water–pyridine (30:6:24:20, v/v).

Radioactive Labeling of the Protein. Labeled S-carboxymethylated monomeric RNase BS-1 was prepared as described in the Results section using as alkylating agent iodo-2-[^{14}C]acetic acid (Amersham, 22.6mCi/mmol), taken to constant specific activity (1600 cpm/nmol) by repeated recrystallizations from chloroform after addition of nonlabeled substance.

Amino Acid Analysis. Samples of protein or of peptide were hydrolyzed for 20 hr at 110° in 6 *N* HCl according to Moore and Stein (1963). Amino acid analyses were performed with a Beckman M116 autoanalyzer.

Other Methods. Ribonuclease activity was assayed with yeast RNA and cytidine 2′,3′-cyclic phosphate (both purchased from Sigma) as described elsewhere (D'Alessio et al., 1972a; Floridi et al., 1972).

Sulfhydryl groups were titrated with Nbs_2 as described by Ellman (1959) using a molar extinction coefficient of 13,600 at 412 nm for calculations (Ellman, 1959).

The preparation of fully reduced and carboxymethylated RNase BS-1 was carried out in 7 *M* Gdn · HCl–0.1 *M* 1,4-dithiothreitol following a procedure suggested by E. Leone et al. (manuscript in preparation).

Results and Discussion

Effect of Thiol Reagents on Dissociation of RNase BS-1. We have previously reported that RNase BS-1 cannot be dissociated by denaturing agents such as urea and Gdn · HCl (Forlani et al., 1967). These experiments, however, were performed in the absence of thiol reagents. RNase BS-1 equilibrated with 2 *M* Gdn · HCl in 0.1 *M* 1,4-dithiothreitol was thus compared by analytical ultracentrifugation to the protein equilibrated with 2 *M* Gdn · HCl in the absence of 1,4-dithiothreitol. The schlieren patterns of the ultracentrifugation runs, presented in Figure 1, show that in the presence of the thiol reagent RNase BS-1 sedimented much more slowly (1.36 S) than in 2 *M* Gdn · HCl alone (2.80 S).

Identical results were obtained from an analogous experiment, where sodium dodecyl sulfate was used as denaturing agent. Previous experiments (D'Alessio et al., 1972b) of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, performed with the sodium dodecyl sulfate–protein complex prepared under standard conditions (i.e., with HSEtOH present in the denaturation mixture), had yielded a molecular weight value for the subunit of RNase BS-1 which corresponded to the value calculated from the amino acid sequence of the subunit chain (Leone et al., 1972). In the present study the protein was denatured with sodium dodecyl sulfate both in the presence and in the absence of HSEtOH in the denaturation mixture. The electrophoretic analysis on polyacrylamide gel in sodium dodecyl sulfate showed that the mobility of RNase BS-1 denatured with sodium dodecyl sulfate in the absence of HSEtOH was much lower than the value found for the subunit of RNase BS-1 (D'Alessio et al., 1972b, and Figure 2 of the present paper). A calibration curve was constructed with a set of standard proteins characterized by their single-chain structure and lack of sulfhydryl groups. No differences were detected in the electrophoretic mobility of any of these proteins (cytochrome *c*, RNase A, chymotrypsinogen, and pepsin) whether they were denatured with sodium dodecyl sulfate in the presence or, respectively, in the absence of HSEtOH. From such a curve (Figure 3) a molecular weight value could be calculated for RNase BS-1 treated with sodium dodecyl sulfate in the absence of the thiol reagent. It was found that

