Seminal RNase (BS-RNase), a ribonuclease from bovine seminal vesicles, is a homodimeric enzyme with a strong cytotoxic activity selective for tumor cells. It displays the unusual structural feature of existing in solution as an equilibrium mixture of two quaternary isoforms. The major one is characterized by the swap between subunits of their N-terminal ends, whereas the minor isoform shows no swap. The tendency of the two isolated isoforms to interconvert into each other has so far made it difficult to attribute the functional properties of BS-RNase to either isoform. Herein, molecular modeling and site-directed mutagenesis were used to engineer the refolding pathway of BS-RNase and obtain a stable variant of its non-swapping isoform. The protein was engineered with two extra disulfide bridges linking the N-terminal helix of each subunit to the main body of the same subunit. Purified as an active enzyme, the BS-RNase variant was found to be very resistant to thermal denaturation. Its functional characterization revealed that the lack of swapping has a negative effect on the cytotoxic activity of BS-RNase.

Seminal ribonuclease (BS-RNase)\textsuperscript{1} is a basic 27-kDa protein produced by bull seminal vesicles and secreted into the semen where it accumulates at concentrations as high as 1–2 mg/ml (see Ref. 1 for a review). The enzyme belongs to the superfamily of pancreatic-type RNases and shares with its cognate members the ability to digest RNA at pyrimidine sites. Because BS-RNase is provided with an effective immunosuppressive activity, it has been suggested that it functions by protecting spermatozoa from the immune system of the female organism (2).

Structurally, BS-RNase is a homodimeric enzyme with a subunit amino acid sequence 83\% identical to that of bovine pancreatic RNase A, the most studied member of the superfamily of pancreatic-type RNases (3). However, RNase A and all other members of the superfamily are monomeric enzymes, whereas BS-RNase is a covalent dimer with the two subunits linked by two disulfides between Cys-31 and Cys-32 of one subunit and Cys-32’ and Cys-31’, respectively, of the partner subunit.

Another singular feature of BS-RNase is that the protein has two quaternary structures. In one of them (termed MXM), determined by x-ray crystallography (4), the two subunits exchange or swap their N-terminal \(\alpha\)-helices (Fig. 1). Each of them folds onto the adjacent protomer, generating composite active sites, i.e. each contributed by both subunit chains. In the second structure (termed M=M), identified through biochemical studies (5) and analyzed by x-ray crystallography,\textsuperscript{2} there is no interchange between subunits (Fig. 1). It has been found that when the two quaternary forms of BS-RNase are isolated, they convert slowly into each other under pseudo-physiological conditions until the equilibrium ratio of 2:1 is reached between the MXM and the M=M forms. Thus, BS-RNase may be described as an equilibrium mixture of MXM and M=M isoforms.

BS-RNase also possesses a strong antitumor action, characterized extensively on both cultured malignant cells and solid tumors induced in mice (reviewed in Ref. 6). This activity consists in a selective cytotoxic action toward cancer cells (7–10) and is exerted by the enzyme in the cell cytosol, where it degrades mRNA, thus leading the cell to death (11).

None of the other mammalian RNases is provided with antitumor activity. This has been attributed (12, 13) to their monomeric structure, which makes them susceptible to inhibition by the cytosolic RNase inhibitor (CRI). CRI is a 50-kDa protein (14, 15) that binds tightly to monomeric mammalian RNases (16, 17) and inhibits competitively their ribonucleolytic activity with \(K\) values in the \(\mu\text{M}\) range. Dimeric BS-RNase cannot bind to CRI (13, 18), hence it preserves its ribonucleolytic activity inside the cytosol. Recently, it has been shown that monomeric BS-RNase becomes cytotoxic when it is engineered into a variant resistant to CRI (19).

The two isolated isoforms of BS-RNase, M=M and MXM, are both endowed with cytotoxic activity toward malignant cells but differ in their potency, with M=M less active than MXM. Given the intrinsic instability of M=M and the relatively long time required for the cytotoxicity tests (at least 48 h), it cannot be excluded that the cytotoxic activity of M=M is because of its transformation into the MXM isoform.

