*Articles*

# Effects of Protein RNase Inhibitor and Substrate on the Quaternary Structures of Bovine Seminal RNase†

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ABSTRACT: The effect of the protein RNase inhibitor (PRI) on the activity of bovine seminal RNase (BS-RNase) was investigated using the isolated quaternary forms, MxM and M=M, of the enzyme reported earlier [Piccoli, R., et al., (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1870−1874]. We found that the inhibitor does not interact with the intact isolated forms but has dramatic, differential effects on the two forms when the assays are performed under reducing conditions. These conditions, which are essential for full activity of the inhibitor, and are typical of its cytosolic localization, also promote monomerization of the M=M form, while under identical conditions the MxM form becomes a noncovalent dimer (NCD). The sensitivity of BS-RNase or that of the isolated quaternary forms under reducing conditions thus appears to be related to differential monomerization of the two forms of the enzyme; monomer being sensitive to PRI. The present study also shows that the interconversion between the two forms in equilibrium occurs at much higher rates in a reducing environment and that PRI further affects the interconversion and alters the equilibrium favoring monomerization of the protein. An opposite effect on the equilibrium between the forms is played by the substrate, which is found to stabilize the NCD form of the protein with a shift in the equilibrium between the two forms towards the dimer. These results are analyzed in the light of the antitumor action of the enzyme which is exerted in the cytosol, i.e., in the compartment housing the PRI and the ribosomal RNA, the molecular target of the enzyme.

In the vertebrate RNase superfamily, for which bovine pancreatic RNase A is the classic prototype, bovine seminal RNase (BS-RNase),[1] which is 80% identical in amino acid sequence to RNase A, holds a unique position, as it is the only dimeric member of the whole superfamily (D'Alessio et al., 1991). This structural feature of BS-RNase appears to be the main basis for the unusual functional properties of this enzyme: its allosteric behavior (Piccoli et al., 1988), its special, i.e., noncatalytic, biological actions (D'Alessio, 1993) and its resistance to PRI, the protein RNase inhibitor (Murthy & Sirdeshmukh, 1992) that can tightly bind and inhibit all other mammalian RNases tested so far (Lee & Vallee, 1993). Monomeric BS-RNase, still active as RNase, in fact superactive with respect to the native dimeric enzyme (Piccoli et al., 1988), has no allosteric properties (Piccoli et al., 1988), no special bioactions (D'Alessio, 1993), and is as sensitive to PRI as is RNase A (Murthy & Sirdeshmukh, 1992).

The BS-RNase dimeric structure is maintained by noncovalent interactions and also by two disulfide bridges (D'Alessio et al., 1975). Furthermore, two dimeric forms have been described for the protein: an MxM form, in which each subunit exchanges with the other its α-helical N-terminal segment (Mazzarella et al., 1993), and an M=M form with no exchange (Piccoli et al., 1992). The two forms are in equilibrium with each other and, upon selective reduction of the intersubunit disulfides, the M=M form readily dissociates into free monomers (M), whereas the MxM form remains a noncovalent dimer, termed NCD (Piccoli et al., 1992).

Recently, the MxM form has been found by different experimental approaches to have the greater antitumor action (Cafaro et al., 1995; Kim et al., 1995; Di Donato et al., 1995). It has also been found (Mastronicola et al., 1995) that BS-RNase must reach the cytosol to exert its antitumor action. This is not only the cell compartment where PRI is located (Lee & Vallee, 1993) but also, given its reducing environment, a compartment in which the MxM and M=M forms of the protein are presumed to generate NCD dimers and free M monomers, respectively.

It thus appeared of interest to investigate the effect of PRI on BS-RNase subforms, expecially upon reduction of their intersubunit disulfides, and to ascertain whether PRI and RNA could have any effects on the equilibrium between the enzyme subforms, with possible consequences on the bio-

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[1] Abbreviations: BS-RNase, bovine seminal RNase; RNase A, bovine pancreatic RNase; MxM, BS-RNase form in which subunits exchange their N-terminal segments; M = M, BS-RNase form in which no exchange occurs; NCD, BS-RNase noncovalent dimers; M, BS-RNase monomers; PRI, protein RNase inhibitor; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; cCMP, cytidine-2′,3′-cyclic phosphate; UpA, urydilyl-3′,5′-adenosine.

