INTERCHAIN DISULFIDE BRIDGES IN RIBONUCLEASE BS-1

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SUMMARY

RNAase BS-1, a dimeric ribonuclease isolated from bovine seminal plasma, is made up of two identical subunits whose amino acid sequence is homologous to the sequence of bovine pancreatic RNAase A. The dimeric structure, resistant to denaturating agents, is sensitive to thiol reagents even in the absence of denaturants. The isolation and characterization of a cystine peptide containing two adjacent ½cystine residues is reported. As the peptide molecular weight is halved after reductive cleavage with dithiothreitol, a structure based on two interchain disulfide bonds between the two adjacent ½cystine of each subunit is proposed. The singularity of such a structure for a small enzymatic protein is discussed.

RNAase BS-1, a ribonuclease isolated from bovine seminal plasma,is a dimeric protein with a molecular weight of 29,000, made up of identical subunits (1,2). The complete amino acid sequence of the subunit chain has been elucidated and found to be strictly homologous to the sequence of bovine pancreatic RNAase A, E.C. 2.7.7.16 (3).

As the dimeric structure of RNAase BS-1 is resistant to denaturating agents, but sensitive to thiol reagents even in the absence of denaturants (4), we have considered the possibility that the two subunits of RNAase BS-1 are linked through interchain disulfide bridges.

The amino acid sequence studies have shown that 8 of the 10 ½cystine residues present in each subunit (the protein does not contain sulfhydryl groups) are placed at sequence positions identical to those of the 8 ½cystines of RNAase A, while the remaining 2, located at positions 31 and 32 of the subunit chain, substitute respectively for a lysine and a serine of RNAase A (3).

On the basis of these results,it may be postulated that the 8 homologous ½cystine residues of RNAase BS-1 pair exactly as they do in RNAase A. The 2 adjacent ½cystine residues at positions 31 and 32 of each subunit

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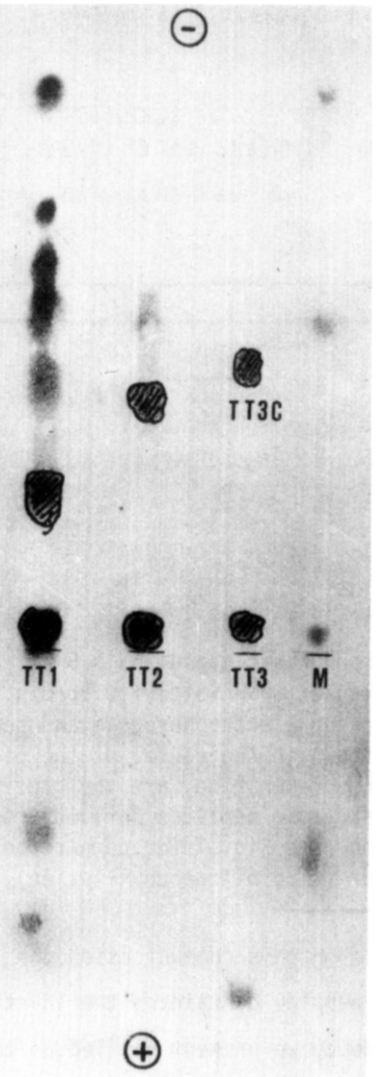


Fig. 1 - High voltage paper electrophoresis at pH 6.5 of fractions TT1,TT2 and TT3 from a thermolytic-tryptic hydrolisate of RNAase BS-1. Electropherogram run for 50 min at 3000 volts and stained with ninhydrin. Pencil marked spots indicate peptides which reacted with the cyanide-nitroprusside reagent on a parallel electropherogram run under the same conditions. A mixture of amino acid and dye markers was spotted in M.

chain would then be linked either one to the other by an intrachain disulfide bond, or, through interchain disulfide, to the corresponding ½cystines of the other subunit.

If the latter alternative is correct, the disulfide bridges formed by the adjacent ½cystine residues of RNAase BS-1 would be responsible for maintaining the protein quaternary structure.