In the present study, molecular modeling and site-directed mutagenesis have been used to alter the refolding pathway leading to dimeric BS-RNase, with the aim to obtain a stable variant of the M=M isoform of BS-RNase, called 24C-BS-RNase, containing an extra disulfide bridge in each subunit, linking the N-terminal arm to the subunit body. The functional characterization of 24C-BS-RNase, with no exchange of N-terminal helices between subunits, has been used to probe the
EXPERIMENTAL PROCEDURES

Materials and General Procedures—Purification of BS-RNase from bull seminal vesicles and isolation of its M−M and MXM isoforms were performed as described (20). Protein sequence analyses were performed by automated Edman degradation of the polypeptide chain with an Applied Bissystems sequencer (model 473A), equipped with a high performance liquid chromatography apparatus for identification of phenylthiohydantoin derivatives. Matrix-assisted laser desorption ionization mass measurements were performed at the Center for Mass Spectrometry (Naples, Italy). The analysis of the quaternary structure of BS-RNase and 24C-BS-RNase by selective reduction of the intersubunit disulfides followed by gel filtration was performed as described (5). For the reaction with divinyl sulfone the procedure proposed in Ref. 21 was followed with modifications (22).

Site-directed Mutagenesis—The mutated DNA coding for 24C-BS-RNase was obtained by PCR. The template for PCR was the expression plasmid pET-BS, which contains a semi-synthetic cDNA coding for BS-RNase, placed under the transcriptional control of T7 phage promoter (20). The segment amplified corresponds to the entire cDNA and contains unique NdeI and HindIII restriction sites at its 5′ and 3′ ends, respectively. The 5′ amplification primer (5′-GAGTATACATATGAAAGAAGCTGTCGTCGAA-3′) introduced the Aln-4 → Cys mutation, whereas the 3′ primer (5′-CTAAAGCTTACTACGAAAGCATCGAAAGTGGCCATGCGACACGAC-3′) introduced the Val-118 → Cys mutation. The PCR product was purified, digested with NdeI and HindIII, and cloned into the corresponding sites of pET-BS (20). The identity of the resulting expression plasmid was confirmed by DNA sequence analysis of the entire coding sequence.

Protein Expression and Purification—The main steps in the preparation of 24C-BS-RNase were as follows: 1) expression in Escherichia coli of the mutated cDNA coding for 24C-BS-RNase, 2) isolation of bacterial inclusion bodies containing the recombinant protein, 3) its refolding to a monomeric species in a glutathione buffer, 4) purification of the monomer by gel filtration, 5) its dimerization by intersubunit disulfide bridging, and 6) purification by gel filtration of the final dimeric species.

These steps were performed as described (20) except for the following. 1) The renaturation step in the glutathione buffer lasted 48 h. 2) Residue Met(-1) of the recombinant polypeptide chain was removed by treatment with Aeromonas proteolytica aminopeptidase (23), performed on the purified monomeric species. In particular, the monomer-containing fractions eluted from the gel filtration were pooled and supplemented directly with 10 μM ZnSO4 and 10 μM aminopeptidase. After 72 h of incubation at 37 °C, the reaction was stopped by addition of Na2EDTA to a final concentration of 10 mM. The processed monomeric protein was then treated with DTT as the first step of the dimerization procedure. 3) The dimeric species, isolated by gel filtration, were concentrated by ultrafiltration and loaded on a high performance liquid chromatography apparatus equipped with a C2 column. Elution was performed with a 60-min linear gradient from 10 to 45% acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min. The main peak of absorbance at 280 nm was collected, lyophilized, and dissolved in 50 mM Tris-Cl, pH 7.0, containing 50 mM NaCl. The purified protein was then incubated at 37 °C for 96 h. The latter treatment is used routinely to equilibrate the two quaternary isoforms of BS-RNase (20).

The protein sample was quantitated by spectrophotometry using a value of E280 of 280 nm of 4.65, equivalent to that of wild-type BS-RNase, and was stored at −20 °C. Yields of 24C-BS-RNase typically ranged between 1 and 2 mg of purified protein per liter of bacterial culture.