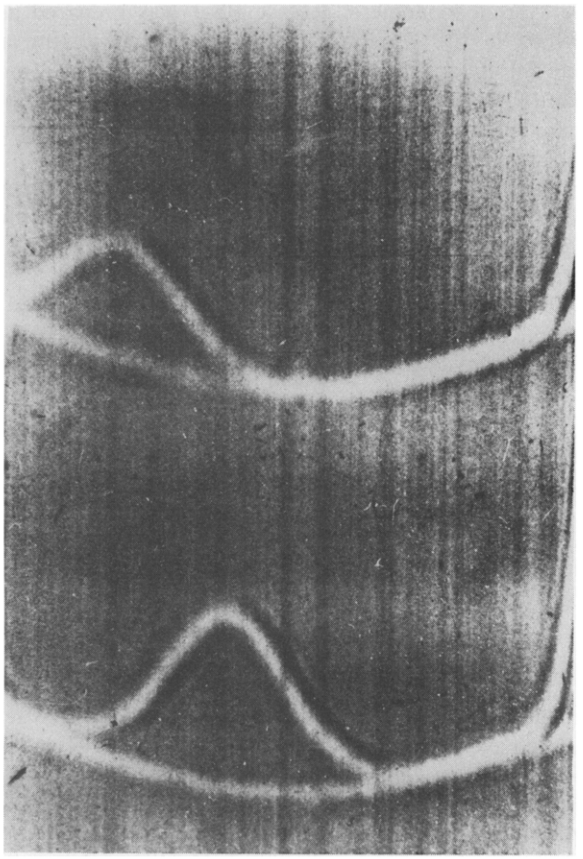

FIGURE 1: Schlieren pattern of RNase BS-1 in 2*M* Gdn · HCl in the presence (top) and in the absence (bottom) of 0.01 *M* 1,4-dithiothreitol. Photograph taken 96 min after reaching the rotor speed of 59,780 rpm at 25°.

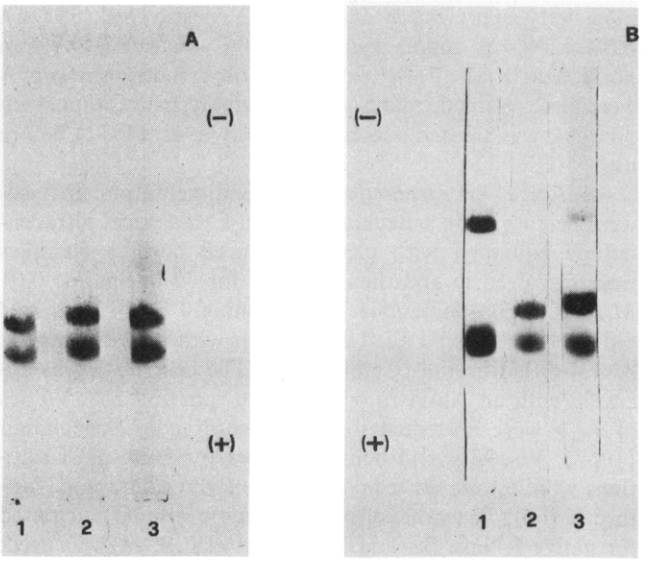

FIGURE 2: Electrophoretic patterns on polyacrylamide gels in sodium dodecyl sulfate of RNase BS-1 (gel 1) and of fraction M (gel 2) and fraction D (gel 3) eluted from Sephadex G-75: (A) in the presence of HSEtOH; (B) in the absence of HSEtOH. The fastest moving band in each gel is the band of the cytochrome *c* marker.

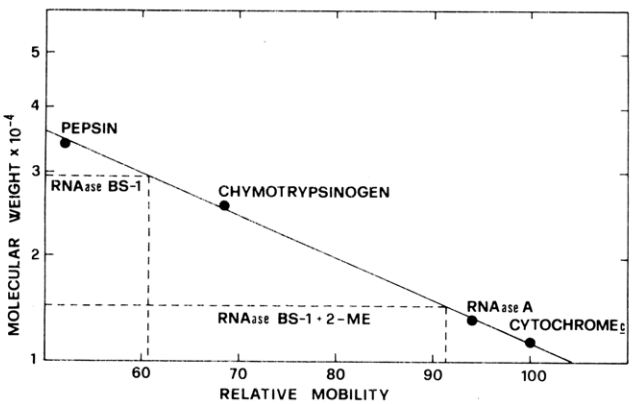

FIGURE 3: Relation of molecular weights to electrophoretic mobilities in sodium dodecyl sulfate–polyacrylamide gels of proteins denatured with sodium dodecyl sulfate in the absence of HSEtOH. The slope of the curve did not change significantly when the standard proteins were denatured with sodium dodecyl sulfate in the presence of HSEtOH (see text). The behavior of RNase BS-1 is shown both for the protein denatured in the absence and in the presence (RNase BS-1 + 2-Me) of 2-mercaptoethanol.

this value was virtually identical with the molecular weight value of the native protein.

