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PROTEOLYTIC ENZYMES AS STRUCTURAL PROBES FOR RIBONUCLEASE BS-1

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Summary

Trypsin, pepsin and subtilisin have been used as conformational probes for the structure of bovine seminal ribonuclease BS-1 by studying, under definite conditions, their effects on the seminal enzyme, a dimeric protein made up of two identical subunits; on bovine pancreatic monomeric ribonuclease A (EC 3.1.4.22) with a polypeptide chain homologous to that of the seminal ribonuclease subunit chain; and on a monomeric, active and stable derivative of seminal ribonuclease. The results show: (1) that the C-terminal regions of the pancreatic and the seminal proteins are very similar as they appear to fit in an identical way to the active site of pepsin; (2) that the resistance of the N-terminal region of ribonuclease BS-1 to subtilisin is not due to the dimeric structure of the protein, but to the conformation of this region, where an essential feature is the presence of a proline residue at position 19; (3) that the monomer of ribonuclease BS-1 is resistant to tryptic action only when bound to the partner monomer in the quaternary structure of the protein. This indicates that dissociation of the seminal ribonuclease makes some potentially susceptible bond or bonds available to trypsin either through a conformational change of the protein subunit, or by simply exposing the protein area hidden at the intersubunit interfaces.

Introduction

Proteolytic enzymes have been used successfully in several studies as structural probes, by relating the susceptibility to various conditions of proteolysis of the protein substrate, to its conformation [1]. This method may be especially useful when a protein can be compared, in its sensitivity to proteolytic action,

Abbreviations: M-CM-RNAase BS-1, monomeric, bis-*S*-carboxymethylcysteine-31,32-ribonuclease BS-1.

with homologous proteins and/or with suitable derivatives obtained by chemical modifications of the parent molecule. We have applied this experimental approach to the study of bovine seminal ribonuclease, RNAase BS-1 [2].

Seminal RNAase is a dimer [3—5] containing two identical 124-amino acid peptide chains (bovine pancreatic RNAase A, EC 3.1.4.22, consists of a single 124-unit amino acid chain with 4 disulfide bonds) linked through 2 interchain disulfide bridges [4,5]. The peptide chain of seminal RNAase has 23 amino acid substitutions compared to the pancreatic enzyme [6,7], but the 8 residues of half-cysteine present in RNAase A (EC 3.1.4.22) are observed at identical positions in the subunit chain of RNAase BS-1, where they pair exactly as in RNAase A (Di Donato, A. and D'Alessio, G., unpublished). The action on RNAase BS-1 of three proteolytic enzymes: trypsin (EC 3.4.21.4) pepsin (EC 3.4.23.1) and subtilisin, (EC 3.4.21.14), was thus studied making use of a comparative system, which included, besides native dimeric RNAase BS-1, monomeric pancreatic RNAase A and a monomeric derivative of RNAase BS-1.

Materials and Methods

RNAase BS-1 was isolated from bull semen and its homogeneity checked as described by D'Alessio et al. [2]. Its monomeric derivative M-CM-RNAase BS-1 was prepared according to the method of D'Alessio et al. [5]. Pancreatic RNAase A, Type XII-A, and subtilisin BPN' were obtained from Sigma Chemical Co. Trypsin-Tos-Phe-CH_2Cl and pepsin were products of Worthington Biochemical Corp. Tryptic hydrolysis was measured on a Radiometer model TTT1 pH-stat equipped with a thermostatically temperature-regulated cell. Protein samples were dissolved in freshly distilled water and thoroughly degassed with nitrogen before the addition of trypsin. Amino acid analyses and high voltage paper electrophoreses were carried out by standard methods as previously described [6].

Results

Action of trypsin

Bovine pancreatic RNAase A is resistant to the proteolytic action of trypsin [8]. Tryptic hydrolysis of this protein has been observed only when the incubation mixture included denaturing agents [8], when hydrolysis was performed above the transition temperature of RNAase A [9], or when a weight ratio of 1 : 1, of trypsin to ribonuclease, was used [10]. Also up to a ratio of 1 : 50 bovine seminal RNAase BS-1 was found to be completely resistant to the action of trypsin (see Fig. 1). Incubations were performed at a protein substrate concentration of 20 mg/ml, measuring with a pH-stat the uptake of 0.05 M NaOH required to maintain a pH of 8.0 at 37° C.