Circular Dichroism Measurements—CD measurements were performed on a JASCO 715 spectropolarimeter equipped with a PTC-348 thermostat and quartz cells of 0.1-cm optical path length. All protein samples were dissolved at 0.3 mg/ml concentration in 10 mM Tris acetate buffer, pH 8.4, containing 0.1 mM NaCl. Far-UV spectra (200–250 nm) were recorded at 20 °C with a response of 18 s, a 0.2-nm band width, and a scan rate of 5 nm/min. Thermal unfolding curves were recorded at 220 nm, in a temperature range from 20 to 90 °C, with a scan rate of 1.0 K/min. Molar ellipticities per mean residue, [θ]220, were calculated from the equation [θ]220 = 1000nm/1/c, where [θ]220 is the mean residue molecular mass (117 Da for BS-RNase), c is the protein concentration. The instrument was calibrated with an aqueous solution of d-10-(+)-camphorsulfonic acid at 290 nm (24).

Assays—Units of ribonucleolytic activity toward yeast RNA were measured according to Kunitz (25), except that reactions were performed in 0.1 mM Tris-Cl, pH 7.5, containing 1 mg/ml RNA. Hydrolytic activity toward cytidine 2′-3′ cyclic phosphate was measured at 25 °C by a spectrophotometric method (26). Reaction mixtures were made of 1 ml of 0.1 mM Tris-Cl, pH 7.3, containing 0.1 mM NaCl, 0.2–0.6 μM enzyme, and 4 mM substrate. One enzyme unit was defined as the amount of enzyme hydrolyzing 1 micromole of substrate per min. Staining for RNase activity of samples run on SDS-polyacrylamide gels was performed according to Ref. 27. Cytotoxicity assays toward cultured SVT2 fibroblasts were performed as described (28). Reported values of enzyme-specific activity and of IC50 values for cytotoxic activity are the mean ± S.E. of three to four determinations.

RESULTS AND DISCUSSION

Design of a Stable Variant of the M=M Isoform of BS-RNase—The isolated M=M isoform of native BS-RNase is unstable. It spontaneously evolves into the MXM isoform until an equilibrium is reached, with the swapping isoform accounting for about two-thirds of the molecules in solution. This transition from a quaternary structure to another also occurs during the refolding pathway of BS-RNase (Fig. 2A). The unfolded polypeptide chain, containing 10 cysteines, first refolds into the monomeric species characterized by four disulfide bridges (29); then monomers associate into non-swapping dimers (M=M isoform) through the formation of two intersubunit disulfides involving the remaining two cysteines. Finally two-thirds of the subunits swap their N-terminal helices to generate the MXM isoform (5).

With the purpose of obtaining a stable variant of the M=M isoform of BS-RNase, we designed the introduction of a trap in the refolding pathway of the protein that would prevent the transformation of M=M into MXM. The strategy consisted in grafting on each subunit of the protein a new disulfide bridge to connect the N-terminal helix of each subunit to the main subunit body. Such a bridge would form during the first refolding step of the protein, in which the unfolded polypeptide chain folds into a monomer (Fig. 2B). In the subsequent refolding step, monomers would associate into non-swapping dimers, but they would not be able to evolve into the MXM isoform because...
of the trap produced by the extra disulfide bridges (Fig. 2B).
Previous biochemical investigations (30) and simulation studies (31) have shown that the first step in the M=M ⇄ MXM interconversion is the unfolding and displacement of the N-terminal arm from each BS-RNase subunit. Thus, the block imposed by the disulfides on the displacement of the N-terminal arm would block the conversion of M=M into MXM completely.