The results of these experiments can be interpreted with several hypotheses: (i) they may suggest the presence of intersubunit disulfide bridges in RNase BS-1 or (ii) they may indicate that in the absence of a mercaptan reducing agent the protein is dissociated, but only partially unfolded by 2 *M* Gdn · HCl or 1% sodium dodecyl sulfate. The differences observed between the sedimentation rate and electrophoretic mobility values of the protein denatured in the presence of the reducing agent and the values observed for the protein treated with the denaturants alone would reflect, rather than a difference in size, a different frictional resistance, which is expected to increase, in a partially unfolded monomer, with respect to a fully reduced and denatured monomer. (iii) Finally, the reported results may also suggest that RNase BS-1 is not dissociated at all by the denaturing conditions employed, in the absence of a reducing agent, because the integrity of some intrachain disulfide bond(s) has a definite stabilizing effect on the quaternary structure of the protein.

It should be noted, however, that the sedimentation coefficient and the molecular weight calculated from the electrophoretic mobility of the protein treated with the denaturants in the absence of the reducing agent are strikingly coincident with the values found for the native protein. This observation would more easily fit into the first hypothesis, of a dimeric structure maintained by intersubunit disulfide bonds. According to this hypothesis, the tightly compact structure of the protein would be minimally, if at all, unfolded by the denaturing conditions employed, but addition of a reducing agent would promote dissociation through the cleavage of intersubunit cross-links. The decrease in size would consequently result in lower values of sedimentation rate and higher values of electrophoretic mobility in sodium dodecyl sulfate gel.

Differential Lability to Reductive Cleavage of Some Disulfide Bonds of RNase BS-1. Preliminary experiments (Malorni et al., 1972) had shown that when RNase BS-1, partially reduced with 1,4-dithiothreitol and alkylated with iodo-2-[^{14}C]acetic acid in Tris buffer, was subsequently fully reduced and alkylated with iodo-2-[^{12}C]acetic acid in 8 *M* urea, more than 80% of the radioactivity was found in the tryptic peptide containing the two adjacent half-cystine residues 31 and 32, with a specific radioactivity one order of magnitude higher than the activity of the other S-carboxy-

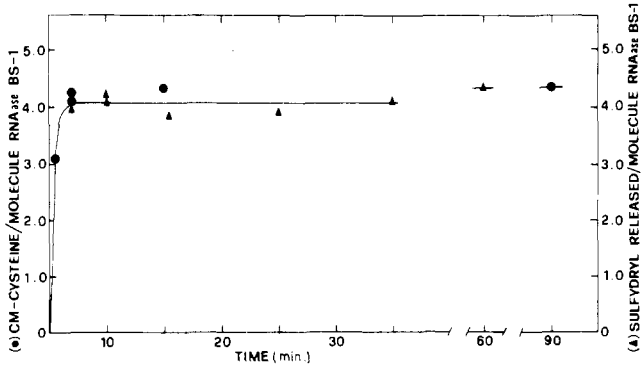

FIGURE 4: Disulfide bonds cleaved per molecule of RNase BS-1 incubated with 1,4-dithiothreitol, expressed as a function of time. The values plotted were obtained either from the number of carboxymethylcysteine residues determined after treatment of aliquots of the incubation mixture with iodoacetic acid (●) or from the number of sulfhydryl groups titrated with Nbs_2 in the presence of arsenite (▲).

methyl peptides. These results indicated a differential reactivity to a thiol reducing agent of the disulfide bonds involving half-cystine residues 31 and 32, and were consistent with the hypothesis of intersubunit disulfides in the protein.

