Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 119-124

BOVINE SEMINAL RIBONUCLEASE DESTABILIZES NEGATIVELY CHARGED MEMBRANES

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Received December 23, 1993

SUMMARY: Bovine seminal ribonuclease (BS-RNase), an antitumor protein selectively cytotoxic for malignant cells, (i) specifically aggregates negatively charged vesicles and modifies the thermotropic behaviour of the phospholipid; (ii) decreases the amplitude of the thermal transition of the phospholipid; and (iii) provokes lipid-mixing between bilayers of negatively charged vesicles. This engenders leakage of the aqueous vesicle contents. Monomeric BS-RNase, devoid of antitumor action, does not produce these effects. These results suggest that the destabilization of the membrane bilayer promoted by BS-RNase may be involved in the antitumor action of the protein.

Bovine seminal RNase (BS-RNase) is a member of the pancreatic-type superfamily, with a selective toxicity for malignant cells (1-3). It has been established that the protein dimeric structure is essential to its antitumor action (2). This would suggest that a cell surface structure discriminates between the native, dimeric protein and its monomeric derivatives, which are catalytically active, but inactive as antitumor agents. However, recent data suggest that binding of BS-RNase to tumor cells does not occur through a cell membrane receptor-like structure (manuscript in preparation). The alternative possibility was thus considered, that BS-RNase permeates tumor cells by altering the cell membrane, as occurs in model membranes treated with a-sarcin, another cytotoxic RNase (4,5). The results described here indicate that BS-RNase may permeate the cell membrane by disrupting its lipid bilayer, as it can specifically aggregate, fuse, and destabilize artificial membranes made up with acidic phospholipids.

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The abbreviations used are: BS-RNase, bovine seminal ribonuclease; DMPC, DMPG, and DMPS, dimyristoylphosphatidyl-choline, -glycerol and -serine, respectively; DPH, 1,6-diphenyl-1,3,5-hexatriene; ANTS, 1,3,6-trisulphonate-8-aminonaphthalene; DPX, p-xylenebispyridinium bromide.

0006-291X/94 $5.00

MATERIALS AND METHODS

Synthetic dimyristoylphosphatidyl-choline (DMPC), -glycerol (DMPG) and -serine (DMPS), and bovine brain phosphatidylserine were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The different lipid vesicles were prepared at 1 mg/mL phospholipid concentration in 30 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, by using a water-bath sonifier for 30 min (the temperature was maintained 5 °C above the phase transition value of the corresponding phospholipid), or by extrusion through 0.1-mm polycarbonate filters (Nucleopore). The average size of the obtained vesicle population was 100 nm, as determined by Coulter counting. Lysophospholipids were not detected in the prepared vesicles as deduced from the chromatographic analysis of the lipid component (6). Lipid vesicles-protein complexes were prepared by adding purified BS-RNase (7) or its monomer form (8) to recently obtained vesicles at the required lipid/protein molar ratios. The protein concentration was determined by absorbance measurements at 278 and 277 nm for native (dimeric) and monomeric BS-RNase respectively, based on the reported extinction coefficients for these two protein forms (7,8).

The aggregation of phospholipid vesicles induced by the proteins was investigated by measuring the kinetics of the change in absorbance at 360 nm on a Beckman DU-8 thermostated spectrophotometer, equilibrated at the required temperature. Samples without protein were used as controls for all measurements.

Fluorescence polarization measurements were performed on a SLM Aminco 8000 spectrofluorimeter equipped with 10 mm Glan-Thompson polarizers. Cells of 0.2 cm optical path were used. The slit widths were 4 nm for both excitation and emission beams. Labelling of the vesicles with 1,6-diphenyl-1,3,5-hexatriene (DPH) was performed as previously described (9). The emission of the fluorescence probe was measured at 425 nm for excitation at 365 nm. The degree of polarization of the fluorescence emission was measured in thermostated cells, equipped with inner temperature probe, after equilibration of the samples for 10 min at each temperature value. Independent experiments demonstrated a negligible contribution of the protein to the degree of polarization of the fluorescence probe. Successive dilutions for each sample were performed in order to check the potential contribution of the sample turbidity to the polarization degree values. This contribution was also negligible for the concentrations and optical paths used.

