The activity on double-stranded RNA of aggregates of ribonuclease A higher than dimers increases as a function of the size of the aggregates

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Stable bovine RNase A aggregates larger than dimers (identified as trimers, tetramers, pentamers and hexamers) were obtained by lyophilization of RNase A from 40–50 % acetic acid solutions. The RNase activity of these aggregates was compared with that of monomeric RNase A on single- and double-stranded polyribonucleotides. Their activity toward poly(U) and yeast RNA

slightly decreases as a function of the size of the aggregates. In contrast, their action on poly(A).poly(U) as substrate progressively increases from a relative activity of 1 for the RNase monomer to 10 for the hexamer. These results are discussed in the light of an already advanced hypothesis about a possible mechanism of RNase attack on double-stranded RNA.

INTRODUCTION

Lyophilization of bovine pancreatic RNase A (EC 3.1.4.22) from 50 % aq. acetic acid produces aggregates, the simplest of which have been characterized as dimers by Crestfield and colleagues [1–4], who proposed a model for the artificial dimeric RNase [3,4]. Dimers of RNase A were also obtained by cross-linkage with dimethyl adipimidate [5] and dimethyl suberimidate [6,7]. Cross-linked dimers of RNase A have, on one hand, a significant anti-tumour action [8,9]; on the other, they show [7], as the aggregated dimers [10,11], a definitely stronger degrading activity than native monomeric RNase toward double-stranded polyribonucleotides and DNA–RNA hybrids. This activity was initially thought to be due to the acquired dimeric structure of the enzyme, the two active sites of the dimers possibly acting simultaneously along the RNA double-helix [10,12]. Later, it was ascribed to the higher density of basic charges of the RNase dimer in respect to the monomer [11]. Several mammalian RNases bearing a higher number of positive charges than bovine RNase A were indeed shown to degrade double-stranded RNAs [13,14] and DNA–RNA hybrids [15] more efficiently than the latter enzyme. This activity was then interpreted as the consequence of the helix-destabilizing activity shown by those basic RNases, which is remarkably higher than that of bovine RNase A [13,14].

By a procedure similar to that outlined by Crestfield et al. [1,2] we have now obtained dimers and higher aggregates which were separated by gel filtration and identified as trimers, tetramers, pentamers and hexamers of RNase A. Their enzymic activity was assayed using single- and double-stranded RNAs as substrates with the following results. The activity toward poly(U) and yeast RNA slightly decreases going from the RNase A monomer to its hexamer, while that on double-stranded poly(A).poly(U) markedly increases as a function of the size of the aggregates.

MATERIALS AND METHODS

Preparation of RNase A oligomers

Bovine ribonuclease A (Type XII-A) was purchased from Sigma. Its concentration in solution was estimated spectrophotometrically on the basis of an $\epsilon_{280}^{1\%}$ of 7.3 [16]. According to the procedure of Crestfield et al. [1], RNase A dimers and higher

aggregates were obtained by lyophilization of the enzyme from 40 or 50 % acetic acid solutions, containing 5 mg of RNase per ml. The lyophilized material was dissolved in a minimal amount of 0.2 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.7: final concentration of RNase A and its aggregates was always between 35 and 40 mg/ml. Aliquots of the dissolved material, corresponding to about 5–6 mg of RNase A, were applied to a Pharmacia Superose 12 HR 10/30 column (in a Pharmacia FPLC System) equilibrated with 0.2 M sodium phosphate buffer, pH 6.7. Elution was performed with the same buffer at a flow rate of 0.05 ml/min at 25 °C (Figure 1). The various RNase aggregated species were identified using proteins of known molecular mass as calibration standards. These were Bio-Rad products: bovine thyroglobulin (670 kDa) and globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B_{12}

Figure 1 Elution pattern of the aggregates of RNase A

An aliquot of the lyophilized material (dissolved in 0.2 M sodium phosphate buffer, pH 6.7) corresponding to 5–6 mg of RNase A was applied to a Superose 12 HR 10/30 column (Pharmacia FPLC system) equilibrated with the same buffer. Elution was performed with 0.2 M sodium phosphate buffer, pH 6.7, at a flow rate of 0.05 ml/min at 25 °C. H, P, TT, T, D and M indicate hexamer, pentamer, tetramer, trimer, dimer and monomer of bovine RNase A, respectively. For details, see the Materials and methods section.

