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Cross-linked trimers of bovine ribonuclease A: activity on double-stranded RNA and antitumor action

Giovanni Gotte[a], Lucia Testolin[b], Chiara Costanzo[a], Salvatore Sorrentino[c], Ubaldo Armato[b], Massimo Libonati[a,*]

[a]*Institute of Biological Chemistry, Faculty of Medicine, The University of Verona, Strada Le Grazie 8, I-37134 Verona, Italy*
[b]*Institute of Anatomy and Histology, Faculty of Medicine, The University of Verona, Strada Le Grazie 8, I-37134 Verona, Italy*
[c]*Department of Organic and Biological Chemistry, The University of Naples, Via Mezzocannone 16, I-80134 Naples, Italy*

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Abstract Trimers of bovine pancreatic RNase A were obtained by cross-linking native RNase A with dimethyl suberimidate. They degrade double-stranded RNA more efficiently than dimers and monomers of RNase A, and display significant cytotoxic and/or cytostatic actions against C4-I cells (a human cell line derived from squamous carcinoma of the uterus cervix). On the same cell line cross-linked dimers of RNase A appear to be ineffective.

Key words: RNase A; RNase A oligomer; Double-stranded RNA; Tumor; Antitumor action

1. Introduction

A distinctive feature of the RNA double-helix is its resistance to digestion by bovine RNase A in 0.15–0.20 M NaCl solutions [1,2]. Under the same conditions dimers and higher aggregates of RNase A obtained by lyophilization from 30–50% acetic acid solutions according to the procedure of Crestfield et al. [3] are instead able to degrade double-stranded RNA (dsRNA) with an efficiency that increases as a function of the molecular mass of the aggregate(s) [4]. A remarkable degrading activity towards dsRNA was also shown by RNase A dimers obtained by cross-linkage with dimethyl suberimidate [5]. These dimers, at difference with those obtained by simple aggregation [3,4] are stabilized by a cross-link formed by the bifunctional reagent between lysine residues of two RNase monomers [5]. The novel catalytic activity acquired by RNase A in form of dimers, as well as the degrading ability shown towards dsRNA by other ribonucleases more basic than native RNase [6–8]A have been interpreted as due to a preferential binding of the various RNase species to short, transiently exposed single-stranded RNA sequences which are then degraded [4,6,7]. The extent of the 'destabilizing' event (and therefore of the degradation) seems to be correlated with the number (or density, as it occurs with aggregated or cross-linked RNase A oligomers), and location of positive charges present on the enzyme protein [4,6–9].

Cross-linked dimers of bovine RNase A have also been shown to display an antitumor action both in vitro and in vivo [10,11]. A similar, but definitely higher antitumor activity [12,13], as well as other biological actions [14,15] are shown by bovine seminal RNase (BS RNase), which is the only native dimeric ribonuclease known thus far [16]. This enzyme is also able to degrade efficiently dsRNA [17] and DNA-RNA hybrids [18]. Recently it was obtained a stable dimeric mutant RNase A, homogeneous and catalytically active, which displayed a selective toxicity for tumor cells similar to, but lower than, that of BS RNase [19].

Aim of the present work was to see whether cross-linked oligomers of bovine RNase A higher than dimers could show, in comparison with native RNase A and its dimers, a higher activity towards dsRNA and possibly an increased antitumor action. Both these features have been verified for trimeric RNase A.