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logical actions of the enzyme.

The results we report here indicate that (i) both isolated MxM and M=M forms are insensitive to the inhibitor, (ii) the noncovalent dimers, produced by reduction of intersubunit disulfides of the MxM form, are also resistant to the inhibitor, (iii) the presence of PRI shifts the equilibrium NCD → 2M toward the PRI-sensitive M monomers, and (iv) the substrate has an opposite effect, as it induces the conversion of M monomers into NCD dimers.

## MATERIALS AND METHODS

*Materials*. BS-RNase was prepared by the method of Tamburrini et al., (1986). BS-RNase subforms were prepared as described (Piccoli et al., 1992). Human placental ribonuclease inhibitor was purchased from Pharmacia Inc. (Uppsala, Sweden) or prepared as described previously (Blackburn, 1979). One unit of the inhibitor is defined as the amount required to inhibit the activity of 5 ng of RNase A by 50%.

*Gel Electrophoresis*. Gel electrophoresis in 15% SDS−polyacrylamide was carried out as described by Laemmli et al., (1970). Proteins were visualized either by 0.02% Coomassie blue (R 250) in a solution containing methanol/water/acetic acid (50:40:10 v/v) or by silver staining (Oakley et al., 1980).

*RNase and Inhibitor Assays.* The RNase and inhibitor assays were carried out by the chromatographic assay method essentially as described previously (Murthy and Sirdeshmukh, 1992). Two micrograms of $^3$H-labeled *Escherichia coli RNA* was incubated with the enzyme, at 37 °C for 15 min, in 15 $\mu$L of buffer A (20 mM Tris-HCl, pH 7.4, containing 100 mM $NH_4Cl$ and 5 mM magnesium acetate) in the absence or presence of PRI. At the end of the reaction, the reaction mixture was subjected to descending paper chromatography on strips of paper using 1:1 mixture of 1 M ammonium acetate and ethanol as solvent. Degradation of RNA was estimated by measuring the radioactivity in the undegraded RNA remaining at the origin. The amount of the enzyme required for 50% degradation of RNA was taken as 1 unit of activity. About 0.8 activity unit (1−2 ng of the enzyme preparations with different specific activities) of the enzyme was used in each assay, unless otherwise stated.

*DTT Treatment.* For selective reduction of intersubunit disulfides (D'Alessio et al., 1975), BS-RNase was treated with at least 10 times molar excess of DTT at 25 or 37 °C for 10 min in 20 mM Tris-HCl or 100 mM Tris-acetate buffer (pH 7.4) unless mentioned otherwise. We earlier observed that incubation of the enzyme with DTT at 0−37 °C for as short as 2 min is adequate for intersubunit disulfide reduction [see Results, Murthy and Sirdeshmukh (1992)]. Also, higher molar ratios of DTT to enzyme were tolerated without any deleterious effect on the enzyme as judged by its specific activity (B.S.M. and R.S., unpublished results).

## RESULTS AND DISCUSSION

*Effects of PRI on the Equilibrium between BS-RNase Quaternary Forms*. It has been shown that BS-RNase is resistant to PRI but becomes sensitive in its monomeric form (Murthy & Sirdeshmukh, 1992). It has also been found that pretreatment of BS-RNase with a reducing agent can render the enzyme partially sensitive to inhibition by PRI (Murthy & Sirdeshmukh, 1992). However, the data illustrated in Figure 1 show that when the pretreatment mixture contains PRI in addition to DTT, the catalytic action of BS-RNase is completely abolished by the inhibitor.

