The antitumor action of seminal ribonuclease and its quaternary conformations

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Abstract **It has been previously shown that the antitumor action of bovine seminal ribonuclease (BS-RNase) is dependent on its dimeric structure. However, two distinct quaternary structures, each in equilibrium with the other, have been described for the enzyme: one in which the two subunits exchange their N-terminal ends, the other with no exchange. Antitumor activity assays, carried out on homogeneous quaternary forms of the enzyme, as well as on dimeric mutants of bovine pancreatic RNase A, reveal that another structural determinant of the antitumor activity of BS-RNase is the exchange of N-terminal ends between subunits.**

Key words: Ribonuclease; Antitumor; Protein conformation; Protein engineering

1. Introduction

Bovine seminal RNase (BS-RNase) is a uniquely dimeric member of the vertebrate superfamily of pancreatic-type RNases, with two intersubunit disulfides linking identical subunits [1]. Along with several members of the superfamily, and RNases of other families from bacteria, fungi, and flowering plants, it belongs to a group of ribonucleases endowed with intrinsic special (i.e. other than catalytic) biological actions (RISBASES), which include such diverse and interesting biological actions as host defense, angiogenesis, pollen control, and antitumor action [2].

The antitumor action of BS-RNase, first discovered by Matousek [3], has been studied in vitro and in vivo on several cell lines and animal models [4,5]. Recently, a powerful antimetastatic activity of the protein has been observed in mice inoculated with Lewis lung carcinoma cells [6]. The mechanism of antitumor action of BS-RNase is still under study, but it has been determined that the dimeric structure of the enzyme protein is essential for its antitumor action [4].

However, two dimeric structures have been described for BS-RNase, which interconvert into each other, so that BS-RNase is an equilibrium mixture of two quaternary conformations [7]. One (termed M × M), is the conformation studied by X-ray crystallography [8], in which the subunits exchange their N-terminal segments; in the other (termed M = M), no exchange occurs. The ratio of M × M to M = M in native BS-RNase is 2:1 [7].

Here we report that the dimeric structure is a necessary, but not sufficient, determinant of the antitumor action of BS-RNase, as another structural determinant has been identified in the exchange of N-terminal ends between subunits.

2. Materials and methods

2.1. Proteins

Native BS-RNase was purified as described [9]. Homogeneous preparations of M × M and M = M subforms of BS-RNase were isolated as previously described [7]. The content of each form was measured [7] by gel-filtration, after selective reduction of the intersubunit disulphides, which liberates free monomers from the M = M form with no exchange, whereas the form with exchange (M × M) remains dimeric, albeit as a non-covalent dimer.

The PLCC-AA dimeric tetra-mutant of RNase A (A19P-Q28L-K31C-S32C-dimeric RNase A) was prepared as described [10]. The CC-AA mutant of the same protein (K31C-S32C-dimeric RNase A) was obtained by site-directed mutagenesis, and expressed and characterized by procedures to be published elsewhere, based on the procedures described for the preparation of recombinant BS-RNase [11] and of the PLCC-AA mutant of RNase A [10].

2.2. Antitumor assay

Protein cytotoxicity was assayed as described [4] on SVT2 cells, a malignant Balb C mouse fibroblast 3T3 line transformed by SV40 (ATCC, Richmond, VA, USA), grown in Dulbecco's modified Eagle's medium, supplemented with 10% FCS, 4 mM glutamine, 400 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were seeded in 24-well plates (2.5×10^4/well) in the presence of the protein to be tested. At 24-h intervals, the percent of cell survival, with respect to control cultures grown in the absence of the protein, was measured in triplicate. Controls on the absence of cytotoxicity of the RNases under study on the parent non-transformed 3T3 cells were routinely run. They consistently revealed no effects of the RNases on these cells.

3. Results and discussion

Homogeneous preparations of the two quaternary forms of BS-RNase, M × M (with exchange of the terminal ends between subunits) and M = M (with no exchange), prepared using an established procedure [7], were tested for antitumor activity. This is defined as selective cytotoxicity on malignant, transformed cells (SVT2-3T3 fibroblasts), with no effects on the parent line before transformation (3T3 fibroblasts). The antitumor activity was measured as the percent of cell survival after 72 h of growth in the presence of increasing concentrations of protein, compared to the survival of cells plated in the absence of the protein. As shown in Fig. 1A, the M × M form was found to affect the survival of malignant cells with about the same potency as the native enzyme, made up of both M × M and M = M forms in a ratio of 2:1. The M = M form instead appeared much less active.