To verify in a quantitative fashion the lability to reductive cleavage of some disulfide bonds of RNase BS-1, the effect of a mild treatment with 1,4-dithiothreitol was investigated as a function of time. Samples of RNase BS-1 were treated with a tenfold molar excess of 1,4-dithiothreitol (final concentration 2.5 m*M*) at 25° in 0.1 *M* Tris-HCl (pH 8.5). At the end of each reaction time the number of disulfides cleaved by 1,4-dithiothreitol was obtained either from the number of carboxymethylcysteine residues determined by amino acid analyses after the appropriate treatment with iodoacetic acid (see Materials and Methods), or from the number of sulfhydryl groups titratable with the Ellman reagent in the presence of arsenite (Zahler and Cleland, 1968). The results of the experiment are illustrated in Figure 4. Under the conditions employed, the initial reduction rate was quite fast, with two disulfide bonds cleaved per molecule of RNase BS-1 in less than 2 min. Then a plateau was rapidly reached and no more disulfides appeared to be sensitive to reduction up to 90 min of incubation. This indicated a marked differential reactivity to reductive cleavage of two disulfide bonds in the dimeric molecule of RNase BS-1.

It should be noted that the procedure proposed by Zahler and Cleland (1968) is not considered generally applicable to the determination of disulfide bonds of proteins. The fact that the method could be successfully applied to the present case was quite probably due not only to the absence of sulfhydryl groups in native RNase BS-1, but also to the lability of the disulfide bonds involved in the assay.

The picture which thus emerges when the results presented above are considered in conjunction with the results reported in the preceding section is that the dimeric structure of RNase BS-1 is easily dissociated when reductive cleavage of disulfide bonds takes place simultaneously to mild denaturation and that two of these bonds are very sensitive to reductive cleavage. If we include in the picture the finding that the other eight disulfide bonds (four per subunit) present in the dimeric molecule pair exactly as the four disulfide bridges of pancreatic RNase (A. Di Donato and G. D'Alessio, manuscript in preparation), and the isolation from a proteolytic digest of the native protein of a dimeric peptide structure made up of two identical peptides linked

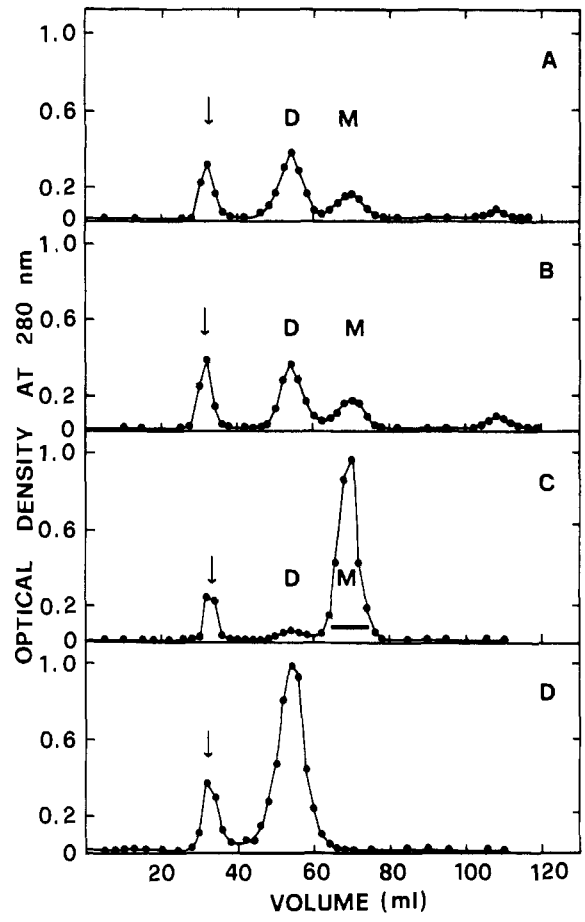

FIGURE 5: Gel filtration through Sephadex G-75 equilibrated with 0.1 *M* ammonium acetate (pH 5) of samples of RNase BS-1 treated as follows: (A) reduction for 20 min with a tenfold molar excess of 1,4-dithiothreitol; (B) reduction as above followed by carboxymethylation; (C) reduction and carboxymethylation as above followed by a gel filtration step through Bio-Gel P-2 equilibrated with 0.1 *M* ammonium acetate containing 5 *M* urea; (D) native RNase BS-1 filtered as the samples above, but with 5 *M* urea added to the equilibration buffer. The arrows indicate the Blue Dextran (Pharmacia) marker for the column void volume.

by two disulfide bonds, it appears evident that all the available information fits into the hypothesis that the dimeric structure of RNase BS-1 is maintained by two interchain disulfide bonds.

