DIMERIC STRUCTURE OF SEMINAL RIBONUCLEASE*

G. D'ALESSIO, A. PARENTE, C. GUIDA and E. LEONE

Laboratorio di Chimica Biologica, Facoltà di Scienze, Università di Napoli, Via Mezzocannone 16, 80134 Napoli, Italy

Received 18 September 1972

RNAase BS-1, the major component of ribonuclease activity in bull seminal plasma, is a basic protein with a molecular weight of 29,000 [1]. Its catalytic properties are very similar to those of bovine pancreatic RNAase A [2], except for a lower k_{cat} and the ability to degrade also double-stranded RNA under conditions in which RNAase A is only slightly active [3]. We wish to report on the subunit structure of this enzyme, the first instance, to our knowledge, of a dimeric ribonuclease. This conclusion rests on several lines of evidence: i) peptide mapping of the protein; ii) quantitation of end groups; iii) estimation of the subunit molecular weight by electrophoresis on polyacrylamide gels in sodium dodecylsulphate; iv) estimation of the number of covalently linked species obtained by amidination of the protein with a cross-linking reagent.

The peptide map of RNAase BS-1 was obtained by tryptic digestion of the protein oxidized with performic acid [4]. A total of 19 ± 2 spots was obtained in several experiments (fig. 1a). From the amino acid composition (28 lysine and 8 arginine residues per molecule), one would expect a number of about 37 peptides for a protein consisting of a single polypeptide chain. The observed value can therefore only be explained if one assumes that the protein is made up of two identical, or very similar, subunits. On the other hand, the possibility of RNAase BS-1 being a dimer of RNAase A is ruled out by a comparison of the peptide maps of the two proteins (fig. 1a and b). Several peptides appear to be different, and this is in line with the differences found in the amino acid compositions of the two proteins [1, 5].

End-group analyses for N-terminal residues were performed by the cyanate method of Stark and Smyth [6]. From two separate experiments a value of 2 ± 0.23 moles of N-terminal lysine per mole of protein was obtained. Yield was calculated by applying a correction factor of 0.82, obtained by processing samples of lysine and of RNAase A (Sigma Type XII) through the complete procedure.

Analyses of the C-terminal residues were carried out by following the rates of appearance of amino acids released by carboxypeptidase A from the performic acid oxidized protein [7]. In table 1 the results of a typical experiment of digestion are summarized along with the results of a parallel experiment performed with RNAase A under the same conditions and employing the same batch of carboxypeptidase A. Two conclusions can be drawn from the results obtained with RNAase BS-1: i) the number of moles per mole of protein approached for all the residues of the degraded sequence is 2; ii) the apparent amino acid sequence at the C-terminal ends of the molecule is identical to the corresponding C-terminal sequence of RNAase A: ... Pro–Val–His–Phe–Asp–Ala–Ser–Val-COOH. This result has been confirmed by a study carried out in this laboratory on the S-carboxymethyl derivative of the protein [8].

The molecular size of the subunit was estimated from the electrophoretic mobility on polyacrylamide gels of the protein dissociated in, and complexed with, sodium dodecylsulphate [9–11]. When tested in this system, together with a set of 7 proteins as standards, RNAase BS-1 gives a single electrophoretic band, with an estimated molecular weight of 14,000 (fig. 2). An identical value was obtained with

* This is paper no. 3 on Bull Semen Ribonucleases. The first two papers have been published elsewhere [1, 2].