A variant of monomeric RNase A with an extra disulfide bridge connecting the N-terminal helix and the main protein body has been obtained already by introducing cysteine residues in positions 4 and 118 of the polypeptide chain (32). The inspection of the three-dimensional structure of BS-RNase, both as MXM dimer with swap (4) and as M=M dimer with no swap, revealed that Ala-4 and Val-118 are in close proximity with each other also in BS-RNase (Fig. 3A). Mutation of these residues to cysteine was then simulated with the aid of the computer program PDB Viewer (33), and the resulting model was subjected to energy minimization with SWISS-MODEL (34). In the final model, the side chains of the newly introduced cysteines were found to be positioned as required for the formation of a canonical disulfide bridge (Fig. 3B). The disulfide would be located outside the active site area and would link the N-terminal helix and one edge of a large β-sheet belonging to the main subunit body. A closer examination of the new disulfide bridges within the two subunits of the model showed that they have virtually identical structures, whose stereochemistry is characterized by the following: 1) CA-CA', CB-CB', and SG-SG' distances of 5.3, 4.2, and 2.0 Å, respectively, 2) CB-SG-SG' and CB'-SG'-SG angles both of 109°, and 3) a right-handed chirality with a chi3 angle of +98°. These values are within the ranges defined by the geometries of disulfide bridges in the crystallographic structures of proteins (35–37).

In conclusion, the modeling session described above suggested that mutation of Ala-4 and Val-118 to cysteines should yield a refolded dimer of BS-RNase endowed with two extra disulfide bridges that link each N-terminal helix to the main body of the same subunit. The protein should be enzymatically active and locked into the non-swapping, M=M conformation. The preparation of this variant of BS-RNase, containing 24 Cys residues per dimer, hence termed 24C-BS-RNase, was subsequently undertaken.

Preparation and Basic Characterization of the Variant 24C-BS-RNase—The cDNA coding for wild-type BS-RNase was mutagenized by PCR to introduce codons for Cys-4 and Val-118. The mutated cDNA was then sequenced and expressed in E. coli under the transcriptional control of the T7 phage promoter. The recombinant protein was then recovered from the inclusion bodies and refolded in the presence of a mixture of glutathione and oxidized glutathione. The resulting monomeric species was then purified by gel filtration.
aminopeptidase to remove the N-terminal Met(-1) residue that characterizes most of the heterologous proteins expressed in E. coli. The processed monomer was then dimerized by favoring the intersubunit disulfide bridging and finally purified by gel-filtration followed by reverse phase chromatography.

The purified 24C-BS-RNase variant was then subjected to structural characterization. First, the protein was analyzed by SDS-PAGE. As shown in Fig. 4, the protein migrates as a single band with an apparent molecular size lower than that of wild-type BS-RNase. A similar result was obtained when the gel was stained for ribonuclease activity (see Fig. 4). These results showed that the purified protein is homogeneous and active enzymatically but raised doubts about the structural integrity of the polypeptide chain, as its unusual electrophoretic mobility could be attributed to unexpected proteolytic cleavages of the polypeptide chain by E. coli proteases or to an incorrect processing by the Aeromonas aminopeptidase. This possible damage was investigated by N-terminal protein sequencing and molecular mass determination. The former analysis revealed an N-terminal Lys-Glu-Ser sequence, as expected for a correctly processed protein. The molecular mass, determined by mass spectrometry, resulted to be 27,274.6 Da, a value that is not significantly different from the expected value (27,283.6 Da), calculated for a variant of BS-RNase with the Ala-4 → Cys and Val-118 → Cys mutations and two extra disulfide bridges. The unusual migration on SDS-PAGE of 24C-BS-RNase was thus taken as an indication that the newly introduced cysteines had indeed formed the expected extra disulfide bridges, thus providing the protein with a more compact structure.

The newly introduced disulfide bridges of 24C-BS-RNase were next analyzed by amino acid sequence determinations performed on the isolated protein variant and after full reduction of its disulfides with DTT followed by modification of the exposed cysteines with 4-vinylpyridine. Before reduction, no amino acid was found by sequence determination at position 4. Given the chemistry of the Edman reaction, this indicated that Cys-4 was not free but linked to another Cys residue in a disulfide bridge. When the protein disulfides were reduced, and the resulting cysteine residues were modified with 4-vinylpyridine, the sequence determinations revealed the expected modified cysteine in position 4 of the polypeptide chain. In conclusion, the results indicate that 24C-BS-RNase is an enzymatically active dimeric protein differing from wild-type BS-RNase by the presence of extra disulfide bridges linking Cys-4 and Cys-118 in each subunit.