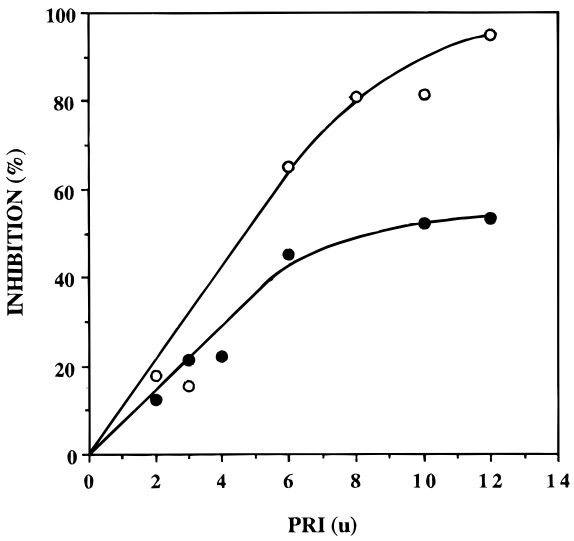


FIGURE 1: Effects of DTT and PRI pretreatment on the sensitivity of BS-RNase to inhibition. BS-RNase was pretreated for 10 min at 25 °C with 5mM DTT at pH 7.4, in the presence (○) or in the absence (●) of PRI, and subjected to inhibition assays. The inhibition assays were started by the addition of the substarte (○), or the substrate plus the inhibitor (●).

Thus BS-RNase, by itself completely resistant to inhibition by PRI (Murthy & Sirdeshmukh, 1992), becomes partially sensitive after a pretreatment with DTT and totally sensitive after a pretreatment with DTT in the presence of PRI.

The analysis of these effects must take into consideration that in native BS-RNase two quaternary conformations coexist in equilibrium with each other (Piccoli et al., 1992): one, denominated MxM, representing about $^2/_3$ of the protein, and characterized by the exchange between subunits of their N-terminal domains; the other (about $^1/_3$ of the protein), denominated M=M, with no exchange of N-terminal domains between subunits. Upon selective cleavage of the intersubunit disulfides, only the M=M form dissociates into monomers, whereas MxM becomes a noncovalent dimer (NCD), as the two subunits are still held together by the noncovalent interactions between the N-terminal domain of one subunit and the main body of the other.

Given the sensitivity of monomeric BS-RNase to PRI (Murthy & Sirdeshmukh, 1992), the partial susceptibility of the protein after the pretreatment with DTT can easily be explained by the effect of PRI on the free monomers readily liberated by DTT from the M=M form. The total sensitivity of BS-RNase preincubated with PRI is more intriguing but can also be explained by assuming that as MxM and M=M are in equilibrium with each other (Piccoli et al., 1992), so are their products in equilibrium after selective reduction of the intersubunit disulfides, NCD and M, respectively. If this were the case, the free monomers M, generated from M=M in the presence of DTT, would be promptly complexed by PRI; but to reestablish the equilibrium between M and NCD, a fraction of NCD should dissociate into monomers, which in turn would be rapidly complexed by PRI, until eventually all the protein would be transformed into monomers and sequestered by PRI.

A first indication in support of these hypotheses is given by the results illustrated in Figure 2, which show that if BS-

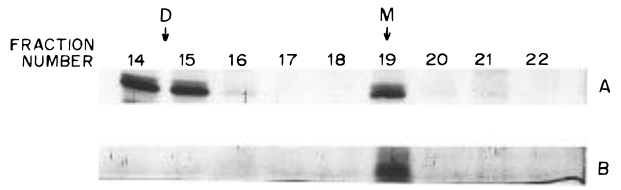


FIGURE 2: Monomerization of BS-RNase treated with DTT in the presence of PRI. Two micrograms BS-RNase was treated with DTT as described in Materials and Methods and further incubated in the absence (A) or in the presence (B) of 1000 units (14 $\mu$g) of PRI in 60 $\mu$L of the same buffer for10 min at 37 °C. The reaction mixture was then treated with 1 mM *p*-chloromercuric benzoate to dissociate the enzyme−inhibitor complex and chromatographed, at 4 °C, on a Bio-Gel P-60 column equilibrated in 0.1 M ammonium acetate, pH 5. The eluate fractions were pooled and analyzed by SDS−PAGE followed by silver staining. The arrows indicate the elution volumes of D, dimeric BS-RNase, and M, monomeric BS-RNase.