RNAase BS-1 is a dimeric protein, M-CM-RNAase BS-1 is a monomeric derivative, stable and catalytically active, obtained by selective reduction, followed by alkylation, of the protein interchain disulfides [5]. Despite the fact that native dimeric RNAase BS-1 and monomeric RNAase A were both resistant to tryptic hydrolysis, monomeric M-CM-RNAase BS-1, showing 80% homology

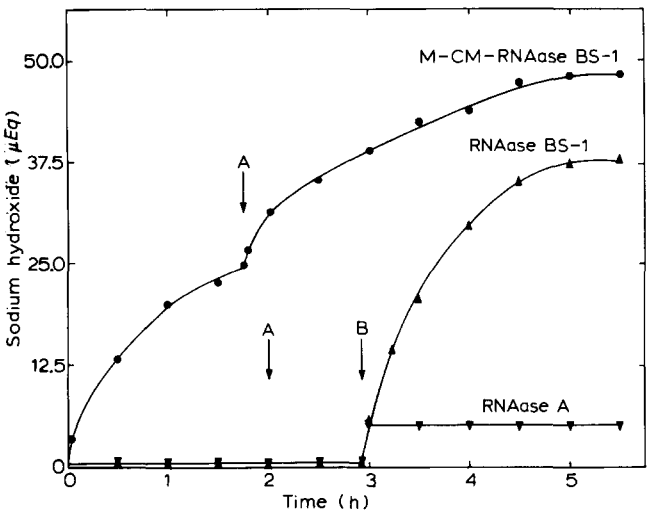


Fig. 1. Action of trypsin on RNAase BS-1, RNAase A and M-CM-RNAase BS-1. Tryptic activity was measured with a pH-stat as the uptake of 0.05 M NaOH required to mantain a pH of 8.0 at 37° C. Incubations were started with a trypsin to protein substrate weight ratio of 1 : 100. The arrows marked A indicate the times when the weight ratio was increased to 1 : 50 by further additions of trypsin. The arrow marked B indicates the time of additions of dithiothreitol.

with RNAase A, was rapidly cleaved by trypsin. The incubation was started with a weight ratio of trypsin to protein substrate of 1 : 100 (Fig. 1). When, after 2 h of hydrolysis, the ratio of trypsin to M-CM-RNAase BS-1 was raised to 1 : 50, a new burst of tryptic activity was observed and in about 2.5 h hydrolysis appeared to be complete.

It seemed then of interest to ascertain whether reduction alone of the interchain disulfides had an effect upon the resistance of RNAase BS-1 to tryptic action. RNAase BS-1 and, in a parallel experiment, RNAase A, were thus incubated with trypsin (up to 1 : 50 of the protein weight). After 3 h of incubation, during which no uptake of alkali was detected for either protein, dithiothreitol was added in a 10-fold molar excess with respect to each protein. This ratio has been found to bring about selective cleavage of the interchain disulfides of RNAase BS-1 [5]. In Fig. 1 it is shown that while RNAase A remained inert to trypsin after the addition of the mercaptan, RNAase BS-1 became immediately sensitive and appeared to be cleaved completely in about 2.5 h. This indicates that reductive cleavage of the intersubunit disulfide bridges makes of RNAase BS-1 at least as good a substrate for trypsin as its stable monomeric derivative M-CM-RNAase BS-1. Moreover, the hydrolysis rate observed for selectively reduced RNAase BS-1 is much higher than the rate measured for the stable monomer M-CM-RNAase BS-1 (See Discussion).

Action of pepsin

Ribonuclease BS-1 was treated with pepsin under conditions in which pancreatic RNAase A is selectively cleaved at the bond between Phe-120 and Asp-121 [11—13]. The incubation mixture contained 5 mg of RNAase BS-1 and 1 μg of pepsin in 0.5 ml of 0.01 M HCl. After 10 min at 37° C the incubate was