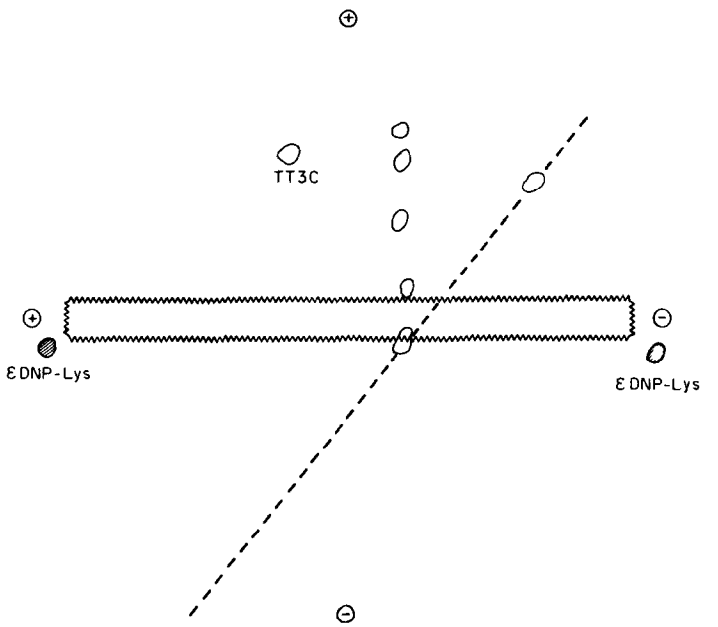


Fig. 2 - Diagonal electrophoresis map at pH 6.5 of fraction TT3. Electrophoreses were run at 3000 volts for 50 min. The strip cut from the first dimension electropherogram and sewn up for the second dimension, is shown in the center of the picture.Off the diagonal, indicated by the broken line, are the peptide of interest (TT3C) and other cysteic acid peptides derived from the neutral band of the TT3 fraction (see Fig. 1 for comparison). The shaded spot is a neutral marker (ε-dinitrophenyl-lysine).

We wish to report in this communication the isolation and characterization of the cystine peptide containing the ½cystine residues 31 and 32 of RNAase BS-1. The evidence we present has led us to the conclusion that the adjacent ½cystine residues of RNAase BS-1 are not linked to each other through intrachain disulfide bonds, but rather form interchain bridges between the two subunits.

MATERIALS AND METHODS

RNAase BS-1 was purified by a modification of the original procedure (1), recently proposed by Floridi and De Prisco (5). Amino acid analysis, high voltage paper electrophoresis and performic acid oxidation were carried out by standard methods as previously described (6). Diagonal electrophoresis and analysis of N-terminal residue with dansyl chloride were performed as suggested by Hartley (7). The sodium nitroprusside-sodium cyanide spray

for cystine detection on paper was prepared according to Toennies and Kolb (8). Carboxypeptidase B (Worthington code COB) was purified immediately before use by gel filtration through a Biogel P2 column equilibrated with 0.1 M ammonium bicarbonate. Protein concentration in the eluate was measured by absorbance at 278 nm (9).