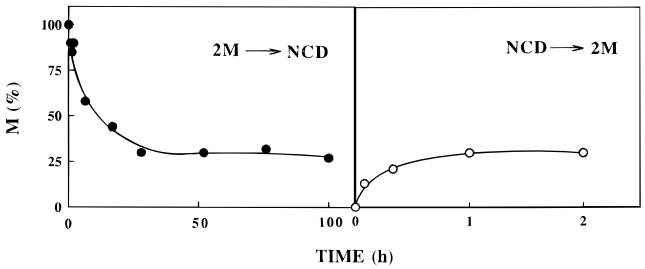


FIGURE 3: Interconversion of the selectively reduced MxM and M=M forms. Homogeneous preparations of M=M (left panel) and MxM (right panel) forms (0.4 mg/mL) were separately incubated at 37 °C in 0.1 M Tris-acetate buffer, pH 7.4, in the presence of 0.25 mM DTT. At the indicated time intervals, 50 $\mu$g aliquots of the incubation mixture were analyzed for the content of monomeric protein by gel filtration in 0.05 M ammonium acetate pH 5, containing 0.3 M NaCl.

RNase is pretreated with DTT for reduction of intersubunit disulfides and further incubated in the presence of PRI under conditions comparable to those of Figure 1, the protein completely dissociated into monomers as observed upon gel filtration.

We then tested the basic prediction of the hypothesis that rapid interconversions occur between the selectively reduced MxM and M=M forms (i.e., between NCD and M) by determining the kinetics of production of NCD from reduced M=M and of M from reduced MxM. It should be noted that the interconversion rates of MxM with M=M under nonreducing conditions are extremely slow, with half-times in the order of days (Piccoli et al., 1992).

Isolated MxM and M=M preparations (0.4 mg/mL) were incubated at 37 °C at pH 7.4 with a 17-fold molar excess of DTT, which is known to produce the selective cleavage of the intersubunit disulfides of BS-RNase (D'Alessio et al., 1975), and of its isolated quaternary forms (Piccoli et al., 1992). To maintain the concentration of reduced DTT when the incubation was prolonged over 24 h, fresh DTT was added every 24 h up to 0.12 mM final concentration. At suitable time intervals, aliquots (50 $\mu$g) of protein were analyzed by gel-filtration on a High Load Superdex G-75 10/30 column (Pharmacia). The monomeric and dimeric fractions were measured by automatic integration of their respective absorbance (at 280 nm) peaks.

The results of this experiment, illustrated in Figure 3, indicate that upon cleavage of the intersubunit disulfides of the MxM and M=M forms, the resulting NCD dimers and M monomers, respectively, transform into each other at much faster rates, compared with those measured in the absence of a reducing agent (Piccoli et al., 1992). In particular, the transformation of NCD into 2M is much faster, with a half-time of 12 min, than the inverse reaction, which occurs with a half-time of 2.4 h. Furthermore, after about 1 h for the NCD → 2M transformation, and after about 50 h for the transformation 2M → NCD, an equilibrium was reached, with a ratio of about 2:1 between NCD and M, corresponding perfectly to the equilibrium ratio between the respective parent forms MxM and M=M (Piccoli et al., 1992). It is difficult to explain why in the presence of PRI a more rapid monomerization of NCD seems to have occurred (see results shown in Figure 2). The titration effect of PRI discussed above and under Figure 4 (see below) could be an important factor; on the other hand, the possibility that PRI has a direct effect on the NCD to M conversion rate cannot be ruled out.

Further support to the above hypothesis was obtained by assaying preparations of isolated MxM and M=M forms for PRI inhibition of enzymatic activity. When the assay was carried out in the absence of a reducing agent in the assay buffer (Figure 4A), the inhibitor activity was significantly reduced even against RNase A, as can be expected in the absence of a reducing environment, but RNase A was still completely inhibited by about 4 units of PRI. Under the same conditions, both MxM and M=M were completely resistant to inhibition by PRI (see Figure 4A). When the assay for PRI inhibition was carried out in the presence of DTT (Figure 4B), (i) the M=M form was found to be highly sensitive to PRI inhibition, even without any pretreatments, and (ii) the MxM form instead was virtually insensitive to inhibition by PRI.