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(1.35 kDa). The pattern of gel filtration experiments was easily reproducible under the experimental conditions defined above.

Substrates

Yeast RNA, purchased from Sigma, was purified according to the method of Blackburn et al. [17]. Poly(A).poly(U) and poly(U) (sodium salt) were also Sigma products.

Enzymic assays

The activity towards yeast RNA was determined by the spectrophotometric assay of Kunitz [18] with 0.5 mg/ml of RNA in 0.1 M sodium acetate/acetic acid buffer, pH 5, at 25 °C. Enzyme concentration was 0.5 µg/ml for all RNase A species. Specific activity values were calculated as Kunitz units/mg of protein [18].

Enzymic degradation of double-stranded poly(A).poly(U) was determined spectrophotometrically according to Libonati and Floridi [10] in 0.15 M sodium chloride/0.015 M sodium citrate, pH 7, at 25 °C. Substrate concentration, calculated at pH 7 on the basis of its absorbance at 260 nm, was 0.11 mM in phosphodiester groups. The concentration of the various enzyme species ranged from 0.6 (hexamer) to 5.5 (monomer) µg/ml.

Enzyme activity toward single-stranded poly(U) as substrate was measured spectrophotometrically as with poly(A).poly(U). The concentration of poly(U) was 0.11 mM in phosphodiester groups; that of the various enzyme species was always 0.5 µg/ml.

All the assay mixtures also contained about 0.5 mM orthophosphate, which was present (see elution buffer composition) in the enzyme solutions used.

Spectrophotometric measurements were performed with a Hitachi U-2000 spectrophotometer. With poly(A).poly(U) or poly(U) as substrates, the changes in absorbance associated with cleavage of the nucleic acid were continuously monitored. The increases in absorbance per min at 260 nm were deduced from the slope of the linear part of the recordings. Enzyme units were calculated (measuring initial velocities under conditions where $[S] \ll K_m$) as change in absorbance per min/total measurable change as described [19–21]. Specific activity was defined as units/mg of protein. The RNase A monomer used in all assays was the enzyme species recovered by gel filtration of the lyophilized material (see above).

Table 1 Identification of bovine RNase A aggregates

RNase A	Protein molecular mass (Da)		Relative amounts (%)‡
	Calculated*	Found†	
Monomer	13 680	12 330 ± 1000	41.73 ± 11.21
Dimer	27 360	27 551 ± 630	13.56 ± 1.93
Trimer	41 040	37 573 ± 2490	24.00 ± 3.15
Tetramer	54 720	52 157 ± 3530	12.25 ± 6.28
Pentamer	68 400	67 142 ± 3500	5.37 ± 2.38
Hexamer	82 080	85 467 ± 2050	3.01 ± 1.12

* Anhydrous molecular mass calculated from the amino acid composition [20]

† Each value is the average (± S.D.) of molecular mass determinations performed in three FPLC experiments (see the Materials and methods section).

‡ Each value is the average (± S.D.) of measurements made in six FPLC experiments (see the Materials and methods section).

Figure 2 Degradation of poly(A).poly(U) by monomeric RNase A and its aggregates

Assay mixtures (0.15 M sodium chloride/0.015 M sodium citrate, pH 7) contained 0.11 mM (in phosphodiester groups) poly(A).poly(U) and amounts of the various RNase A species ranging from 5.5 µg/ml for the monomer to 0.6 µg/ml for the hexamer. Spectrophotometric assay at 25 °C was performed as described [10]. Units are defined as change in absorbance/min divided by total measurable change. The data represent means (± S.D.) of two independent experiments (performed with different enzyme preparations) with incubation mixtures prepared in duplicate.