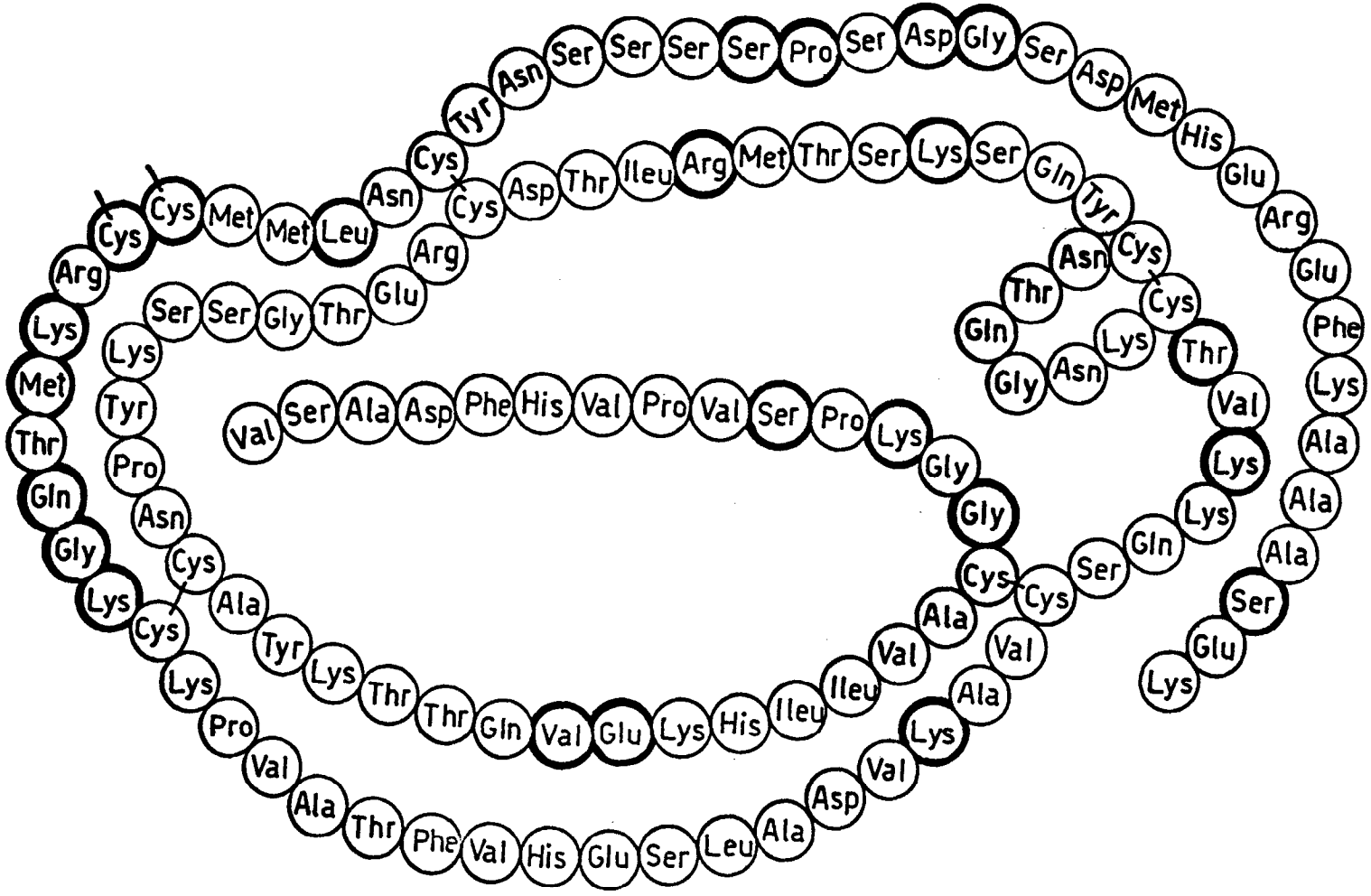
RESULTS

Native RNAase BS-1 was fragmented into peptides by digestion with thermolysin and trypsin according to a procedure which will be described elsewhere (Di Donato, A. and D'Alessio, G., manuscript in preparation). Care was taken to minimize disulfide interchange by lowering the incubation pH to 6.5. Fractionation of the peptide mixture was achieved by gel filtration through a Biogel P4 column equilibrated with 0.1 N acetic acid. The elution pattern, monitored at 235 nm, consisted of several peaks; however, when the eluate was assayed for its disulfide content (10), three main peaks could be identified. They were marked TT1, TT2 and TT3.

The eluate fractions corresponding to these peaks were combined and lyophilized. Two electrophoretic runs at pH 6.5 were then carried out in parallel with aliquots from each peak. After electrophoresis, one paper was stained with the ninhydrin reagent, while the other was treated with the cyanide-nitroprusside reagent. This procedure resulted in the identification in each peak of two main fractions of cystine containing material: fractions TT1N, TT2N and TT3N with no net charge at pH 6.5 and fractions TT1C, TT2C and TT3C, which moved towards the catode (Fig. 1).