Quaternary Structure of 24C-BS-RNase—The quaternary structure of 24C-BS-RNase was investigated by two different methods. In the first method (5), the two intersubunit disulfide bridges are reduced selectively by limited amounts of DTT, and the reaction products are analyzed by gel filtration. If the protein is entirely in the M=M form, i.e. its quaternary structure is stabilized only by the two intersubunit disulfides, the procedure yields monomers. If the protein is in the MXM form, reduction of the intersubunit disulfides yields non-covalent dimers, whose quaternary structure is stabilized by the swap of the N-terminal helices.

When a sample of native BS-RNase (naturally occurring as a mixture of about 30% M=M and 70% MXM) is subjected to selective reduction of its intersubunit disulfides and analyzed by gel filtration, two products are observed (Fig. 5): a monomeric species deriving from M=M and a dimeric species deriving from MXM. It is noteworthy that the areas of the two peaks reflect the relative amounts of M=M and MXM present originally in the protein sample. When 24C-BS-RNase was reduced selectively and analyzed, the gel filtration revealed only one peak, with the molecular size of a monomer. This result indicates that 24C-BS-RNase behaves as the M=M form of BS-RNase, i.e. it consists entirely of a non-swapping dimer.

The second method employed to investigate the quaternary structure of 24C-BS-RNase is based on the use of a bifunctional reagent, divinyl sulfone, which reacts simultaneously with the enzyme catalytic residues His-12 and His-119 (21). When the active site of a dimer is composite (as in the case of MXM), with His-12 from one polypeptide chain and His-119 from the other, the cross-link joins the two chains, yielding a cross-linked dimer. If the active site of a dimer is not composite (the M=M case), divinyl sulfone acts as it does in a monomeric RNase, linking two histidines from the same polypeptide chain.

24C-BS-RNase and two controls, BS-RNase and RNase A, were treated with divinyl sulfone, and the reaction products were analyzed by SDS-PAGE under reducing conditions to distinguish cross-linked from disulfide-linked dimers (Fig. 6). As expected, RNase A migrated as a monomer, whereas BS-RNase showed two bands arising from the MXM and M=M isoforms. The only detectable product of reacted 24C-BS-RNase was a monomer, thus providing an additional indication that the engineered protein is provided with the non-swapping conformation only.

Thermal Stability of 24C-BS-RNase—The thermal stability of the 24C-BS-RNase variant was investigated by circular dichroism. Far-UV CD spectra were recorded at 20 °C for BS-RNase, its MXM and M=M isoforms, and for 24C-BS-RNase (Fig. 7). As described previously (38), the BS-RNase spectrum has an intermediate intensity between those of MXM and M=M, with M=M being the isoform showing a lower content of secondary structure. Furthermore, the spectra of the isolated
isomers are distinguished readily from that of native BS-RNase, the mixture of the two isomers, by the lack of distinct minima and shoulders. The spectrum of 24C-BS-RNase was found to be very similar to that of M-like non-swapping dimer.

A melting curve for the temperature-induced denaturation of 24C-BS-RNase was obtained, in parallel with the curve for isolated M=M, by raising the temperature from 20 to 90 °C gradually and recording the molar ellipticity at 220 nm. The results, illustrated in Fig. 8, show that for both proteins the molar ellipticity at high temperature is lower than zero: \[ \theta_220 = -4500 \text{ and } -4600 \text{ deg dmol}^{-1} \text{ cm}^2 \] for M=M and 24C-BS-RNase, respectively. This indicates that at high temperatures the two proteins contain residual secondary structure even though most parts of the polypeptide chain are unordered. The denaturation temperature of the M=M isomorph of BS-RNase was confirmed to be 63 °C (38); that of 24C-BS-RNase was instead much higher, at 72 °C (see Fig. 8).

The data of a higher stability of the 24C-BS-RNase variant, as indicated by the CD analyses, are in line with, and shed light on, the results of the SDS-PAGE runs described above (see Fig. 4), in which the protein showed a higher electrophoretic mobility. It can be inferred that the higher mobility of 24C-BS-RNase can be attributed confidently to the much more compact structure of the variant, compared with the native protein.