2. Materials and methods

2.1. Preparation of RNase A cross-linked oligomers

Bovine RNase A (type XII-A), purchased from Sigma, was cross-linked with dimethyl suberimidate under conditions identical with those described by Wang et al. [5], who have carefully characterized the dimers produced in the reaction, also mentioning the presence of higher unresolved oligomers. The reaction was carried out for 15 min at pH 8.0 and 21°C with 1.25 mol equiv. of the diimido ester and a protein concentration of 6%. The unreacted reagent was quenched by the addition of 20 equiv. of ammonium acetate (as 0.2 M solution) per initial imido ester group. The mixture was then applied to a Pharmacia Superdex 75 HR 10/30 column (in a Pharmacia FPLC System) equilibrated with a 0.15 M NaCl/0.05 M sodium phosphate buffer, pH 7. Elution was performed with the same buffer (flow rate, 0.1 ml/min at 25°C). Monomeric, dimeric, trimeric RNase A, as well as a small amount of tetrameric RNase A comprised in an unresolved mixture of higher oligomers, were identified (Fig. 1) using proteins of known molecular mass as calibration standards. These were Sigma products: bovine serum albumine (66 kDa), carbonic anhydrase (29 kDa), horse heart cytochrome C (12.4 kDa) and bovine lung aprotinin (6.5 kDa). To the mixture Dextran Blue (2000 kDa) was also added. The various RNase A species were also analyzed and identified by SDS-PAGE. Aggregated dimers (for which a molecular model has been proposed by Fruchter and Crestfield [20,21]) and trimers of bovine ribonuclease A were also prepared according to the procedure already described [3,4]. The concentration of RNase A was estimated spectrophotometrically on the basis of $\varepsilon_{280}^{1\%}$ of 7.3 [3].

2.2. Substrates

Yeast RNA, purchased from Sigma, was purified according to Blackburn et al. [22]. The double-helical poly(A).poly(U) (sodium salt) was also a Sigma product.

2.3. Enzymic assays

The activity towards yeast RNA was determined by the spectrophotometric assay of Kunitz [23], with 0.5 mg/ml of RNA in 0.1 M sodium acetate/acetic acid buffer, pH 5.0, at 25°C. Enzyme concentration was 0.5 μg/ml for all RNA species. Specific activity values were calculated as Kunitz units/mg protein. Enzymic degradation of poly(A).poly(U) was determined spectrophotometrically [16] in 0.15 M sodium chloride/0.015 M sodium citrate, pH 7, at 25°C. Substrate concentration was 0.11 mM in phosphodiester groups. The concen-

*Corresponding author. Fax: +39 (45) 8098170.
E-mail: Libonati@borgoroma.univr.it

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tration of the various enzyme species ranged from 3 μg/ml (trimer) to 12 μg/ml (monomer). Spectrophotometric measurements were performed with a Beckman DU 650 spectrophotometer. The changes in absorbance following poly(A).poly(U) cleavage 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 $[S] \ll Km$) as change in absorbance per min/total measurable change [3,6]. Specific activity was defined as units/mg protein. The RNase monomer used in assays was always the enzyme species recovered from the gel filtration experiment.

2.4. Cell cultures

C4-I cells – a human cell line derived from squamous carcinoma of the uterus cervix [24] – were purchased from ATCC, Rockwille, MD, USA. They were maintained in DME (Sigma) culture medium supplemented with 5% (vol/vol) heat-inactivated (30 min at 56°C) fetal bovine serum (FBS, Hyclone, Celbio, Milan, Italy), and the antibiotics Penicillin-G (200 U/ml) and Streptomicin (200 μg/ml) (Gibco BRL, Milan, Italy). Cells were kept in a 5% CO_2/95% air incubator at 37°C, and 48 h before treatment were seeded in 24 well-plates (Costar Italia, Milan, Italy) at a density of 6×10^4 cells/well in 1 ml of culture medium. 100 μg/ml of the various RNase A oligomers (in DME) were added to the cells and their effects (cytotoxicity and/or cytostaticity) monitored every 24 h, up to 72 h.

2.5. Cell viability

Viability of both treated and control-untreated cells was assessed by both Trypan Blue exclusion and the MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide), slightly modified, assay [25]: every 24 h following treatment, 250 μl of the MTT solution (from a stock solution of 5 mg/ml in PBS) were added to the wells containing 1 ml of culturing DME medium. The cells were incubated at 37°C for 2 h to allow reaction to take place. Following the addition of 1 ml of lysing buffer (20% SDS in H_2O/50:50 dimethyl formamide, 2.5% of 80% acetic acid and 2.5% of 1 N HCl, pH 4.7), the MTT dyed cultures were incubated at room temperature for 15 min. Cell viability was estimated by measuring optical density in an ELISA scanner at 570 nm wavelength (vs. 630 nm).