After a series of dose–response experiments, values of IC_{50}, i.e. of the protein concentration producing an inhibition of 50%

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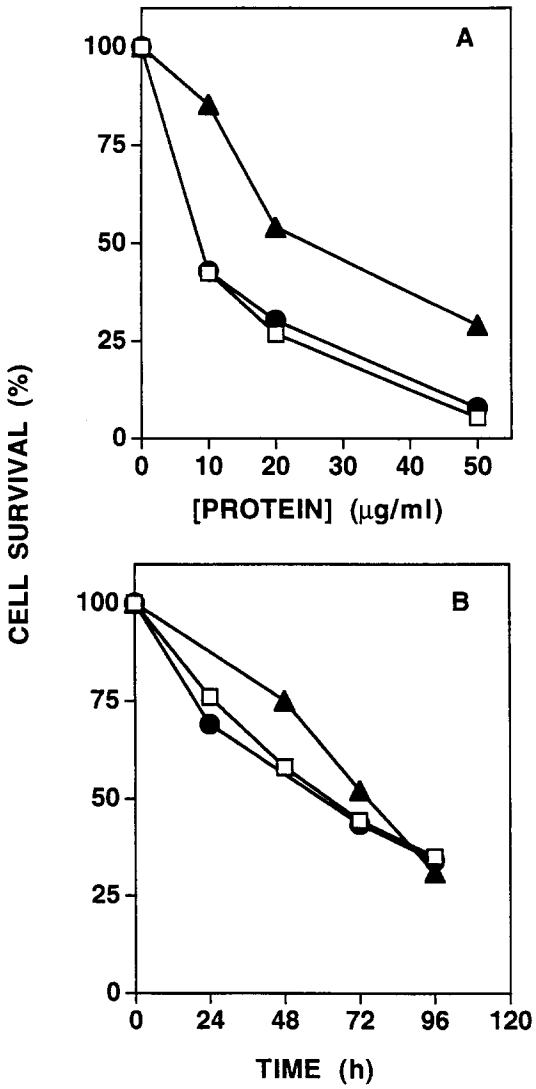

Fig. 1. Survival of SVT2 fibroblasts (A) grown for 72 h in the presence of increasing concentrations of BS-RNase (□), and of its M × M (●) and M = M (▲) forms, (B) determined from growth curves obtained in the presence of the proteins as in A, at a concentration of 30 mg/ml. Survival is expressed as percent of cell survival measured in the absence of added proteins.

of cell survival, were determined for the M = M and the M × M forms, and found to be 19 ± 3.2, and 8.7 ± 0.3 μg/ml, respectively. Under the same conditions the IC_{50} for BS-RNase was found to be 7.8 ± 0.7 μg/ml.

These results could indicate that the M = M form has a lower antitumor activity than that of the M × M form, or of native BS-RNase. However, a clearer picture was obtained when the antitumor activities of the M = M and of M × M forms were determined from growth curves obtained in the presence of 30 μg/ml of each protein. The results of these experiments, illustrated in Fig. 1B, show that while the activity of M × M is confirmed to be similar to that of native BS-RNase, that of M = M increases with time until at 96 h it approaches that of native BS-RNase, i.e. of the equilibrium mixture of M = M and M × M forms.

In fact, it has been found [7] that isolated, homogeneous preparations of metastable M = M and M × M forms interconvert into each other under the conditions of assay (several days at 37°C, pH 7), so that: (i) the homogeneous M = M form is increasingly converted into the M × M form; (ii) the homogeneous M × M form fully equilibrates within 24 h with the M = M form, thus generating the equilibrium mixture typical of native BS-RNase. Thus, under the assay conditions, (i) the activity of M × M would be the same as that of BS-RNase, as its partial transformation into M = M would render the resulting mixture undistinguishable from native BS-RNase, and (ii) the M = M form could have an activity even lower than that observed, or no activity at all, as only a continuously decreasing fraction of the original M = M form would be present at any time in the cell culture.