Preparation of Selectively S-Carboxymethylated Monomeric RNase BS-1. A final check to this hypothesis would then have been the isolation, after selective reduction of the interchain disulfides, of the monomeric subunit of the protein.

RNase BS-1, 20 mg/ml, was thus incubated under a nitrogen barrier with a tenfold molar excess of 1,4-dithiothreitol in 0.1 *M* Tris-HCl (pH 8.5). After 20 min of incubation at 25°, the incubate was filtered through a column of Sephadex G-75 (1.5 × 72 cm) equilibrated with 0.1 *M* ammonium acetate (pH 5). The elution profile, shown in Figure 5A, consisted of two protein peaks, eluted respectively at the elution volumes expected for RNase BS-1 (fraction D, 70% of total protein) and for its subunit (fraction M, 30% of total protein). When the protein eluted in each peak was analyzed with the Ellman reagent a value of two sulfydryl groups per subunit chain was found for both protein species. The experiment was then repeated, but this time, before gel filtration, the partially reduced protein was treated with iodoacetic acid (final concentration 17.5 m*M*) for 60 min in

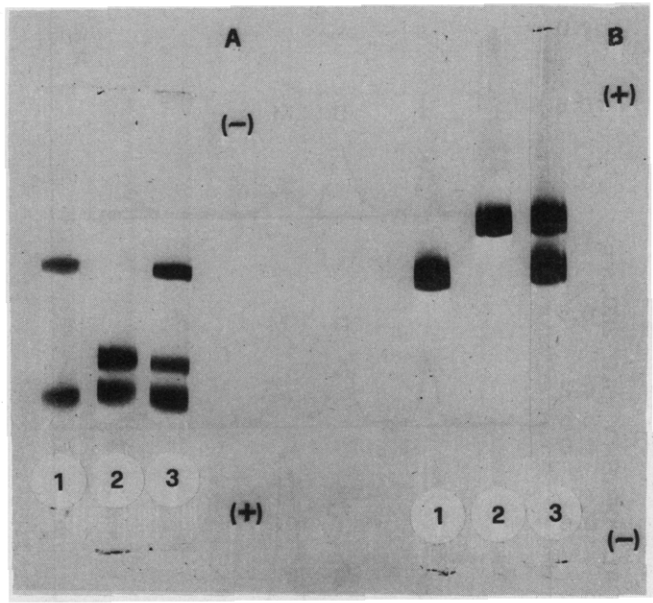

FIGURE 6: Electrophoretic patterns on polyacrylamide gels of RNase BS-1 (gel 1) and monomeric carboxymethylated RNase BS-1 (gel 2). Gel 3 is a sample prepared with a mixture of the two proteins: (A) electrophoresis in the presence of sodium dodecyl sulfate; the fastest moving band in each gel is the band of the cytochrome *c* marker; (B) electrophoresis at pH 4.5.

Table I: Amino Acid Compositions of RNase BS-1 and of S-Carboxymethylated (CM) Derivatives of the Protein.[a]

	Native Protein	Fully Reduced and CM Protein	Selectively Reduced and CM Protein
CM-cysteine		10.20	2.17
Lysine	14	13.87	14.40
Histidine	4	3.98	3.88
Arginine	4	4.08	4.02
Aspartic acid	11	11.10	10.85
Threonine	9	9.10	9.01
Serine	15	15.00	15.02
Glutamic acid	11	11.02	10.76
Proline	5	5.10	5.05
Glycine	6	5.96	6.13
Alanine	8	7.87	8.05
Valine	10	9.91	10.28
Methionine	5	4.46	4.45
Isoleucine	3	3.20	2.76
Leucine	2	2.25	2.04
Tyrosine	4	3.76	4.03
Phenylalanine	3	3.13	3.14

[a] The compositions are expressed as moles of residues per mole of protein subunit.

the dark. The elution profile virtually did not change, with the fraction D, eluted earlier, representing again about 70% of the total protein (Figure 5B). Amino acid analyses carried out on samples of each protein peak revealed a content of 5.3 and 4.01 carboxymethylcysteine residues per 29,000 g, respectively, in fraction D and fraction M.