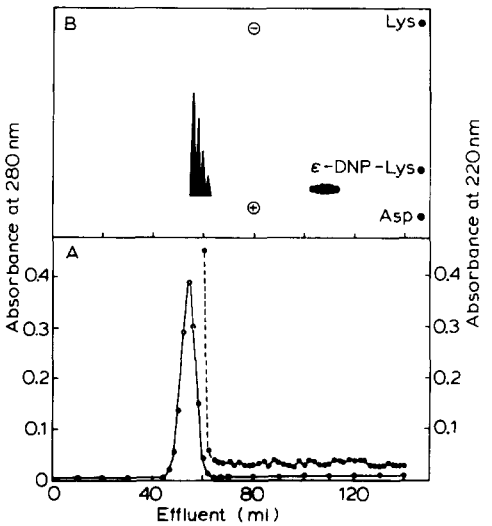


Fig. 2. Gel-filtration on Sephadex G-25 of RNAase BS-1 digested with pepsin. After incubation with pepsin (see text) RNAase BS-1 was applied to a 1.5 × 100-cm column of Sephadex G-25 equilibrated with 0.16 M ammonium bicarbonate. Elution was carried out with the equilibrating solvent collecting 2-ml fractions at a flow rate of 10 ml/h. A: elution profile at 280 (○———○———○) and at 220 nm (•———•———•). B: High-voltage paper electrophoresis at pH 3.6 of 100 μl aliquots from the fractions eluted from 52 through 140 ml of eluate. Electrophoresis was carried out for 50 min at 3000 volts. Lysine (Lys), ε-dinitrophenyl-lysine (ε-DNP-Lys), and aspartic acid (Asp) were used as markers. The electropherogram was stained with ninhydrin.

adjusted to pH 7 by small additions of 1 M NaOH and taken to a 1.5 × 100-cm column of Sephadex G-25, equilibrated with 0.15 M ammonium bicarbonate. A protein peak, presumably containing pepsin-modified ribonuclease and native enzyme, was detected by its absorbance at 280 nm (Fig. 2). No other ultraviolet-absorbing material was detected in the eluate. Aliquots (100 μl) were then withdrawn from all the remaining fractions, dried over P_2O_5 in a desiccator, resuspended in 10 μl of pyridinium acetate buffer at pH 3.6 [14] and subjected to high-voltage paper electrophoresis. After ninhydrin staining it was found that, besides the fractions eluted at the void volume of the column, also another zone of the eluate contained ninhydrin-positive material (see upper part of Fig. 2). The corresponding fractions were combined and lyophilized. An aliquot of the resulting material was hydrolyzed in 6 M HCl for 20 h at 110°C and its amino acid composition determined. The following composition resulted, relative to alanine and neglecting the residues present in relative amounts lower than 0.1: Asx, 0.89; Ser, 0.99; Ala, 1.00; Val, 0.96. An aliquot of this peptide was subjected to high-voltage paper electrophoresis at pH 6.5. The resulting electrophoretic mobility [15] relative to aspartic acid, allowed the assignment of a negative charge to the peptide and its identification with the C-terminal tetrapeptide, which is identical in RNAase A and RNAase BS-1. [3,7].

Action of subtilisin

When bovine pancreatic RNAase A is treated with subtilisin under controlled conditions a highly selective proteolytic cleavage occurs [16]. Subtilisin BPN'

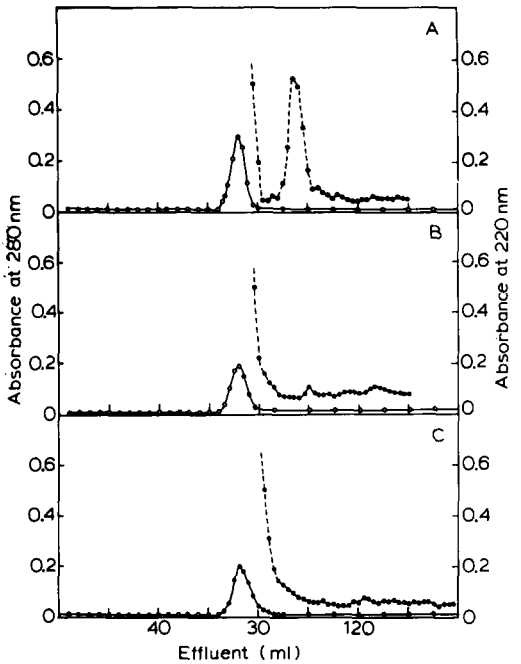


Fig. 3. Gel-filtration on Sephadex G-50 of RNAase A, RNAase BS-1 and M-CM-RNAase BS-1 treated with subtilisin BPN'. RNAase A (A), RNAase BS-1 (B) and M-CM-RNAase BS-1 (C) after incubation with subtilisin (see text for details) were applied to a 2.5 × 32.5-cm column of Sephadex G-50 (fine) equilibrated with 0.05 M HCl. Elution was carried out with the equilibrating solvent collecting 2-ml fractions at a flow rate of 10 ml/h. Elution profiles are shown at 280 (○——○——○) and 220 nm (●——●——●).

splits the bond 20—21 and, to a lesser extent, the bond 21—22, at 5°C and using a subtilisin to RNAase A weight ratio of 1 : 1000 [17]. Under identical conditions RNAase BS-1 is completely resistant, up to a weight ratio of 1 : 20 [8].