However, a definite conclusion as to whether the M = M form has low antitumor activity, or no activity at all, could not be reached, given the difficulty in setting up a rapid antitumor assay within a time frame in which little interconversion of forms occurs. Therefore, an alternative approach was employed, based on the results obtained with a mutant of pancreatic RNase A, a protein which shares more than 80% of its primary structure with BS-RNase subunit. In this study monomeric RNase A was rendered dimeric by introducing into its chain four residues present in the sequence of BS-RNase, and considered essential or important for the dimeric structure of the protein [8]. The residues introduced into the RNase A chain were a Pro, a Leu, and two Cys, at sequence positions 19, 28, 31, and 32, respectively. The dimeric mutant of RNase A (denoted PLCC-AA) also possessed a strong antitumor action [10].

The insertion of two Cys residues and of Leu-28 was clearly intended to provide the mutant with the ability to form intersubunit disulfides, and a strong hydrophobic interaction at the subunit interface, respectively. The insertion instead of Pro at

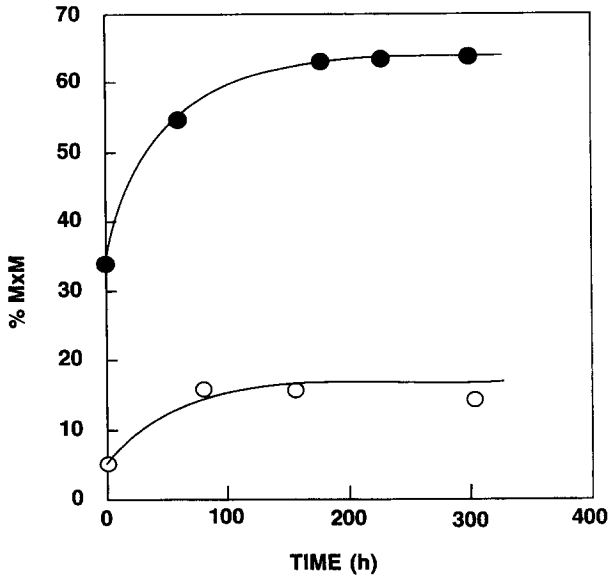

Fig. 2. Kinetics of the transformation at 37°C in 0.1 M Tris-acetate, pH 7, of the M = M quaternary form of RNase A mutants PLCC-AA (●) and CC-AA (○) into the M × M form. The percent of M × M form was obtained at the indicated time intervals by measuring by gel-filtration aliquots of the incubation mixture for the amount of monomer liberated from M = M by selective reduction of its intersubunit disulfides.

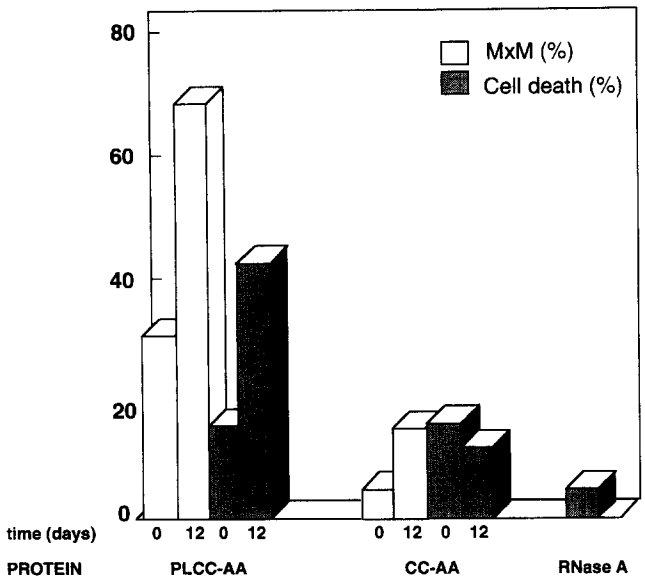

Fig. 3. Comparison between the antitumor activity (filled bars) and the content of the M × M quaternary form (empty bars), of RNase A mutants PLCC-AA and CC-AA, with monomeric RNase A as a negative control. The antitumor activity was determined by incubating SVT2 fibroblasts for 72 h in the presence of 50 μg/ml of each protein, and expressed as the percent of cell death with respect to untreated cultures. The content of M × M form was assayed as in the legend to Fig. 2. Assays were carried out on freshly prepared mutants and after an incubation of 12 days under the conditions described in Fig. 2.