This indicated that the inability to obtain a yield higher than 30% of apparently monomeric fraction M could not be ascribed to reoxidation of cleaved disulfides after the removal of 1,4-dithiothreitol. On the other hand the relatively high carboxymethylcysteine content of fraction D could suggest that reductive cleavage of some intrachain disulfide bonds had occurred, possibly followed by disulfide interchange reactions. This possibility, however, was found to apply only to a small amount of fraction D when samples of fractions M and D were analyzed by sodium dodecyl sulfate gel electrophoresis and the gels were scanned with a photodensitometer. Both fractions, like the untreated protein, gave a single band with a molecular weight of about 15,000 when HSEtOH was present in the sodium dodecyl sulfate denaturation mixtures (Figure 2A). Omission of HSEtOH (Figure 2B) did not change the electrophoretic pattern of fraction M, while fraction D was found to be heterogeneous, with about 90% of monomeric and 10% of dimeric protein.

These results indicated that partial reduction and carboxymethylation yielded as a main product a protein derivative which did not require the action of a thiol reagent for dissociating in sodium dodecyl sulfate. However, more than 50% of this derivative was composed of protein subunits still held together in a dimeric structure. In conclusion, reduction of two disulfide bonds on the dimeric molecule of RNase BS-1 was apparently not only necessary but also a sufficient condition for promoting dissociation. It was not sufficient, however, for complete dissociation. The reason for this could be that besides disulfide bonds, noncovalent bonds also participated in maintaining the quaternary structure of RNase BS-1. The partial dissociation of about 30% of the selectively reduced and carboxymethylated protein could be explained in terms of an equilibrium between noncovalently associated and dissociated forms of monomeric carboxymethylated (MCM) protein, such as MCM · MCM $\rightleftharpoons$ 2MCM, shifted toward the left side.

A third experiment was thus performed with RNase BS-1 reduced and then alkylated as described above, but in this experiment the reduction–alkylation mixture, before gel filtration through Sephadex G-75, was previously filtered through a column of Bio-Gel P-2 (0.9 × 60 cm) equilibrated with 0.1 *M* ammonium acetate (pH 5) containing 5 *M* urea. By this procedure, more than 90% of the total incubated protein was recovered from the Sephadex G-75 column at the elution volume of the RNase BS-1 subunit (see Figure 5C), thus indicating that also noncovalent bonds were involved in maintaining the dimeric structure of RNase BS-1. In Figure 5D a control is shown of untreated RNase BS-1 filtered through the Sephadex G-75 column equilibrated with 0.1 *M* ammonium acetate (pH 5), in 5 *M* urea.

Characterization of Monomeric Carboxymethylated (MCM) RNase BS-1. The protein fractions eluted from the Sephadex G-75 column were combined as shown in Figure 5C and analyzed. Upon gel electrophoresis in sodium dodecyl sulfate, the protein derivative, denatured with sodium dodecyl sulfate in the absence of HSEtOH, migrated as a single band with a molecular weight of approximately 15,000 (Figure 6A). It was found to be homogeneous also when analyzed by gel electrophoresis without sodium dodecyl sulfate, at pH 4.5 (Figure 6B). The amino acid composition of MCM RNase BS-1 is presented in Table I, where it is compared to the compositions of the native protein and of the fully reduced and carboxymethylated derivative. The composition of selectively reduced and carboxymethylated RNase BS-1 was identical with that of the native protein but for the presence of four residues of carboxymethylcysteine residues per molecule of dimeric protein.