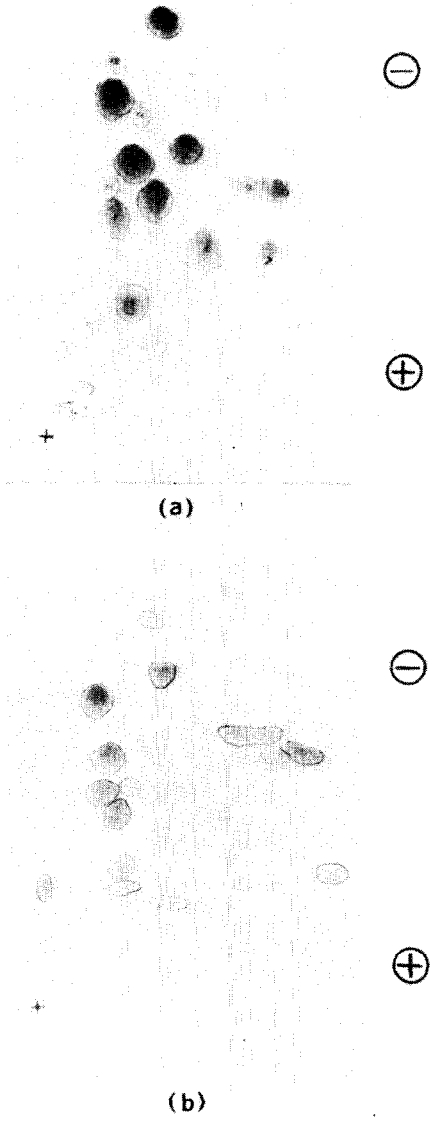

Fig. 1. Peptide maps of RNAase BS-1 (a) and RNAase A (b) obtained by trypsin digestion of the performic acid oxidized proteins. Trypsin (Worthington TRTPCK) digestion was carried out in 0.5% ammonium bicarbonate at 37° for 5 hr at a trypsin to protein ratio of 1 to 100 (w/w). Chromatography in n-butanol–acetic acid–water–pyridine (30:6:24:20) for 24 hr followed by ionophoresis in pyridine–acetic acid, pH 3.6, at 50 V/cm for 60 min. The peptides were detected by ninhydrin staining. Very faintly staining peptides are marked by dotted circles.

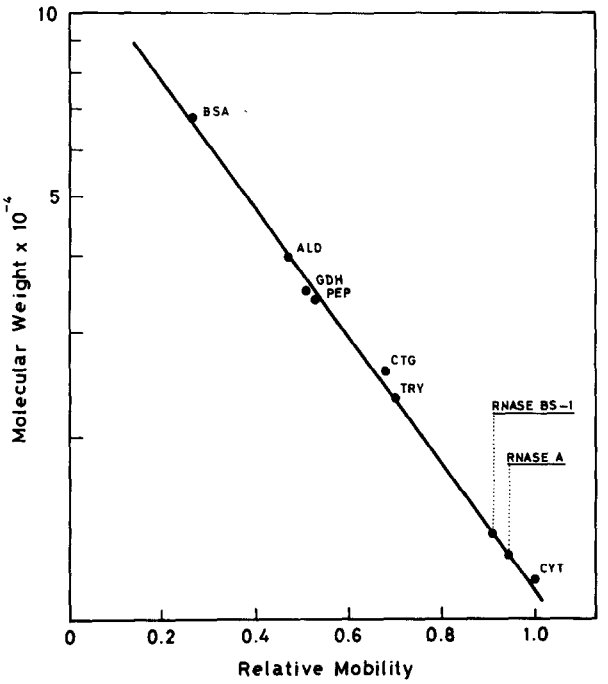

Fig. 2. Molecular weights of SDS-complexes of RNAase BS-1 as determined by electrophoresis on polyacrylamide gels in SDS. Preparation of SDS-complexes was carried out as described by Dunker and Ruekert [11], in the presence of 1% 2-mercaptoethanol. Electrophoreses were performed according to Weber and Osborn [10]. To each sample cytochrome *c* was added as reference marker and mobility was measured relative to the cytochrome band.

Molecular weights of standard proteins (or protein subunits) used for constructing the calibration curve were as quoted by Weber and Osborn [10]; bovine serum albumin (BSA), 68,000; aldolase (ALD), 40,000; glyceraldehyde-3-phosphate dehydrogenase (GDH), 35,000; chymotrypsinogen (CTG), 25,700; trypsin (TRY), 23,000; cytochrome *c* (CYT), 11,700. For pepsin (PEP), a molecular weight of 34,000 was used (Mills and Tang [17]).