Intermixing of membrane lipids was measured by resonance energy transfer (RET) assays (10). A vesicle population containing as fluorescence lipid analogues 0.6% N-(lissamine rhodamine B sulphonyl)-diacylphosphatidylethanolamine (Rh-PE, acceptor) and 1.0% N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine (NBD-PE, donor), was mixed with unlabelled vesicles at 1:9 molar ratio. Steady-state emission spectra were recorded on a SLM Aminco 8000 spectrofluorimeter for excitation at 450 nm. The efficiency of the energy transfer (%RET) is defined as: $(\%RET) = [1-F/F_0] \times 100$, where F and F_0 are the fluorescence emission intensities at 520 nm in the presence and in the absence of Rh-PE (acceptor), respectively. The lipid mixing produced by the proteins was estimated from the increase in the NBD-PE fluorescence emission (decrease in the %RET value) produced. Normalization of the measurements was performed by independent experiments in the presence of 1% Triton X-100 (4, 10).

Leakage of the lipid vesicle contents induced by BS-RNase was measured by the ANTS/DPX system (11). Bovine brain phosphatidylserine liposomes contained 12.5 mM ANTS (1,3,6-trisulphonate-8-aminonaphthalene), 45 mM DPX (p-xylenebispyridinium bromide), 20 mM NaCl and 10 mM Tris buffer, pH 7.5. ANTS/DPX-containing vesicles were equilibrated in the Tris buffer containing 100 mM NaCl and 1 mM EDTA. Fluorescence emission at 510 nm for excitation at 386 nm was measured. The fluorescence emission measured after totale vesicle lysis, obtained with a detergent (4 mM Triton X-100), was taken as indicative of 100% leakage.

RESULTS AND DISCUSSION

BS-RNase produces aggregation of DMPS vesicles as deduced from the results given in Fig.1. Addition of the protein to a DMPS vesicle preparation resulted in an increase of absorbance at 360 nm, due to the scattered-light of the enlarged particles, and dependent on

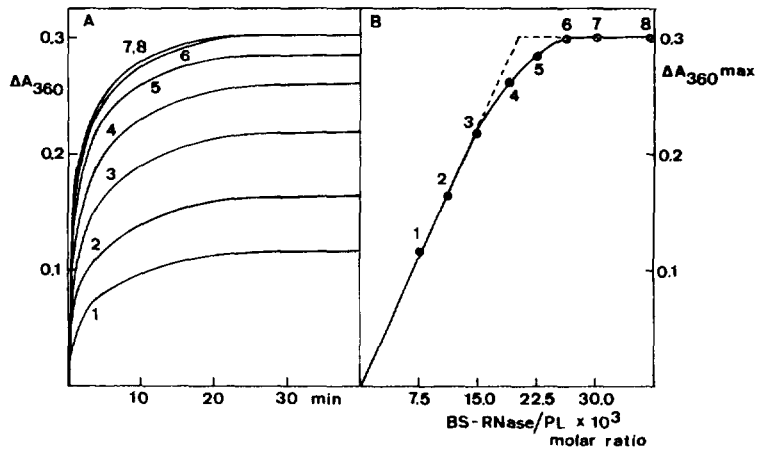


FIGURE 1. (A) Kinetics of the absorbance increase at 360 nm of DMPS vesicles/BS-RNase mixtures at different protein/phospholipid molar ratios (numbers in curves as in part B). (B) Maximum increase of the absorbance at 360 nm *versus* BS-RNase/DMPS molar ratio. Total phospholipid concentration was 30 mM. The intersection of the dotted lines corresponds to the saturating protein/lipid molar ratio.

both time and protein concentration (Fig.1A). About 30 min were required for completion of the aggregation process at the lowest protein concentration tested, as observed from the kinetic analysis of the absorbance variation. The plot of maximum absorbance variation *versus* protein concentration was hyperbolic (Fig.1B) with saturation at about 50:1 phospholipid to protein molar ratio.

The same experiment was carried out with DMPG and DMPC vesicles. BS-RNase produced similar effects on DMPG vesicles, but no absorbance variation was observed for DMPC vesicles in the protein concentration range considered above. All of these analyses were performed at neutral pH. Under these conditions phosphatidylserine and phosphatidylglycerol exhibit a net negative charge whereas phosphatidylcholine behaves as a zwiterionic molecule. These data suggest that an electrostatic component is inherent in the interaction between BS-RNase and phospholipid vesicles.