RESULTS

RNase A aggregates

By gel filtration of RNase A lyophilized from aq. 40–50 % acetic acid through a calibrated Superose FPLC column, several species of aggregates were separated and identified besides the well-known dimers characterized by Crestfield and colleagues [1–4]. We found trimers, tetramers, pentamers and hexamers of RNase A (Table 1 and Figure 1). They were eluted (see the Materials and methods section) in about 5 h total, the elution of the highest aggregate occurring after approximately 3.5 h, when about 10.5 ml of the eluent had flowed through (Figure 1). The separation of the various aggregates from one another was rather good, the dimer and trimer being the only species whose resolution was not always quite satisfactory. The relative amounts of the aggregates decrease from the monomer to the hexamer, with the exception of dimeric and trimeric RNase A. The relative quantity of the former was indeed constantly about 40 % lower than that of the latter (Table 1). Anyhow, the relative amounts of the aggregates could be a variable depending on experimental conditions (mainly enzyme protein concentration), and is presently under investigation.

Stability of the aggregates

The stability of RNase A aggregates is quite high, at least at low temperature. The lyophilized material, dissolved in 0.2 M sodium phosphate buffer, pH 6.7, at a concentration of 35–40 mg/ml, can be kept at 4 °C for more than 1 month or frozen (–20 °C) for more than 2 months without any change of its gel filtration pattern. Also, under the same conditions the enzyme activity of each RNase species did not change.

Figure 3 Activity of bovine RNase A monomer and its aggregates on poly(U) or yeast RNA

RNase activity, expressed as units/mg of protein, on poly(U) was determined as described in the legend to Figure 2. Enzyme concentration was 0.5 μ g/ml for all RNase species. RNase activity on yeast RNA, expressed as units/mg of protein, was determined according to Kunitz [18]. Enzyme concentration was 0.5 μ g/ml for all RNase species. The data are means of two independent experiments (performed with different enzyme preparations) with incubation mixtures prepared in duplicate.

Catalytic properties of the RNase A aggregates

The activity of monomeric RNase A was compared with that of its aggregates in side-by-side assays performed with single- or double-stranded polymeric substrates.

Figure 2 shows the results obtained by using the double-helical poly(A).poly(U) as substrate for the RNase monomer and its aggregates. Enzymic activity increases remarkably as a function of the size of the aggregates, varying, in terms of relative activity, from 1 for the monomer to 10 for the hexamer. The action of the various RNase species on single-stranded poly(U) and yeast RNA as substrates is shown in Figure 3. While the enzyme activity of the smaller aggregates on poly(U) is similar to that of monomeric RNase A, that of the larger aggregates (pentamer and hexamer) seems to be lower. This trend of the enzyme specific activity values to decrease going from RNase A monomer to the higher aggregates becomes more definite when yeast RNA is used as substrate. Indeed, the activity of tetramer, pentamer and hexamer is about 52% that of monomeric RNase A.

DISCUSSION

Bovine RNase A samples may contain aggregates [1,22], the amount of which can be markedly increased by lyophilization of the enzyme from 50% acetic acid solutions [1,23]. By this procedure Crestfield and colleagues [1–4] obtained and characterized RNase A dimers. By the same procedure we have now obtained and identified, besides the dimers, RNase trimers, tetramers, pentamers and hexamers, the activity of which was assayed toward single- and double-stranded RNAs. The results were that, while a slight decrease could be observed in enzyme activity against single-stranded poly(U) and a more definite one against yeast RNA as a function of the size of the aggregates (Figure 3), degradation of double-stranded poly(A).poly(U) markedly increases going from the RNase A monomer to its hexamer (Figure 2). The peculiar enzymic properties of the RNase A aggregates are clearly not explainable as simply due to

the additive effect of single active-site activities. On one hand, the lower activity on single-stranded RNAs of the aggregates in comparison with monomeric RNase A could be explained by a decreased catalytic efficiency (per active centre) of the oligomeric enzyme, the active sites of which may not all be necessarily available. On the other hand, the activity of the RNase aggregates on poly(A).poly(U), increasing as their size increases, cannot be due either to a simple addition of active-site actions. It will therefore be discussed in some detail. The observation that bovine RNase A acquires a significant ability to degrade double-stranded polyribonucleotides when the enzyme is in the form of aggregated dimers is not new [10]. This observation, together with the finding that native dimeric bovine seminal RNase is capable of efficiently cleaving double-helical RNA [10] and DNA–RNA hybrids [15], led us to propose that an efficient degradation of double-stranded RNA by RNases non-specific for this RNA species could require a dimeric enzyme, the two active sites of which could act simultaneously along the two strands of the RNA duplex [10,12]. However, subsequent observations invalidated this hypothesis. The first one was that artificially monomerized bovine seminal RNase maintained about 40–50% of the degrading ability of the native dimer [24]. A second observation was that other mammalian RNases, structurally and catalytically similar to bovine RNase A but sharing with bovine seminal RNase the property of bearing a higher number of positive charges than RNase A, showed an activity toward double-stranded RNA significantly higher than that of the latter enzyme [13]. This finding was strengthened by experiments performed by Wang and Moore with a highly basic derivative of bovine RNase A, prepared by linking with dimethyl suberimidate eight spermine residues to the RNase molecule [16]. This polyspermine-ribonuclease was 115 and 382 times as active as the parent enzyme toward poly(A).poly(U) and the hybrid substrate poly(dA).poly(rU), respectively [16].