The half-cystine residues of RNase BS-1 involved in the selectively reduced and alkylated disulfide bonds were iden-

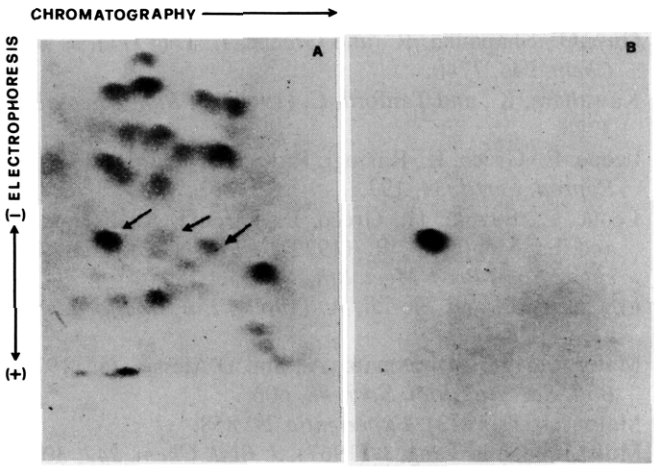

FIGURE 7: Fingerprint of RNase BS-1 selectively reduced and carboxymethylated. A thermolytic hydrolysate of the protein was chromatographed in a 1-butanol–acetic acid–water–pyridine solvent system and then electrophoresed in the second dimension in pyridinium acetate (pH 3.6) at 3000 V for 50 min: (A) fingerprint stained with ninhydrin; the arrows indicate the radioactive peptide spots identified through the autoradiogram shown in B.

tified by preparing S-carboxymethylated monomeric RNase BS-1 as described above, but with iodo-2-[^{14}C]acetate as alkylating agent. A 10-mg sample of the radioactively labeled protein derivative was then incubated with thermolysin (0.12 mg) in 1 ml of 0.1 *M* ammonium bicarbonate (pH 8) at 37° for 16 hr. A fingerprint was obtained by subjecting a 1-mg aliquot of the thermolytic digest to paper chromatography and then to high voltage paper electrophoresis in pyridinium acetate buffer (pH 3.5). The fingerprint and its autoradiographic replica are shown in Figure 7. Almost all the radioactivity appears to be confined to a single spot, with two minor radioactive spots hardly detectable. The remaining 9 mg was then run by electrophoresis at pH 6.5 as a 7-cm band. A single radioactive band, with no net charge, was identified by autoradiography, cut out, and inserted on a second sheet of paper. After a second electrophoresis at pH 3.5, again a single radioactive band was obtained, but moving toward the anode. This band was also cut out from the paper and inserted on a new sheet of paper, which was chromatographed. The autoradiography of the chromatogram revealed three radioactive bands, which were eluted from the paper (TL-1, TL-2, and TL-3). Amino acid analyses of the eluates gave the compositions presented in Table II. All three peptides appear to belong to the sequence region of RNase BS-1 subunit which goes from Met-29 through Met-35. The only detectable difference among the three peptides lies in their different contents of Met, which is very likely due to different degrees of susceptibility to thermolytic action of the peptide bonds involved.

The product of selective reduction and alkylation of RNase BS-1 was thus identified as bis-*S*-carboxymethylcysteine-31,32-ribonuclease BS-1 (MCM RNase BS-1). When assayed on yeast RNA and on cytidine 2′,3′-cyclic phosphate, MCM RNase BS-1 was found to be catalytically active, with specific activities comparable to those of the native enzymic protein.

The stability of the monomeric derivative of RNase BS-1 was checked by gel filtration on calibrated columns of Sephadex G-75 as described above. No reaggregation to a dimeric structure was detected in preparations kept for 2 months at 4° or more than 6 months at −20°.

Table II: Amino Acid Compositions of Radioactive Thermolytic Peptides from Selectively Reduced and Carboxymethylated RNase BS-1.

	Peptide		
	TL-1	TL-2	TL-3
CM-cysteine	*2.00*	*2.00*	*2.00*
Lysine	1.07	1.14	1.08
Arginine	1.03	0.90	1.41
Methionine	0.89	1.90	3.25

[a] Compositions are expressed as molar ratios relative to the content of carboxymethylcysteine. Amino acids present in quantities lower than 0.1 mol/mol of carboxymethylsteine were not included.