3. Results

3.1. Cross-linked oligomers of RNase A

A gel filtration pattern of the cross-linked RNase A oligomers is shown in Fig. 1. The separation obtained is an example of one out of many similar, highly reproducible, experiments performed with the product of six different preparations of cross-linked RNase A oligomers. Molecular mass values and relative amounts of the different RNase A species appear in Table 1. Besides monomeric, dimeric and trimeric RNase A, some higher and poorly resolved oligomers were also present, among which an RNase A tetramer could be identified, but in very small amount and heavily contaminated by other species. Only the fractions around the peak of each RNase oligomer were collected and used in the following experiments, after SDS-PAGE analysis. The results obtained by this procedure were coherent with those of the gel filtration experiments (see Fig. 1 and Table 1). Single bands were detected whose positions corresponded to those expected for monomers, dimers and trimers of RNase A on the basis of the migration pattern of the proteins used as calibration standards (see Section 2). A band corresponding to a tetrameric form of RNase A was also present, although contaminated by other oligomeric species, as mentioned above.

Table 1
Identification of RNase A oligomers

RNase A	Protein molecular mass (Da)		
	Calculated[a]	Found[b]	Rel. amounts (%)[c]
Monomer	13820	13895 ± 1160	77.10 ± 2.18
Dimer	27640	29150 ± 2380	16.95 ± 1.65
Trimer	41460	42780 ± 3320	4.58 ± 0.41
Higher oligomers			1.39 ± 0.13

[a]Anhydrous molecular mass calculated from the amino acid composition, and increased by the presence of *n* residues of dimethyl suberimidate (M.W. of each residue, 140 Da; *n* equal to the polymerization degree).
[b]Each value is the average (± S.D.) of molecular mass determinations performed in 18 FPLC experiments (see Section 2).
[c]Each value is the average (± S.D.) of 7 measurements.

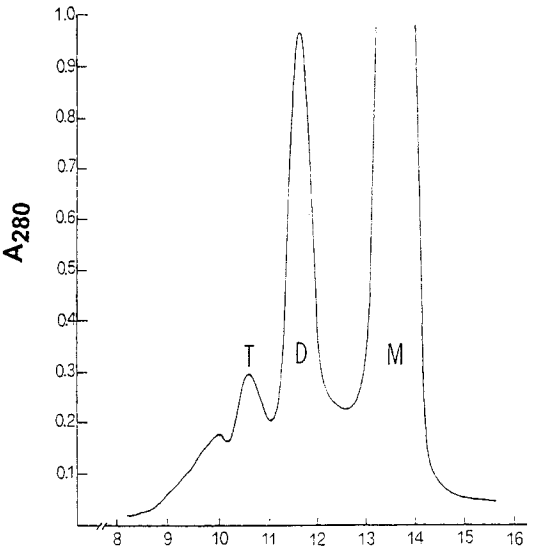

Fig. 1. Elution pattern of cross-linked oligomers of bovine RNase A. An aliquot (5.2 mg proteins) of a preparation of RNase A oligomers was applied to a Superdex 75 HR 10/30 column (Pharmacia FPLC System) equilibrated with 0.15 M NaCl/0.05 M sodium phosphate buffer, pH 7. Elution was performed with the same buffer at a flow rate of 0.1 ml/min at 25°C. T, D, M indicate trimer, dimer and monomer of RNase A. Higher, unresolved oligomers can also be noticed.