These data indicate that, in the absence of DTT, both MxM and M=M would remain dimeric and therefore inhibitor insensitive, but even on short exposure to DTT (in the assay mixture), the M=M form may be converted into free monomers which are readily complexed by PRI and inhibited; the MxM form changes into NCD form in the presence of DTT and, being dimeric, still remains insensitive to PRI inhibition. Upon preincubation of the isolated forms for 10 min with DTT (Figure 4C), i.e., after a longer treatment with the reducing agent, the effect on M=M was that of a higher degree of inhibition, but also MxM was found to lose its resistance to PRI, an effect even more dramatic when the preincubation mixture included PRI (see Figure 4C).

The main conclusion which can be drawn from these data is that even the noncovalent dimer was resistant to the action of PRI. Only after a prolonged pretreatment with DTT, especially when carried out in the presence of PRI, NCD became susceptible to PRI inhibition, clearly through transformation toward equilibrium into monomers.

The large difference in equilibration rate between the 2M → NCD and NCD → 2M transformations (see Figure 3) explains why only the M form is comparatively stable, hence readily complexed by PRI, in the time scale of the assays. It also explains why the unstable NCD form, which can partially dissociate into monomers within minutes, appears to be partially sensitive to PRI, even without pretreatment with PRI.

It can thus be concluded that all the data available are in line with the hypothesis that the partial sensitivity to PRI inhibition of BS-RNase pretreated with PRI in the presence of DTT is based on a rapid transformation under reducing

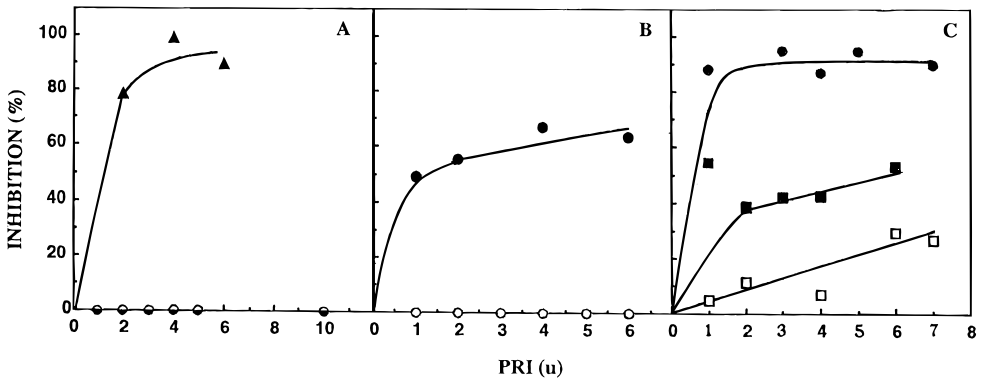


FIGURE 4: Sensitivity of isolated MxM and M=M forms to PRI.The inhibitor assays were carried out as described in Materials and Methods. The inhibition assays were carried out in the absence (A) or presence (B) of 5 mM DTT. In both A and B, the reaction was started by the addition of the enzyme: the M=M form (●), the MxM form (○), or RNase A (▲) which served as control. (C) shows inhibition curves of the MxM form after preincubation with 5 mM DTT (□) or with DTT plus PRI (■), and of M=M form (●) preincubated with only 5 mM DTT. Assay conditions were as reported in Figure 1.

conditions of the NCD generated from MxM into free monomers, which in the presence of PRI are titrated out and removed from the equilibrium.