Similar and contrasting results have been obtained when extra disulfide bridges were introduced into proteins by site-directed mutagenesis (39, 40). In some cases the protein structure was stabilized; in others the mutant proteins were found to be less stable (41–43). A proposal originated from these studies (42) was that an increase of protein stability is obtained when the novel disulfide is introduced in a flexible region of the protein.

The 4–118 disulfide grafted into BS-RNase is not located in a flexible region of the protein. It links the N-terminal α-helix with the 116–124 strand of a large β-sheet (see Fig. 3A). However, this α-helix is connected to the main body of the subunit through a linker peptide spanning residues 16–22. By x-ray crystallography, it has been found that this peptide linker is certainly flexible, as it assumes two distinct conformations in the MXM isomorph of BS-RNase (4), and is totally disordered in the M=M isomorph and in a monomeric derivative of the protein. It is tempting to surmise that the newly inserted 4–118 disulfide can stabilize the linker peptide, thus conferring stability to a large region of the protein.

Enzymatic Activity of 24C-BS-RNase—Pancreatic-type RNases digest RNA by a two-step transphosphorolytic-hydrolytic reaction. In the first step, the RNA is depolymerized to nucleoside or oligonucleotide 2’,3’-cyclic phosphates. This step is quite fast and follows standard Michaelis-Menten kinetics. In the second step, the products of the previous reaction are hydrolyzed to nucleoside- or oligonucleotide-3’-phosphates.

The enzymatic activity of 24C-BS-RNase was tested in a spectrophotometric assay toward yeast RNA, a conventional RNase substrate, to monitor the first step of the reaction. The engineered enzyme exhibited a specific activity of 84 ± 5 units/mg, a value that is about one-half of the activity of the wild-type protein (192 ± 6 units/mg). A similar decrease in catalytic activity, with respect to that of the native enzyme, was found when 24C-BS-RNase was tested with cytidine 2’,3’-cyclic phosphate (C>p), a substrate for the second, hydrolytic reaction step. At 4 mM C>p concentration, the enzyme was found to have a specific activity of 17 ± 4 units/mg, i.e. about one-half that of the native enzyme (36 ± 6 units/mg) assayed under identical conditions.

The reduced catalytic activity of the 24C-BS-RNase variant may not be surprising, for several reasons. It has been mentioned above that the insertion in the molecule of the extra disulfides has a dramatic effect on its stability, with an obvious

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increase in structural rigidity of the whole molecule. Then, in particular, we should consider the domain motions that BS-RNase (44), as RNase A (45), undergoes upon ligand binding. The new links imposed between Cys-4 in the N-terminal helix and Cys-118 in the nearest b-sheet from the main subunit body may well have a detrimental effect on these dynamics primed by quasi-substrate binding. Furthermore, although the new disulfide is located outside the subunit active site as defined by the catalytic triad (His-12, His-119, Lys-41), it is immediately adjacent with its Cys-118 to one member of the triad. It is known that in RNase A (46), but also in BS-RNase (4), the side chain of His-119 may assume two alternative conformations, a clear indication of local flexibility in that region of the active site. In fact, the active site of these RNases comprises a rigid and a flexible region, the former made up of His-12, Asn-44, and Thr-45, and the latter made up of His-119, Asp-83, and Asp-121. Clearly, the presence of the novel disulfide within 3 Å from the position of His-19 can affect the flexibility of the latter region dramatically, thus hindering the dynamics primed by substrate binding in the enzyme molecule.

It may be mentioned that Matsumura and Matthews (47) succeeded in switching off the activity of bacteriophage T4 lysozyme by engineering a disulfide in the active site of the enzyme. Recently, Park and Raines (48) inserted a redox switch in RNase A by introducing a new disulfide bond in the N-terminal helix of the protein, not far from the active site. When the disulfide was in the oxidized state, catalytic activity was reduced by 70%.