3.2. Enzyme activities of monomeric, dimeric and trimeric RNase A

RNase A monomer, dimer and trimer were dialysed against 2×10^{-4} M NaCl, and concentrated. The action of these stable, cross-linked oligomers of RNase A towards poly(A).poly(U) was assayed in parallel with that of the corresponding oligomers obtained by simple aggregation [3,4] (Fig. 2a). Degradation of dsRNA increased as a function of the molecular mass of the RNase A oligomers, but the cross-linked species were about 30% less active than the corresponding aggregated oligomers. While with the double-helical substrate cross-linked or aggregated dimers and trimers of RNase A are definitely more active than the RNase A monomer, the contrary is true with yeast RNA as a substrate (Fig. 2b), in agreement with what previously reported with aggregated RNase A oligomers [4].

3.3. Action of RNase A oligomers on C4-I cells

The action of trimeric RNase A on C4-I cells is shown in Fig. 3. Experiments were performed by adding 100 μg/ml of the various RNase A species to the cell culture. The biological effects were determined every 24 h by Trypan Blue exclusion. As shown, treatment of C4-I cells with both the monomeric (M) and dimeric (D) forms of RNase A as well as with native

RNase A (A) did not induce significant changes in cell survival: up to 72 h the number of alive treated cells – similar to that of the control (C) – remained close to 100%. On the contrary, cross-linked trimers of RNase A (T) induced a rapid and massive increase in the number of Trypan Blue positive cells. 24 h after treatment was started, alive cells were about 68%, this indicating a cytotoxic effect of the RNase A trimer on C4-I cells. However, 48 and 72 h following treatment only a small further decrease in the number of alive cells could be observed, which could be interpreted more as a cytostatic-like effect. Cell viability was also assessed by the MTT assay (data not shown), which did not show a decrease in cell metabolic activity. The same was true for C4-I cells treated with monomeric and dimeric RNase A or with native RNase A. It has also to be mentioned here that one out of six preparations of RNase A oligomers, although catalytically active on poly(A).poly(U) and yeast RNA, was found to be ineffective on C4-I cells.

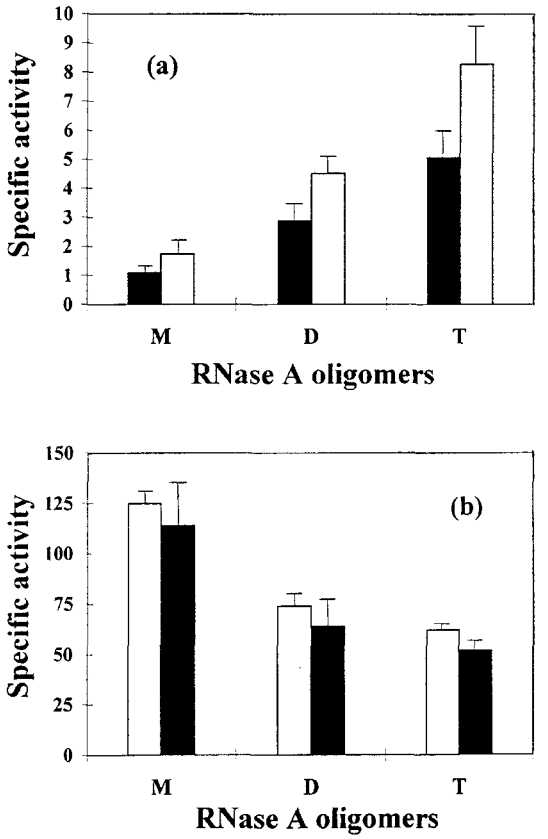

Fig. 2. Activity of cross-linked oligomers of RNase A on double- and single-stranded RNAs. a: Monomers and cross-linked dimers and trimers of RNase A (dark bars) were assayed towards double-stranded poly(A).poly(U) side-by-side with the corresponding aggregated species (empty bars). Standard deviations are indicated. Substrate concentration, 0.11 mM in phosphodiester groups. Enzyme concentration: trimer, 3 μg/ml; dimer, 5.5 μg/ml, monomer, 12 μg/ml. Temp., 25°C. Spectrophotometric assay performed as described [17] (see also Section 2). The data with cross-linked oligomers are means of 7 independent experiments; those with the aggregated oligomers [3] are means of 5 independent experiments. b: Activity (Kunitz assay [23]) of monomeric RNase A and cross-linked (dark bars; means of 4 independent experiments) or aggregated (empty bars; means of 3 independent experiments) RNase A oligomers on yeast RNA. Standard deviations are indicated. Substrate concentration, 0.5 mg/ml. Enzyme concentration, 0.5 μg/ml. Temp., 25°C.