When the material from peak TT3 was subjected to diagonal electrophoresis, it appeared that the cationic fraction TT3C produced a single peptide spot off diagonal, which was not contaminated by other nearby peptides. (Fig. 2). Furthermore, the presence of this single peptide off diagonal indicated the possibility that in this case the cysteic acid residues formed upon performic acid oxidation of TT3C were contained in a single peptide. Therefore, peptide TT3C could well correspond to the cystine peptide containing the two adjacent ½cystine residues located at positions 31 and 32 in the RNAase BS-1 sequence.

Peptide TT3C was thus isolated by a single electrophoretic run (at



Amino acid sequence of the Ribonuclease BS-1 subunit. Heavy circles denote the 23 positions where substitutions have been found with respect to pancreatic ribonuclease A. These data will be presented in a manuscript now in preparation by E. Leone and his coworkers.

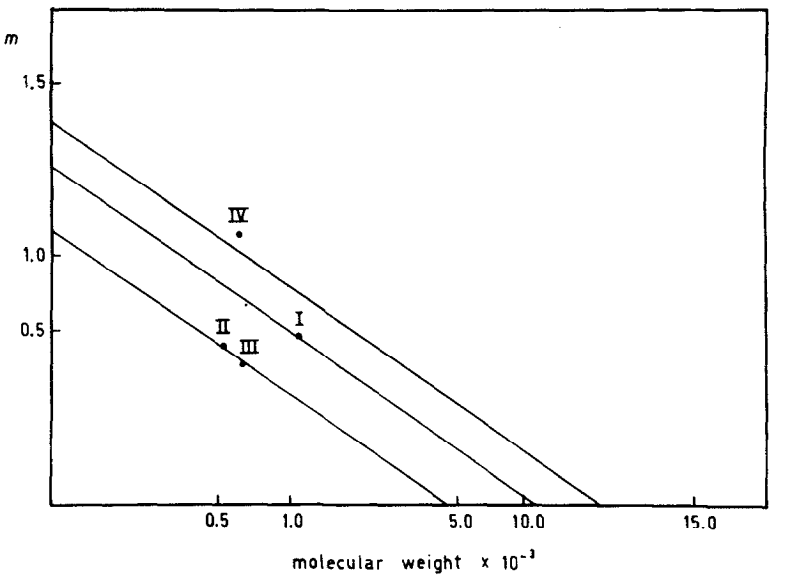


Fig. 3 - Relative electrophoretic mobility at pH 6.5 of peptide TT3C: as isolated (I), after reduction (II), after reduction and carboxymethylation (III), and after reduction and aminoethylation (IV). The curves were redrawn after the original Offord graph (11). The experimental points were measured as described in the text on electropherograms run for 50 min at 3000 volts.

pH 6.5) of the material eluted with peak TT3, using both diagonal electrophoresis and the cyanide-nitroprusside test, performed on side strips, for identification. The amino acid composition of the peptide, after performic acid oxidation, was the following: arginine, 1.20, cysteic acid, 2.00, methionine sulphone, 1.02. Its amino terminal residue, identified with the dansyl chloride method, was found to be methionine. Incubation for 1 hour at 38° in 0.2 M ammonium bicarbonate with carboxypeptidase B, with an enzyme: substrate ratio of 1 enzyme unit per 100 nmoles of peptide TT3C, produced free arginine and a peptide which was neutral upon electrophoresis at pH 6.5.

All these data led us to conclude that the amino acid sequence of peptide TT3C is MET-CYS-CYS-ARG and that it contains residues 30 through 33 of the RNAase BS-1 subunit sequence.

As electrophoretic mobility of peptides can be used, according to Offord (11), to estimate their molecular weights, this parameter was measu-

red for peptide TT3C: (i)as isolated, (ii)after disulfide reduction, and (iii)after disulfide reduction followed by alkylation.

Disulfide reduction was carried out in 0.2 M ammonium bicarbonate by incubating the peptide with an excess of dithiothreitol (5 moles of reagent per mole of peptide) for 30 min at room temperature.

Alkylation with iodoacetic acid was accomplished by adding to the peptide, reduced as described above, an excess of recrystallized and neutralized iodoacetic acid (2 moles of reagent per mole of sulfydryl). After 30 min of incubation at room temperature, the incubate was directly applied onto the electrophoresis paper.