As RNAase A also becomes resistant to subtilisin when it is artificially dimerized by lyophilization from acetic acid [19], one of the possible reasons for the resistance of naturally dimeric RNAase BS-1 could be its quaternary structure [18,19].

An experiment was thus carried out by submitting the monomeric derivative of seminal ribonuclease, M-CM-RNAase BS-1 to hydrolysis by subtilisin under conditions in which RNAase A is selectively cleaved, whereas RNAase BS-1 and artificially dimerized RNAase A are completely resistant. Incubations were performed in 0.1 M Tris · HCl, pH 8, with a weight ratio of subtilisin BPN' to protein substrate of 1 : 100. After 2 h at 0°C the incubates were acidified by small additions of 0.5 M HCl and applied to a 2.5 × 32.5-cm column of Sephadex G-50 (fine) equilibrated with 0.05 M HCl. The results of experiments carried out in parallel on RNAase A, RNAase BS-1 and on M-CM-RNAase BS-1 are shown in Fig. 3. Only RNAase A appears to be a substrate for subtilisin under the conditions chosen. The peptides isolated from the subtilisin digest of RNAase A were identified by amino acid analysis as mixture of the S-peptides which are known to be cleaved from RNAase A by subtilisin BPN' [16,17]. The eluates of the columns to which the incubates of subtilisin with RNAase

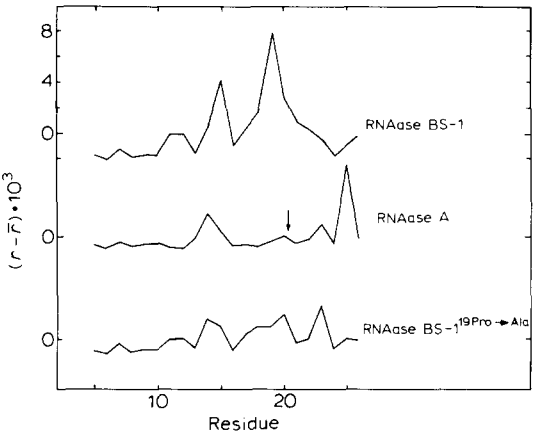


Fig. 4. Bend probability profiles of N-terminal sequences of RNAase BS-1, RNAase A and RNAase BS-1$^{19Pro\rightarrow Ala}$. The probability curves calculated for the N-terminal ribonuclease sequence (r) were plotted as $(r-\bar{r})$, where $\bar{r} = (\Sigma r)/n$. A bend is predicted whenever $r > \bar{r}$.

BS-1 and with M-CM-RNAase BS-1 had been applied, were checked for ninhydrin-positive material after high-voltage paper electrophoresis at pH 3.6. No traces of peptide spots were detected.

Another reason for the resistance of RNAase BS-1 to subtilisin could be the presence, in the sequence around the presumptive point of cleavage, of amino acid incompatible with the substrate-binding site of subtilisin, with resulting differences of conformation in the region of interest [20,21]. In the N-terminal sequence of RNAase BS-1, up to position 26, there are the following amino acid replacements, with respect to the sequence of RNAase A: Ser for Thr at position 3; Gly-Asp for Ser-Thr at position 16 and 17; and Pro-Ser for Ala-Ala at positions 19 and 20 [6,7].

An empirical method to predict β-bends using a nonamer correlation has been applied to the N-terminal sequences of a number of ribonucleases [20,21]. The profiles obtained for the ribonucleases which can be cleaved by subtilisin generally resemble each other and show a low bend probability in the region of cleavage, whereas bends are predicted for the resistant ribonucleases [20,21].

In Fig. 4 the prediction for seminal ribonuclease is compared with that calculated for pancreatic ribonuclease. A high bend probability was observed in the N-terminal sequence of RNAase BS-1, whereas in the corresponding region of RNAase A, where cleavage occurs, the profile shows a trough.