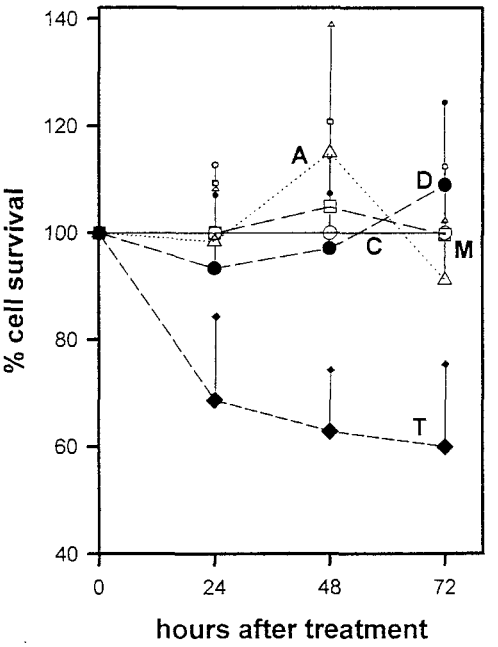

Fig. 3. Antitumor activity of cross-linked RNase A oligomers. To cultures of C4-I cells 100 μg/ml of native RNase A (△, A), monomeric RNase A recovered from the gel filtration column (□, M), dimeric (●, D) and trimeric (◆, T) RNase A were added; (○, C = Control) untreated cells. Cells were harvested every 24 h up to 72 h, and their viability was assessed by Trypan Blue exclusion. The data represent means (±S.E.) of 11 experiments performed with the various RNase A species obtained in 5 different preparations of RNase A oligomers.

4. Discussion

Trimers of bovine RNase A, prepared by cross-linkage with dimethyl suberimidate, show a definite activity, higher than that of RNase A dimers and monomers, towards dsRNA. They also show a remarkable antitumor action in vitro. With the cell line (C4-I) used by us, dimeric RNase A appears instead to be ineffective (see Fig. 3), contrary to what reported by others, but with different cell lines [10,11].

4.1. Catalytic activity of RNase A trimers towards dsRNA

It is known that the RNA double-helix in its canonic form protects dsRNA from base-catalyzed cleavage since the stereochemical requirements for the transesterification reaction cannot be met [2,26]. DsRNA is indeed resistant to digestion by pancreatic ribonuclease A at physiological ionic strength. However, aggregated oligomers (from dimers up to hexamers) of bovine RNase A – the prototype of single-strand-preferring RNases (6,7) – cleave dsRNA with an efficiency that increases exponentially as a function of the molecular mass of the aggregates [4]. RNase A in form of cross-linked dimers was also shown to acquire a significant activity towards dsRNA [5] and DNA-RNA hybrids [27]. On the other hand, several mammalian single-strand-preferring ribonucleases of the pancreatic type, monomeric but endowed with a higher basicity than bovine RNase A, are also able to degrade dsRNA with a remarkably high efficiency [6–8]. These results have been explained on the basis of the following arguments. A correlation was demonstrated between the number (or density) and location (in the region of the active site) of the positive charges