All these data confirm the proposal that the resistance of BS-RNase to PRI is essentially based on its dimeric structure (Murthy & Sirdeshmukh, 1992). Support to this proposal has also emerged from the recent description of the three-dimensional structure of the PRI−RNase A complex (Kobe & Deisenhofer, 1995). An inspection of the model (Shapiro et al., 1995) readily reveals that a dimeric RNase such as BS-RNase, either in its MxM or in the M=M form, could not be accommodated into the horseshoe cavity of the inhibitor. In particular, residues of RNase A, which directly interact with PRI in the PRI-RNase complex, such as Gln-28, Lys-31, and Ser-32 (Kobe & Deisenhofer, 1995), have all been replaced in BS-RNase sequence by residues (Leu-28 and Cys-31 and -32, respectively), which are located at the subunit interface in BS-RNase (Mazzarella et al., 1993) and have been identified as essential determinants of BS-RNase dimeric structure (Di Donato et al., 1994,1995; Mazzarella et al., 1995).

*The Effect of Substrate on the Interconversion of NCD and M*. It has been demonstrated that a nucleotide inhibitor (Lee et al., 1989) and UpA, as a model substrate (Vicentini et al., 1990), inhibit in a competitive fashion the action of PRI on homologous RNases, such as angiogenin (Lee et al., 1989) and pig liver RNase (Vicentini et al., 1990), respectively. It has also been reported that RNA, the enzyme substrate, affects the response of BS-RNase to PRI inhibition, by rendering it less susceptible to inhibition (Murthy & Sirdeshmukh, 1992). We thus considered the possibility that the coexistence of the two quaternary forms of the enzyme also played a role in determining modulation of PRI inhibition by the substrate.

BS-RNase was thus treated with DTT in the presence or in the absence of cCMP, the substrate for the second reaction step. To avoid any subsequent reoxidation and also to quench the excess of DTT used in the reaction, the sulfhydryls were blocked with iodoacetamide at the end of the reaction, and the reaction products were analyzed by SDS−PAGE (Figure 5A). We found that, in the absence of substrate, the intersubunit disulfides were completely reduced with 5 mM DTT both at pH 5.75 and 7.4 (lanes 2 and 3). In the presence of the added substrate, reduction of intersubunit disulfides was partial up to a DTT concentration

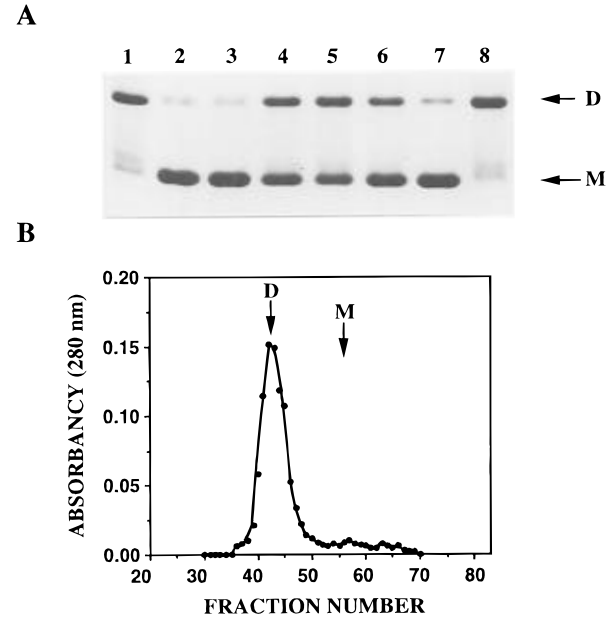


FIGURE 5: Effect of the substrate on the quaternary structure of BS-RNase. (A) 100 μg of BS-RNase (3.4 nmol) was treated with the specified concentrations of DTT at 37 °C for 10 min in 25 μL of 20 mM Tris-HCl buffer, in the absence or presence of 0.5 mg of cCMP (1.5 μmol). Excess DTT was quenched by immediately treating the reaction mixture with 100 mM iodoacetamide, the enzyme was electrophoresed in 15% SDS−polyacrylamide gel without 2-mercaptoethanol or DTT, and the gel was stained with Coomassie blue. Lanes 1−3 are controls: (Lane 1) Native BS-RNase; (lanes 2 and 3) BS-RNase treated with 5 mM DTT at pH 7.4 and at 5.75 (adjusted with phosphoric acid), respectively. Lanes 4−7 are BS-RNase treated with 2.5, 5, 10, and 20 mM DTT (60−500 nmol), respectively, in the presence of cCMP. Lane 8 shows absence of any reduction of intersubunit disulfides in BS-RNase treated with 20 mM DTT in the presence of iodoacetamide. (B) Bio-Gel P-60 gel filtration chromatography of BS-RNase treated with 20 mM DTT in the presence of cCMP as in lane 7, above. The chromatography was carried out as in Figure 2.