Conclusions

The results reported in the present paper indicate that the dimeric structure of RNase BS-1 is maintained: (i) by two disulfide bonds linking half-cystine residues in positions 31 and 32 of one subunit chain to the corresponding half-cystine residues of the other subunit chain; (ii) by noncovalent forces. They also show that under appropriate conditions it is possible to prepare, through selective reduction of the interchain disulfide bridges, followed by alkylation of the exposed sulfhydryl groups, a monomeric derivative of the protein, which is catalytically active.

The biological function of seminal RNase is not known. Recently, however, it has been discovered that the enzyme is capable of important biological actions in vivo on the germ line cells (Dostál and Matoušek, 1972; Leone et al. 1973), and on tumor tissues (Matoušek, 1973) and in vitro on virus transformed cells (Leone and Amati, personal communication).

The knowledge of the nature of the bonds which stabilize the quaternary structure of the protein and the availability of an enzymically active monomeric derivative may be useful to the evaluation of its biological role. In fact, the most meaningful structural difference between the seminal enzyme and pancreatic ribonuclease, which is totally devoid of the above-mentioned biological actions, lies in the fact that seminal RNase has a quaternary structure, while the pancreatic enzyme is a single chain protein.

But besides its biological actions, RNase BS-1 shows quite interesting enzymic properties, such as the ability to degrade double-stranded polyribonucleotides (Libonati and Floridi, 1969) as well as the ribonucleic chain of DNA–RNA hybrids (Taniguchi and Libonati, 1974), and a peculiar turnover number (Floridi et al., 1972), which appears to be much lower than the value expected for an active site presumably very similar to the active site of pancreatic RNase. With respect to the studies of the enzymic functions of RNase BS-1, and of the mechanism of action of ribonucleases in general, the monomeric active derivative of the protein may be regarded not only as a useful tool per se, but also as the fourth component of an experimental system of ribonucleases, where one native monomer (pancreatic RNase A) and one native dimer (seminal RNase BS-1) pair, respectively, with artificially dimerized RNase A (Crestfield et al., 1962; Bartholeyns and Moore, 1974) and artificially monomerized RNase BS-1.

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The Role of Protein and Lipids in Stabilizing the Activity of Bovine Heart Succinate Dehydrogenase†

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ABSTRACT: When incubated in an air atmosphere, solubilized succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) quickly loses the capability to recombine with membrane components to catalyze mitochondrial related electron transport activities. At 0° the loss in reconstitution capability is a first-order process; the half-life of the enzyme is 1.6 hr at this temperature. The enzyme is stabilized by recombining it with submitochondrial particles or with a cytochrome *b* preparation-phospholipid mixture. The presence of the cytochrome *b* preparation in the succinate dehydrogenase-cytochrome *b*-phospholipid complex is obligatory, indicating that protein-protein interactions between succinate dehydrogenase and other membrane components are important in stabilizing the capability of the flavoprotein to transfer electrons to other respiratory components. Treatment of this complex with phospholipase C results in loss of most of the succinate-dichlorophenolindophenol reductase activity and almost complete hydrolysis of phospholipid. Succinate dehydrogenase maintains its capability to participate in mitochondrial electron transport for several hours if the phospholipase treated complex is reconstituted with lysolecithin at the time of assay. Phospholipids are therefore not required for the stabilization process, but rather for formation of an active reductase complex. A lipophilic environment, if required for stabilization, can be provided by diglycerides. Diglycerides also can provide an environment conducive to electron transfer from succinate to ubiquinone but do so less efficiently than intact phospholipids.

The properties of membrane associated enzymes are controlled or modified by interaction with other components of the membrane. A notable example is the mitochondrial ATPase which requires phospholipid for activity and is oligomycin sensitive when associated with the membrane (Racker, 1963; Kagawa and Racker, 1966). This enzyme when solubilized from the membrane is not inhibited by oligomycin and does not require phospholipid for activity (Pullman et al., 1960). The importance of both protein-protein and protein-lipid interactions in conferring the mem-

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