The straight line shown in the diagram was calculated by a least square procedure from the results of 5 experiments.

the protein denatured by performic acid oxidation and with the S-carboxymethyl derivative of the protein.

The experiment described above was then repeated on the protein previously amidinated with diimidoesters used as cross-linking reagents [12]. It has been shown by Davies and Stark that when the cross-linking reaction is performed at low protein concentrations, amidination occurs predominantly within

Table 1
Amino acids released (moles/mole of protein) from performic acid oxidized RNAase BS-1 and RNAase A by carboxypeptidase A.

Amino acid	After 60 min of incubation at 25°		After 60 min of incubation at 25° plus 120 min at 37°		After 60 min of incubation at 25° plus 20 hr at 37°	
	RNAase BS-1	RNAase A	RNAase BS-1	RNAase A	RNAase BS-1	RNAase A
Val	1.91	0.88	1.95	1.31	2.14	1.27
Ser	1.32	0.71	1.82	1.07	2.09	1.07
Ala	0.13	0.59	0.71	0.96	2.16	1.08
Asp	0.12	0.57	0.57	0.98	2.03	1.07
Phe	0.12	0.51	0.43	0.91	1.88	1.01
His	0.12	0.38	0.44	0.82	2.08	0.85

Experimental conditions: an enzyme–substrate mole ratio of 1/100 was used, and incubation was carried out at 25° for 1 hr followed by 20 hr at 37° in *N*-ethylmorpholine acetate buffer at pH 8.4 [7]. Carboxypeptidase A was Worthington COADFP. The released amino acids were separated from the protein by addition of sulphosalicylic acid to a final concentration of 6% and determined with a Beckman–Spinco Model 116 amino acid analyzer. For quantitation of results, internal standards (norleucine and L-α-amino-β-guanidinopropionic acid) were added to the incubation mixtures.

oligomers. This promotes, in oligomeric proteins made up of identical subunits, the formation of a number of molecular species which equals the number of subunits present in the protein. Thus for a protein made up of two identical subunits, one would expect two species: the unreacted protomer and the cross-linked dimer. This is what was found for RNAase BS-1, as it is shown in fig. 3.

The results presented above clearly point to the conclusion that RNAase BS-1 is a protein made up of two chains, and that the two chains are probably identical. This conclusion has also been reached by an independent X-ray crystallographic study [13].

The dimeric structure makes RNAase BS-1 rather unique. It is indeed well known that none of the large number of ribonucleases described so far has been shown to consist of subunits [14]. This property of RNAase BS-1 can be related to its ability to hydrolyze both single- and double-stranded RNA, another unusual feature for a nuclease with a phosphotransferase mode of action. The relationship in fact may be more than casual, as it has been found that synthetic dimers of RNAase A can also hydrolyze both types of substrates [15].

Another important feature is the presence in both chains of RNAase BS-1 of two histidine residues in sequence positions equivalent to His-12 and His-119 of RNAase A. Besides the histidine included in the C-terminal sequence reported in this paper, and equivalent to His-119 of RNAase A, a histidine in position 12 has also been located [16]. It would appear, therefore, that the seminal enzyme contains in both chains structural elements which have been found to be essential for the active site of RNAase A (see [14] for a review).

On the other hand, as the value of k_{cat} for RNAase BS-1 is lower than for RNAase A, at least three hypotheses can be made: i) the enzyme contains two potential active sites, their availability depending on the structure of the substrate; ii) the two active sites catalyze hydrolysis at a lower rate because of structural constraints imposed by the protein dimeric structure; iii) the two chains are very similar, but not identical, and only one has all the structural elements which make up an active site.