Alkylation with ethylenimine was carried out in an analogous way by incubating the reduced peptide with an excess of freshly redistilled reagent. The pH of the incubation mixture was adjusted to 8.6 with dilute ammonia and ethylenimine was added in two portions, up to a final ratio of 100 moles of reagent per mole of sulfydryl present. After a total incubation time of 30 min at room temperature, the incubate was directly applied onto the electrophoresis paper.

For each experiment a control was prepared, consisting of peptide incubated without reagent, and electrophoresed along with the treated samples. All electrophoreses were run at pH 6.5 and mobilities, measured from a neutral marker (ε-dinitrophenyl-lysine) to the leading edge of the peptide spot, were expressed relatively to the aspartic acid mobility.

The data obtained in these experiments were included in the log-log plot proposed by Offord, where peptide mobilities, relative to the mobility of aspartic acid are plotted against peptide molecular weights (11). Fig. 3 shows that the data fit quite well to the curves as redrawn after the original Offord graph. The molecular weight and net charge values assigned to peptide TT3C and its derivatives are listed in Table 1, where the observed relative mobilities are compared to the correspondent values calculated graphically from the Offord plot.

It is apparent that on the basis of these data the structure (Ib), with an intrachain disulfide bridge between the two adjacent ½cystine residues, can be excluded. Such a structure should in fact confer to peptide TT3C an electrophoretic mobility which is only observed after reductive cleavage of the peptide disulfide.

TABLE 1

		molecular weight	net charge	electrophoretic mobility expected[a]	found
Ia	MET-CYS-CYS-ARG | | MET-CYS-CYS-ARG	1018	+2	+0.480	+0.474[b]
Ib	MET-CYS-CYS-ARG └──┘	509	+1	+0.480	
II	MET-CYS-CYS-ARG | | H H	511	+1	+0.430	+0.422[c]
III	MET-CYS-CYS-ARG | | CM CM	625	-1	-0.380	-0.368
IV	MET-CYS-CYS-ARG | | AE AE	597	+3	+1.050	+1.210

[a]from the Offord plot; [b]average of 3 determinations (±0.004); [c]average of 4 determinations (±0.011); CM = carboxymethyl; AE = aminoethyl.

DISCUSSION

Quaternary structure of proteins is mainly determined by non covalent bonds. Among the best studied exceptions to this simplifying rule are immunoglobulins, whose chains are covalently linked through disulfide bonds (12); fibrinogen, where the three polypeptide chains participate to the "disulfide knot" structure (13); and keratins (14).

As for enzymatic proteins, interchain disulfide bonds have been occasionally hypothesized on the basis of circumstantial evidence, and subsequently disproved. A special case in fact is that of zymogen derived pro-

teins, as the interchain disulfides present in these molecules originate from intrachain bonds of the parent molecules.

More recently, however, it was reported that interchain disulfide bonds are responsible for maintaining the quaternary structure of threonine deaminase from Salmonella typhimurium (15) and glucose oxidase from Penicillium amagasakiense (16) and Aspergillus niger (17). It was in fact observed that necessary condition for the dissociation of these oligomeric proteins was the reductive cleavage of some of the disulfide bridges present in these molecules. A similar observation has also been made for RNAase BS-1, whose dimeric structure can be dissociated by thiol reagents, even in the absence of denaturants (4).

Yet it can be argued that all these observations may be interpreted with two alternative hypotheses: the protein subunits are linked through interchain disulfide bridges; or, subunits are linked through non covalent bonds, but the availability of these bonds to the denaturating agents depends on the integrity of some intrachain disulfides of the protein subunits.

Direct evidence for the presence of interchain disulfide bonds in proteins would therefore be produced only through actual isolation and characterization of the cystine peptide(s) containing the interchain disulfide bond(s).

In this paper we have described the isolation from RNAase BS-1 of a dimeric structure made up of two identical peptides linked by two disulfide bonds. These findings provide for the first time direct evidence for the presence of interchain bonds in an oligomeric enzymatic protein, which is not derived from a zymogen.

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