This interaction was also studied by considering the effect of the protein on the phase transition profile of the phospholipid, using DPH-labelled vesicles. The phase-transition profile of the lipid vesicles was determined by measuring the steady-state fluorescence anisotropy of the DPH-labelled vesicles. No changes in the thermotropic behaviour of the phospholipid were observed with DMPC vesicles. This result was not surprising, since no aggregation was observed upon addition of the protein to DMPC vesicles. On the other hand, the protein considerably modified the phase transition profile of DMPS vesicles with a decrease of the amplitude of the thermal transition, but no significant change in the phase transition temperature for the phospholipid (Fig.2A). The protein also modified the phase transition profile of DMPG vesicles (Fig.2B). These results may be interpreted in terms of phospholipid molecules being removed from participating in the gel-to-liquid phase transition. They also suggest a significant hydrophobic component in the interaction between the protein and acidic phospholipid vesicles.

Resonance energy transfer (RET) measurements showed that BS-RNase promotes lipid-mixing between bilayers of negatively charged vesicles. A DMPS vesicle population containing NBD-PE (fluorescence donor) and Rh-PE (fluorescence acceptor) was incubated in the presence of a large excess of non-labelled vesicles. The fluorescence resonance energy transfer measured under these conditions represents the RET efficiency of the system. Lipid

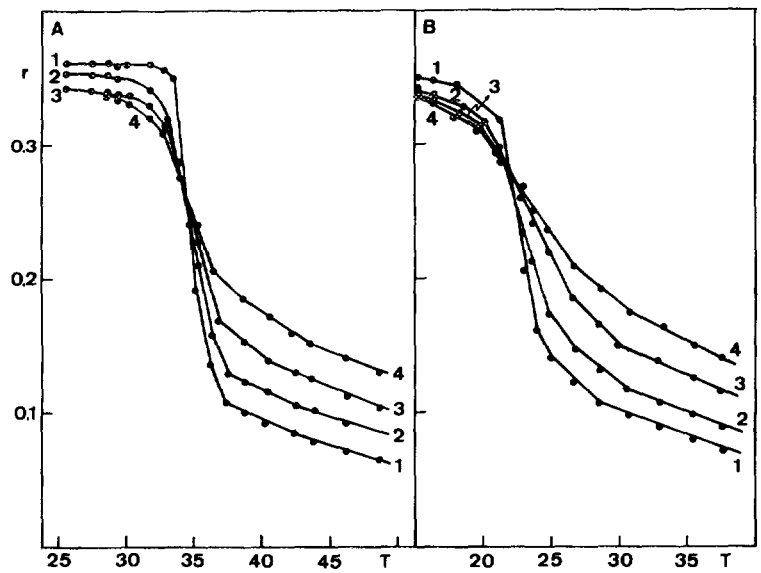


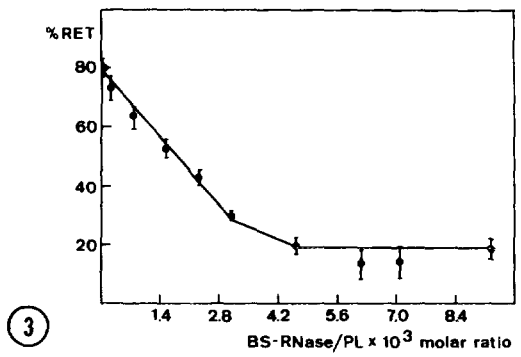
FIGURE 2. Steady-state fluorescence anisotropy variation *versus* temperature (°C) of DPH-labelled vesicles of (A) DMPS and (B) DMPG in the presence of BS-RNase: (1) phospholipid alone; (2), (3) and (4), 80:1, 25:1 and 7.5:1 phospholipid/BS-RNase molar ratios, respectively. Experimental points represent the average of three different determinations.

mixing between vesicle populations results in a decrease of the fluorophore surface density, which is reflected in a less efficient RET. BS-RNase was found to decrease the RET efficiency from about 80% to about 20% (Fig.3). This corresponds to a decrease of about 9-fold in the surface density of the energy acceptor (10). Considering the proportion between fluorescence labelled/unlabelled vesicle, this result is consistent with a BS-RNase induced fusion between the labelled and the unlabelled vesicles, followed by lateral diffusion of the fluorescence probes in the plane of the newly enlarged membranes. Simple aggregation of the vesicles would not result in such a change in RET. No lipid-mixing was observed when DMPC vesicles were tested under identical experimental conditions and protein concentration range.