Discussion

The structural relationships between seminal RNAase BS-1 and pancreatic RNAase A are immediately evident when one considers that RNAase BS-1 is composed of two identical peptide chains which are homologous to the chain of RNAase A. It could be argued that the structural differences between the two proteins would be minimized were the comparison made between RNAase BS-1 and a dimeric derivative of RNAase A [24,25], or alternatively between

RNAase A and a monomeric derivative of RNAase BS-1 [5]. These structural relationships are reflected in the similarities between the catalytic properties of pancreatic and seminal RNAases [26]. The active sites of the 2 enzymes however, should not be considered functionally equivalent [27]. Furthermore, the active site of RNAase BS-1, at difference with RNAase A, is active on double-stranded RNA and DNA-RNA hybrids [28,29], even when the protein is monomerized [30,31].

As for the structural similarities of the monomer of RNAase BS-1 towards RNAase A it should be reasonable to suppose that they must be limited by the quaternary structure interactions that the monomer has to have with its partner monomer.

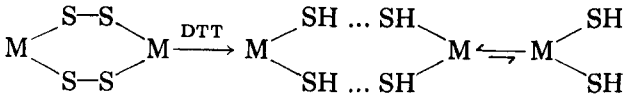
The observations reported in this paper indicate that while in some portions of the two molecules no differences can be detected, in other regions quite distinct conformations occur. Whereas in fact the C-terminal regions of the pancreatic and the seminal enzymes appear to be very similar, and probably identical, as they fit in an identical way to the active site of pepsin, this is not true for the N-terminal regions of the molecules, as was found using subtilisin as a proteolytic probe.

The possibility that the resistance of RNAase BS-1 to subtilisin, under conditions in which RNAase A is sensitive, is due to the dimeric structure of the seminal enzyme [19], should now be discarded. RNAase BS-1 maintains its resistance even after monomerization. On the other hand the bend probability curve calculated for the N-terminal sequence of RNAase BS-1 allows the prediction of a distinct conformation for the region where cleavage would be expected, different from the conformation of the corresponding region of RNAase A and of several other pancreatic ribonucleases also sensitive to selective subtilisin attack [21]. In particular it can be suggested that the replacement in RNAase BS-1 at position 19 of Pro for Ala plays a significant role in making this portion of the molecule incompatible with the active site of subtilisin (see bottom curve of Fig. 4).

When trypsin is used as a proteolytic probe, an apparently different picture results. The monomer of RNAase BS-1 is resistant to proteolysis as long as it holds to its partner monomer in the dimeric structure, but becomes sensitive immediately the covalent intersubunit bonds are cleaved. Hydrophilic residues are generally exposed in proteins and the resistance of certain lysyl and arginyl bonds to trypsin can be cue to hydrogen bonding between their peptide carbonyls and other side chains. This has been suggested in order to explain the resistance of RNAase A to tryptic hydrolysis [32] and may be true for native RNAase BS-1 as well. The sensitivity to trypsin of the monomeric derivative of RNAase BS-1 could thus be ascribed to a conformational change brought about by dissociation upon the structure of the RNAase BS-1 subunit. Furthermore the results would indicate that the conformation of the isolated monomer of RNAase BS-1 is distinct from that of the homologous RNAase A.

An alternative explanation for the susceptibility to trypsin of the protein upon dissociation may be that potentially susceptible bonds are hidden at the subunit-subunit interfaces in the dimeric structure. These bonds would become exposed and available to trypsin only after dissociation. As for the observation that the rate of hydrolysis is higher for the selectively reduced dissociated pro-

tein than for the stable monomeric derivative (see Fig. 1), this is very probably due to the concerted action of trypsin and of dithiothreitol. The selective reduction of the intersubunit disulfides of RNAase BS-1 has been described as follows [5]:



where M is the monomer of RNAase BS-1 and DTT is dithiothreitol. The products are a monomeric derivative and a non-covalent dimer stabilized by secondary forces, in equilibrium. As the monomeric form is sensitive to trypsin, the proteolytic action would have two effects: it would withdraw M-$(SH)_2$, shifting the equilibrium to the right; it would also make the intrachain disulfides of M-$(SH)_2$ available to cleavage by dithiothreitol. This would in turn promote unfolding of the protein, rendering it even more sensitive to tryptic action, with a resulting enhancement of the overall rate of the hydrolytic reaction.

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