of 10 mM and complete only at 20 mM DTT (lanes 4−7) where the DTT to enzyme molar ratio was 160. This suggests that the susceptibility of the intersubunit disulfides of BS-RNase was significantly altered in the presence of the substrate. One possible explanation for this could be a drop in the pH caused by the protons liberated from the transformed substrate. However, this drop in the pH per se appeared to be irrelevant as the reduction of the intersubunit disulfides observed at pH 5.75 and 7.4 was found to be

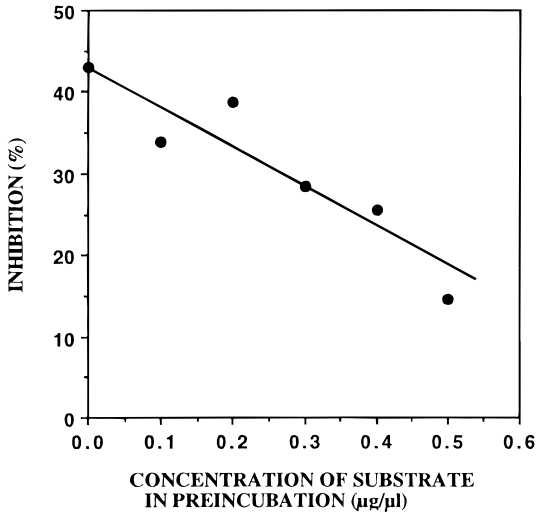


FIGURE 6: Effect of RNA on PRI sensitivity of BS-RNase M=M form under reducing conditions. The M=M form was preincubated, at 37 °C for 2 min, with 5 mM DTT in the absence (control) or in the presence of varying amounts of the RNA as described earlier for BS-RNase (Murthy & Sirdeshmukh, 1992). The preincubated enzyme was then quickly diluted to lower the concentration of RNA to a minimum (see Results) and immediately assayed as described in Materials and Methods with freshly added RNA (2 $\mu$g) either in the absence or presence of excess (16 units) of PRI. Inhibition is plotted as a function of the amount of RNA used in the preincubation.

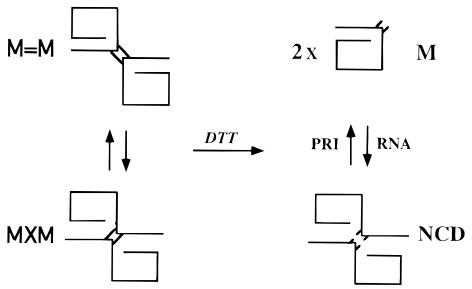


FIGURE 7: Schematic representation of the effects of PRI and RNA on the equilibrium between the quaternary BS-RNase forms.

comparable (controls; lanes 2 and 3). Further, when BS-RNase treated with 20 mM DTT in the presence of a 450-fold molar excess of cCMP (as in lane 7, Figure 5A) was analyzed by gel filtration in the absence of a denaturant, the protein was found to elute as a dimer, even after total cleavage of intersubunit disulfides (Figure 5B). As selectively reduced BS-RNase, i.e., the resulting NCD and M products, are stable under the gel filtration conditions (D'Alessio et al., 1975; Piccoli et al., 1992), these data indicate that when selective reduction of BS-RNase occurs in the presence of the substrate, the only product is the NCD form. The substrate thus seems to have a stabilizing effect on the MxM and NCD conformation, and hence an inhibitory effect on intersubunit disulfide reduction and the NCD → 2M conversion. In addition, it seems to induce or accelerate conversion of the monomers, dissociated from M=M, into NCD.