**Antitumor Activity of 24C-BS-RNase**—When 24C-BS-RNase was tested for cytotoxic activity on tumor cells, the engineered protein was found to be able to inhibit the proliferation of malignant SVT2 fibroblasts. The inhibition was dose-dependent with an IC50 value of 61 ± 9 µg/ml, a value about 3-fold higher than that of native BS-RNase (20 ± 5 µg/ml) assayed in parallel experiments. These results indicate that the swapping of the N-terminal helices between subunits is not essential for the antitumor activity of BS-RNase; however, they also indicate that the absence of swapping decreases the cytotoxic activity of the protein. These data are in agreement with previous results suggesting that the isolated M=M isoform of BS-RNase was less active than the MXM form on malignant cells (49). We can now conclude that M=M is endowed with an intrinsic cytotoxic activity, which may not be ascribed to its partial conversion to the MXM isoform under the conditions of the assay (48 h in culture medium at 37 °C).

The possibility that the lower cytotoxic activity of 24C-BS-RNase is because of its lower catalytic activity is very unlikely. When 24C-BS-RNase reaches the cytosol, its RNase activity, 10²- to 10³-fold higher than that of onconase, another effective antitumor RNase (6), is certainly adequate to initiate degradation of rRNA, hence to block protein synthesis and kill the cell.

On the other hand, the finding of cytotoxic activity in 24C-BS-RNase, as in M=M, would not be in line with current data and hypotheses (12, 13, 19), which assign a key role of control against exogenous RNAses to CRI. According to this model, monomeric RNases cannot be active as cytotoxic agents, as in the cytosol they are neutralized by CRI. In fact, when monomeric RNases are rendered resistant to CRI, they acquire cytotoxic activity (12, 19). Thus, 24C-BS-RNase and M=M would be expected to be devoid of cytotoxic activity, given that the intersubunit disulfides should be cleaved in the reducing environment of the cytosol, and the dimers should dissociate into monomers. It has been shown (50, 51) that the intersubunit disulfides of BS-RNase are cleaved readily when incubated in a mixture of glutathione and oxidized glutathione, which closely mimics the reducing environment of cytosol.

However, it should be considered that disulfide cleavage in the cytosol may not affect the entirety of the RNase molecules entering the cytosol instantaneously. The small fraction of 24C-BS-RNase that remains dimeric soon after the protein enters the cytosol can escape CRI and damage rRNA, hence the protein synthesis machinery. The transient survival of a dimeric fraction of the RNase molecules that reach the cytosol explains the resistance to CRI of 24C-BS-RNase and MXM, and their cytotoxicity. These considerations also explain why 24C-BS-RNase, and M=M, are much less cytotoxic when compared with the MXM isoform, which maintains its dimeric, albeit non-covalent, structure fully, and hence is fully resistant to CRI.

**CONCLUSIONS**

BS-RNase has a very complex folding pathway comprising the oxidation of 20 cysteines (8% of the total number of residues). 16 of them form eight intra-subunit disulfide bridges, whereas the remaining four cysteines are responsible of the formation of two intersubunit disulfides. Despite this complexity, the present study has shown that four other cysteines can be introduced in BS-RNase dimers. The engineered variant 24C-BS-RNase was obtained as an active enzyme with each N-terminal helix linked covalently to the main protein body of the same subunit. The covalent link blocks the displacement of the N-terminal arm from the subunit body, a necessary step in the refolding pathway that leads to a partial conversion of the first folded quaternary conformation, with no swap (M=M), into the conformation with swap (MXM). The protein was found to be very stable and represented a valuable tool for probing the role of the swapping in the enzymatic and antitumor activities of BS-RNase.

The results on the enzyme activity of the engineered variant have shown that the extra disulfides affect, but not dramatically, the catalytic activity of the enzyme. This is interpreted in terms of the increased rigidity imposed on the protein active site by the extra disulfides.

With regard to the antitumor activity, the results show that the transition from non-swapping dimer (M=M) to dimer with swap (M=M) generates an increase in the enzyme cytotoxic activity of about 3-fold. The lower cytotoxic activity of 24C-BS-RNase and of the isolated M=M isoform, can be attributed to the absence of swap in these protein forms, which remain only fractionally dimeric in the reducing environment of the cytosol. Therefore, the dimeric structure of BS-RNase by itself is not essential for cytotoxicity but is very important as a basis for the formation of swapping dimers with enhanced antitumor activity.

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