A third observation was that RNases more basic than RNase A, as well as RNase A dimers, are capable of destabilizing double-stranded DNAs more efficiently than native bovine RNase A [14,25,26].

On the basis of these facts, the idea was advanced that the efficiency of the RNase attack on secondary structures of RNA could be ascribed to the helix-unwinding action of these basic RNase molecules (higher than that shown by bovine RNase A, and a function of the number of positive charges present on the RNase protein) as an event necessary for their activity on double-stranded RNA [13,27]. Bovine RNase A, as suggested by Jensen and von Hippel [28], can destabilize the DNA double-helix by 'trapping' open single-stranded loops generated by spontaneous thermal fluctuations, and it has been shown that this action is related to the multiplicity of phosphate-binding subsites of the enzyme protein [29]. It must be pointed out here that spontaneous base-opening events also occur in double-helical RNA and involve a small number of base pairs [30]. It might be reasonable therefore to think that enzymic cleavage of double-stranded RNA could occur as the consequence of a preferential binding of the RNase molecule to short transiently exposed single-stranded sequences of the RNA duplex [27]. The enzyme could also bind single nucleotides, wound off the double-helix because of thermal fluctuations, and cleave the relative phosphodiester bond, as proposed by Yakovlev et al. [31,32]. In conclusion, an efficient degradation of double-stranded RNA could be ascribed to the number and co-operativity of the electrostatic interactions between the phosphates of the substrate and specifically located positive groups of the RNase molecule. Indeed, human pancreatic-type RNases (the enzymes from pancreas and semen), which show a very remarkable activity on

double-stranded RNA and a conspicuous destabilizing action on DNA [14,19], have their positive amino acid residues localized in the vicinity of their active site, where therefore a strong positive electrostatic potential can develop [19]. In particular, these enzymes have three basic residues (Arg-4, Lys-6, Arg-32) where in RNase A three neutral amino acids are present instead [33,34]. In contrast, human non-pancreatic-type RNases (liver RNase, ECP, EDN), although highly basic proteins [34,35], have their basic amino acid residues localized in a region which is distant from the active site. Accordingly, these enzymes do not show any activity on double-stranded RNA nor a destabilizing action on DNA [19–21]. The high DNA helix-unwinding action and the efficient activity against double-stranded RNA shown by other mammalian pancreatic-type RNases more basic than bovine RNase A [13,26] could be explained in a similar manner.

We think that the data obtained with poly(A).poly(U) as substrate in the experiments described fit the frame outlined above rather well. It is not easy to envisage a model for RNase A aggregates higher than dimers which could fit that proposed by Fruchter and Crestfield for dimeric RNase A [3,4]. However, the positive electrostatic potential of an oligomeric RNase molecule in its interaction with a double-helical RNA is undoubtedly higher than that developing in the interaction between that substrate and a monomeric RNase. Moreover, it can increase as a function of the number of the subunits composing the aggregate. The increasing activity of the artificial RNase oligomers on poly(A).poly(U) observed as their size was increased could therefore be the consequence of the higher binding efficiency of the aggregates to the transiently exposed single-stranded loops of the double-stranded substrate.

In conclusion, the activity of RNase A aggregates on double-stranded poly(A).poly(U) could fall within the phenomena interpreted by the 'rule' that a high activity of a RNase towards secondary structures of RNA can be related to a high number of positive charges localized in the active-site region of the enzyme [19,27].

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