This conclusion appeared to be supported by the results of the experiment reported in Figure 6, in which the effect of RNA substrate was tested in terms of the susceptibility of the isolated M=M form treated with DTT in the presence of the substrate to PRI inhibition. Under these conditions, M=M is very sensitive to PRI inhibition, whereas MxM is resistant (see Figure 4B,C). The addition of increasing concentrations of RNA in the preincubation with DTT instead lowered the susceptibility of the M=M form. The concentrations of RNA differred only in the preincubation step and not during the actual assay in the presence of PRI as the preincubation mixture was diluted before the assay such that the RNA from enzyme additions was less than 5% (0.1 $\mu$g) of the total amount (2 $\mu$g) used in the assay. Thus the possibility of competition between the substrate and PRI is very unlikely. Further, the DTT to enzyme molar ratio in this and other earlier assays was >160; under this condition, the reduction of intersubunit disulfides of the enzyme is expected to be complete even in the presence of the substrate (see Figure 5). Therefore, a more likely explanation for the above observation can be in terms of an enhanced concentration of NCD in the assay mixture engendered by the presence of RNA. Taken together, the results of the experiments of Figures 5 and 6 suggest that in the presence of the substrate the conversion of M to NCD is favored, as the substrate stabilizes the NCD conformation. This stabilizing effect is likely based on the occupation of the active sites of the enzyme by the substrate, which can freeze the conformation with "exchange" of the N-terminals and hinder the NCD → 2M conversion. The overall result would be a shift in the equilibrium between M and NCD opposite to that observed for the effect of PRI.

*A Role for PRI and RNA in the Mechanism(s) of BS-RNase Bioactions.* Both the effects of PRI and of the substrate on the equilibrium between the selectively reduced quaternary forms of BS-RNase may well have biological significance. BS-RNase is a RISBASE, i.e., an enzyme endowed with special biological actions (D'Alessio, 1993), including an antitumor action (Vescia et al., 1980; Laccetti et al., 1992; Laccetti et al., 1994), which is not shared by the enzyme monomeric form (Vescia et al., 1980). PRI is an intracellular factor (Lee & Vallee, 1993) which is fully active in a reducing environment, such as that of the cytosol . On the other hand, it has been shown that in tumor cells the enzyme reaches the cytosol, where it degrades rRNAs, blocks protein synthesis, and provokes cell death (Mastronicola et al., 1995). It should be noted that the highly reducing potential of the cytosol, in which high ratios of glutathione to oxidized glutathione have been found (Hwang et al., 1992; Gilbert et al., 1990), would readily reduce the intersubunit disulfides of BS-RNase (Smith & Schaffer, 1979).

It could thus be envisaged, on the basis of these data, and of the results reported above, that in the reducing environment of the cytosol of tumor cells the M=M form dissociates into monomers and is promptly neutralized by PRI, whereas the MxM form, still dimeric as NCD and resistant to PRI, can degrade rRNA and kill the cell. In fact, it has been demonstrated by different experimental approaches (Cafaro et al., 1995; Kim et al., 1995; Di Donato et al., 1995) that the MxM form is more cytotoxic toward tumor cells than the M=M form. The propensity of NCD to rapidly convert into monomers in the presence of PRI, as indicated by the results reported above, may not defeat its cytotoxic action: (i) because the conversion of NCD into monomers is not instantaneous, and any remaining fraction of NCD can continue its action on rRNA; (ii) the substrate RNA including the degradation fragments generated would stabilize NCD with a further increase in RNA degradation.

Thus the RNase inhibitor and RNA can have decisive roles in the ratio between BS-RNase NCD and M forms in the

cytosol environment (Figure 7), and modulate the RNase activity, which is essential for any of the special biological actions of BS-RNase (D'Alessio, 1993). The findings that the RNase inhibitor is expressed at different levels in different mammalian cells and tissues (Lee & Vallee, 1993) have been recently confirmed with more direct methodologies (unpublished data). Thus, the role of PRI in the mechanism(s) of the special bioactions of BS-RNase certainly deserves further investigation.

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