position 19 was based on the proposed role of this residue in favoring the exchange of N-terminal ends in BS-RNase [12]. Thus a mutant of RNase A, in which a Pro residue was not present at position 19, could be dimeric without, or with limited, N-terminal exchange. This mutant, denoted CC-AA, in which only the two substitutions of Cys at sequence positions 31 and 32 were introduced (the insertion of a Leu at position 28 was also eliminated for the sake of simplification), was cloned and expressed in *Escherichia coli*. After extraction from inclusion bodies, purification and refolding, the mutant CCAA, like both PLCC-AA [10] and recombinant BS-RNase obtained in the same expression system [11], was found to be a mixture of dimeric forms, made up mainly of the M = M-type of dimers, with a low fraction of M × M-type dimers.

Upon incubation at 37°C in 0.1 M Tris-acetate, pH 7 [7] (see Fig. 2), the M = M form of PLCC-AA partially converted into the M × M form, until an equilibrium ratio of 2:1 between M × M and M = M was established, the same ratio found between the M × M and M = M forms of BS-RNase. In the case of the CC-AA mutant, however, the equilibrium ratio between the two forms was of about 1:9. This indicated that in the CC-AA mutant the extent of exchange, although not absent, was very limited, and suggested that Pro-19, as proposed [12], plays an important role in determining the exchange of N-terminal ends in BS-RNase.

Fig. 3 shows the results of antitumor assays carried out on mutants PLCC-AA and CC-AA, before and after the establishment of equilibrium between the two quaternary forms M = M and M × M for each mutant. Native monomeric RNase A was used as a negative control. In the case of PLCC-AA, in which equilibration between the two forms consisted in a significant increase in the M × M fraction, this resulted in a significant increase in antitumor activity; in the case of the CC-AA mutant, however, the increase in M × M form content was moderate, and no increase in antitumor activity was registered. Thus in mutant RNases with different degrees of N-terminal exchange the extent of exchange correlates unequivocally with the level of antitumor activity.

These results, and those described above, obtained with isolated BS-RNase forms M × M and M = M, suggest that the apparent antitumor activity of the M = M form from natural and mutant dimeric RNases can be ascribed to its metastability, i.e. to its transformation into the M × M form occurring during the lengthy bioassay. On the other hand, the activity of the M × M form could actually be higher than that measured with the available bioassay. Hence the exchange of subunit N-terminal ends is another important structural feature, besides the dimeric structure, in determining the antitumor action of BS-RNase. However, the possibility that a limited, small antitumor activity is also present in the M = M forms of dimeric RNases can not be totally excluded at present. A definite, conclusive answer could be obtained from the construction of a mutant of BS-RNase, or of a dimeric mutant of RNase A, in which no exchange of parts between subunits takes place at all. The results presented here favor the prediction that such a mutant would be totally devoid of antitumor action.

What is the mechanistic meaning of these observations is not clear. It has been proposed [13] that a higher antitumor activity of the M × M form of BS-RNase may be due to a higher affinity of this form for cellular binding sites. Another possibility is that the two forms of BS-RNase may be driven along different intracellular paths. It is known that RNase A, the tertiary structure of which is extremely similar to that of BS-RNase monomer in the M = M form [8], can be endocytosized by tumor cells without any adverse effects on the cells, and is directed toward the lysosomal district [14]. Possibly this pathway is accessible to M = M, which would be monomerized by the reducing potential of the cytosol, but not accessible to M × M, even after reduction of the intersubunit disulfides, as its dimeric structure would be maintained by non-covalent interactions [7]. It may also be considered that monomeric, dissociated BS-RNase is sensitive to the cytosolic RNase inhibitor, whereas dimeric BS-RNase is resistant [15]. Thus it is possible that the intracellular level of inhibitor is sufficient to sequester and inactivate the enzyme monomers as dissociated from M = M by the cytosolic redox potential.

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