Acknowledgements

Most of the work described in this paper was carried out at the Stazione Zoologica, Napoli, whose hospitality is gratefully acknowledged. We wish to thank Dr. G. Stark for helpful discussion and suggestions. We also wish to acknowledge the assistance of Dr. G.B. Demma in performing some of the experiments reported in this communication. This research

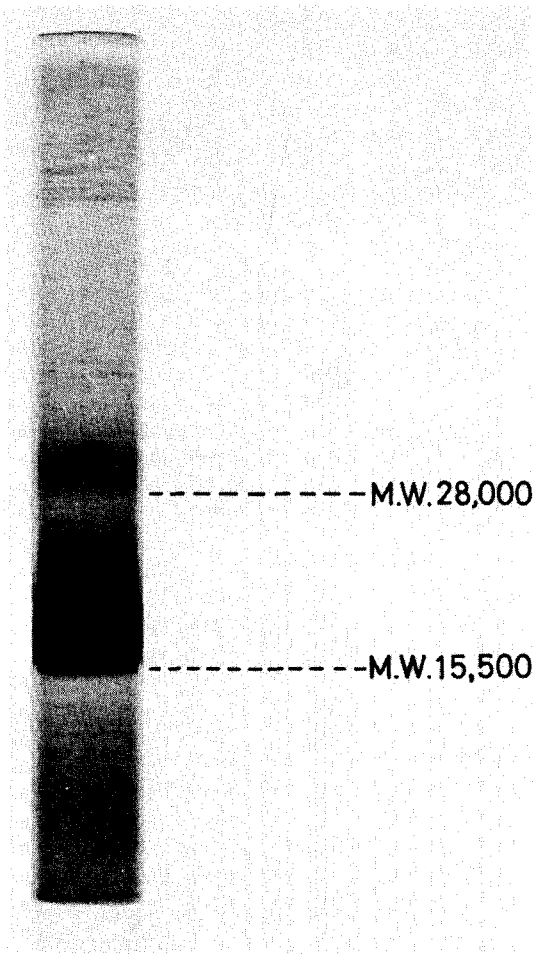

Fig. 3. Electrophoresis on polyacrylamide gel of RNAase BS-1 previously reacted at a concentration of 0.1% with a 0.4% solution of dimethylsuberimidate according to Davies and Stark [12]. Reaction with sodium dodecylsulphate and electrophoresis were carried out as described in the legend to fig. 2.

has been supported by grant N. 6902178 from the Consiglio Nazionale delle Ricerche, Italy.

References

[1] G. D'Alessio, A. Floridi, R. De Prisco, A. Pignero and E. Leone, European J. Biochem. 26 (1972) 153.
[2] A. Floridi, G. D'Alessio and E. Leone, European J. Biochem. 26 (1972) 162.
[3] M. Libonati and A. Floridi, European J. Biochem. 8 (1969) 81.
[4] C.H.W. Hirs, J. Biol. Chem. 219 (1956) 611.
[5] C.H.W. Hirs, W.H. Stein and S. Moore, J. Biol. Chem. 211 (1954) 941.
[6] G.R. Stark and D.G. Smyth, J. Biol. Chem. 238 (1963) 214.
[7] R.P. Ambler, in: Methods in Enzymology, Vol. XI, ed. C.H.W. Hirs (Academic Press, 1967) p. 155.
[8] E. Leone, H. Suzuki, L. Greco, A. Parente, B. Farina and R. La Montagna, FEBS Meet. Abstr., 8th, Amsterdam 1972.
[9] A.L. Shapiro, E. Vinuela and J.V. Maizel, Biochem. Biophys. Res. Commun. 28 (1967) 815.
[10] K. Weber and M. Osborn, J. Biol. Chem. 244 (1969) 4406.
[11] A.K. Dunker and R.R. Ruekert, J. Biol. Chem. 244 (1969) 5074.
[12] G.E. Davies and G.R. Stark, Proc. Natl. Acad. Sci. U.S. 66 (1970) 651.
[13] S. Capasso, F. Giordano, L. Mazzarella and A. Ripamonti, J. Mol. Biol. 64 (1972) 311.
[14] E.A. Barnard, Ann. Rev. Biochem. 38 (1969) 677.
[15] M. Libonati, Biochim. Biophys. Acta 288 (1971) 440.
[16] G. D'Alessio, A. Parente, B. Farina, R. La Montagna, R. De Prisco, G.B. Demma and E. Leone, Biochem. Biophys. Res. Commun. 47 (1972) 293.
[17] J.N. Mills and J. Tang, J. Biol. Chem. 242 (1967) 3093.