The effect of BS-RNase on promoting lipid-mixing of negatively charged membranes becomes saturated at about 275:1 phospholipid/protein molar ratio. Therefore, completion of this effect required about 5-fold less protein than vesicles aggregation. This may be interpreted in terms of a further electrostatic interaction of the excess protein with the mixed membranes.

Native dimeric BS-RNase also induced leakage of the aqueous contents of phosphatidylserine vesicles (Fig.4). This has been deduced by measuring the ANTS fluorescence emission, upon relief of the DPX quenching due to the release of coencapsulated ANTS/DPX (11).

BS-RNase is a homodimeric protein with two intersubunit disulfides (1). A monomeric preparation can be obtained by reduction of these bonds and alkylation of the resulting thiols (12). When monomeric BS-RNase was analyzed for its potential interaction with lipid vesicles, it was found to promote aggregation of negatively charged lipid vesicles to a much lower extent than the native dimeric enzyme. While the maximum absorbance variation produced by native BS-RNase at 360 nm was about 0.30 absorbance unit, the absorbance increase measured with a double molar concentration of monomeric BS-RNase was of only 0.06 unit. The thermotropic behaviour of DMPG vesicles was not significantly modified, and a very slight



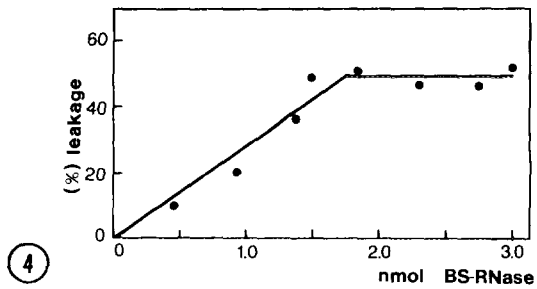


FIGURE 3. Variation of the percentage of fluorescence energy transfer (%RET) ± SD *versus* BS-RNase/phospholipid molar ratio for DMPS vesicles containing 0.6% NBD-PE (donor) and Rh-PE (acceptor). %RET calculations were performed after three independent determinations from the fluorescence intensity values at 520 nm for excitation at 450 nm.

FIGURE 4. Extent of vesicle leakage induced by native dimeric BS-RNase. The (%) leakage was calculated from the fluorescence emission of ANTS resulting from the release of the aqueous contents of the vesicles at 37°C with 115 mM phospholipid concentration. The emission obtained in the presence of 4 mM Triton X-100 (total vesicle lysis) was taken as 100% leakage.

increase (a maximum of 0.02 unit) was produced in the steady-state fluorescence anisotropy of DPH-labelled DMPS vesicles at temperatures above the phase transition of this phospholipid. Monomeric BS-RNase did not produce any lipid-mixing in the negatively charged vesicles considered, and had no effects on DMPC vesicles.

Concluding Remarks

The main conclusion that can be drawn from the results above is that BS-RNase destabilizes lipid bilayers, and specifically bilayers of negatively charged phospholipids. Since BS-RNase is a basic protein, with a pI of 10.4 (7), the interaction observed between the protein and negatively charged vesicles could be explained by its high positive net charge. However, monomeric BS-RNase does not exhibit this behavior, although the charge of the protein is not significantly modified by monomerization. In fact the weak electrostatic interaction, and the resulting low aggregation observed for negatively charged vesicles tested with monomeric BS-RNase may be likely accounted for its net positive charge. Native, dimeric BS-RNase was instead found to be involved in hydrophobic interactions with the bilayers. Hence, the dimeric structure of native BS-RNase can be considered as essential for the main interactions of the protein with lipid vesicles.

Thus native BS-RNase, fully active as an antitumor agent, can disrupt and permeate membranes. On the other hand monomeric BS-RNase, inactive as antitumor agent (2), is incapable of significantly affecting membrane vesicles. These data lead us to propose that the specific interaction of BS-RNase with tumor cell membranes may be the basis of its selective toxicity toward tumor cells. This conclusion is not surprising, since it has long been established that plasma membranes from tumor cells are remarkably different than their counterparts from normal cells, and in particular, that they have higher negative net charges (13).

ACKNOWLEDGMENTS

The skill of Ms Dominique Melck in the preparation of BS-RNase is gratefully appreciated. This work was in part financed with grants from C.N.R. (PF-BTBS 92.01185), and from MURST, Italy, and from DGICYT (Grant PB90/0007), Spain.

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