present on various RNases and their ability to destabilize double-helical polydeoxyribonucleotides [6–9]. This destabilizing action (in the terms proposed by Jensen and von Hippel [28] for RNase A), that can be envisaged to also occur with dsRNA, would in this case represent the necessary event for the enzymic cleavage of the resulting single-stranded RNA sequences [4,6,7]. Here we show that a cross-linked trimer of bovine RNase A has an activity towards dsRNA higher than that of cross-linked dimers. This fact can easily be explained in the light of the hypothesis mentioned above. RNase A oligomers, and pancreatic-type RNases more basic than RNase A could indeed be regarded as nucleic acid-melting proteins which can bind short sequences of dsRNA transiently made single-stranded by the dynamic 'breathing' of the RNA double-helix [2,6,7]. A complementary model, consisting in the binding by the RNase of single, wound off ribonucleotides, has been proposed by Yakovlev et al. [29]. As for the lower activity, in comparison with that of monomers, of cross-linked dimers and trimers of RNase A on yeast RNA, it agrees with what was observed with aggregated RNase A oligomers [4] and could be ascribed to a lower catalytic efficiency (per active center) of the oligomeric form of the enzyme, whose active sites might not all be simultaneously available.

4.2. *Cytotoxic and cytostatic action of RNase A trimers on C4-I cells*

Bartholeyns and Baudhuin [10], and others [11] have shown that cross-linked dimers of RNase A have a significant cytostatic action against tumor cells both in vitro and in vivo, similar but definitely lower than that displayed by BS RNase [12,13], which is the only known ribonuclease dimeric in nature [16]. Recently, Di Donato et al. [19] have prepared an RNase A mutant that spontaneously dimerizes forming molecules structurally similar to BS RNase, and endowed with a similar although lower antitumor activity.

In this work we have shown that cross-linked trimers of RNase A, added to a human cell line derived from squamous carcinoma of the uterus cervix, display a remarkable cytotoxic effect in the first 24 h following treatment. From 24 h up to 72 h a cytostatic-like effect could instead be observed. No change in the metabolic activity of C4-I cells could be measured by the MTT assay. However, this could just mean an increase in the metabolic activity of the surviving cells. All together these results cannot be easily explained, particularly if one takes into account that, contrary to what was reported using different cell lines [10,11], dimeric RNase A seems to be ineffective on C4-I cells. At the moment we lack any information about the fate of cross-linked RNase A trimers in the cell culture, and the manner in which they interact with the cells. Moreover, we also do not know whether their cytotoxic and cytostatic actions could in some way be related to their activity towards dsRNA. Therefore, the hypotheses already advanced to explain the biological actions of BS RNase [30,31], the dimeric RNase A mutant [19] or the cross-linked dimers of bovine RNase A [10,11] cannot be easily applied to the results reported here. However, one point seems to be clear, i.e. that the cytotoxic and/or cytostatic actions of those ribonucleases cannot be ascribed (besides other possible determinants) to the dimeric nature of the enzymes. More than the dimeric structure of the proteins in itself, the insensitivity of the dimeric biologically active form of BS RNase and of the dimeric mutant of RNase A to the RNase inhibitor is a feature that could correlate with the antitumor action of these enzymes [19,30–32]. The RNase inhibitor binds indeed tightly to the monomeric forms of these RNases. Thus, a particular dimeric structure of the enzyme could represent a sterical obstacle for the specific amino acid residues responsible for the binding of the ribonuclease to the RNase inhibitor. This idea could indeed be extended to the cross-linked trimers of bovine RNase A obtained by us. An example pertinent to this point could be the observation that onconase (a *monomeric* ribonuclease from *Rana pipiens* oocytes [33–35]) is resistant to the RNase A inhibitor, and displays a strong antitumor action [34,35]. Therefore, the intrinsic absence of proper residues necessary for the binding of the ribonuclease to the RNase inhibitor, as it occurs in onconase [35], or their sterical masking seem to be important prerequisites for ribonuclease cytotoxicity. With regard to these points and to the biological actions of ribonucleases in general [14,15], interesting can also be the observation [15] that angiogenin, a *monomeric* [36,37] member of the ribonuclease superfamily [38] shows an immunosuppressive activity that is quite similar to that displayed by the dimeric BS RNase.

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