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A New Mutant of Bovine Seminal Ribonuclease with a Reversed Swapping Propensity†

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ABSTRACT: Bovine seminal ribonuclease (BS-RNase) is made up of two identical subunits bridged through two disulfide bonds. In solution, it exists as a 2:1 equilibrium mixture between two forms, with (M×M) and without swapping (M=M) of the N-terminal arms. The swapping endows BS-RNase with some special biological functions, including antitumor activity, since M×M retains a dimeric structure even under reducing conditions, thus evading the cytosolic ribonuclease inhibitor. To investigate the structural basis of domain swapping in BS-RNase, we have obtained several mutants by replacing selected residues with the corresponding ones of its monomeric counterpart, bovine pancreatic ribonuclease (RNase A). We have already shown that, in contrast with all other cases of swapped proteins, the swapping propensity of BS-RNase does not depend on the specific sequence of the 16−22 hinge loop, which connects the main body to the dislocating arm. In this paper we report the design, the expression, and the structural characterization of two mutants obtained by replacing Arg80 with Ser either in BS-RNase or in the mutant already containing the 16−22 hinge sequence of RNase A. NMR and circular dichroism data indicate that, in the monomeric form of the latter mutant, Ser80 acts as a switch for the conformation of the hinge region. Accordingly, in the dimeric form of the same mutant the M×M:M=M equilibrium ratio is inverted to 1:2. Overall, these data suggest that the presence of Arg80 triggers the swapping of N-terminal ends and plays a relevant role in the stability of the swapped form of BS-RNase.

In the mammalian ribonuclease family, bovine seminal ribonuclease (BS-RNase)[1] is the only dimeric protein made up of two identical subunits cross-linked by two disulfide bridges between Cys31 from one subunit and Cys32 from the other, and vice versa. Each subunit of BS-RNase is a homologue of bovine pancreatic ribonuclease (RNase A), showing more than 80% sequence identity. The native protein exists as an equilibrium mixture between two forms with different tertiary structure: a swapped form (dubbed M×M), characterized by the exchange of the N-terminal α-helices between the subunits, and an unswapped one (dubbed M=M), with no exchange (*1*). Under physiological conditions, the equilibrium ratio between M×M and M=M is about 2:1. The swapping of N-terminal arms has relevant biological implications, since it has been found that, upon selective reduction of the interchain disulfide bridges, the unswapped form readily dissociates into two monomeric derivatives (*1*), whereas the M×M form is converted into a noncovalent dimer (dubbed NCD), which retains a dimeric structure owing to the exchange of N-terminal tails (*1*). This structural feature prevents the interaction with the cytosolic RNase inhibitor (cRI) and endows BS-RNase with some additional biological functions, including antitumor activity (*2*, *3*).

X-ray structures of both M=M and M×M dimers (*4*, *5*) indicate a strong similarity except for the 16−22 loop region, the so-called hinge peptide, connecting the dislocating helix to the main body of the protein. Interestingly, the 16−22 hinge region of BS-RNase (***GN*S*PS***SS) contains the highest number of substitutions with respect to RNase A (***ST*S*AA***SS), which can be considered the monomeric counterpart of BS-RNase, suggesting a possible driving role played by this region in the swapping process. A similar hypothesis is also supported by the general observation that manipulation of the hinge loops affects substantially the swapping propensity of many other proteins (*6*). However, as we have already shown, in the variants obtained by substituting either only Pro19 (*7*) or even all hinge residues (*8*) with the corresponding ones of RNase A, the swapping process takes place at the same rate as in the native enzyme and reaches the same

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[1] Abbreviations: BS-RNase, bovine seminal ribonuclease; CD, circular dichroism spectroscopy; DVS, divinyl sulfone; hA-BS-RNase, G16S/N17T/P19A/S20A variant of BS-RNase; hA-mBS, G16S/N17T/P19A/S20A variant of mBS; HSQC, heteronuclear single-quantum coherence spectroscopy; mBS, monomeric Asn67Asp variant of BS-RNase with cysteines 31 and 32 linked to glutathione moieties; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser enhancement; RNase A, bovine pancreatic ribonuclease; S_{80}-BS-RNase, R80S variant of BS-RNase; S_{80}-hA-BS-RNase, R80S/G16S/N17T/P19A/S20A variant of BS-RNase; S_{80}-hA-mBS, R80S/G16S/N17T/P19A/S20A variant of mBS; S_{80}-mBS, R80S variant of mBS; TOCSY, total correlation spectroscopy.

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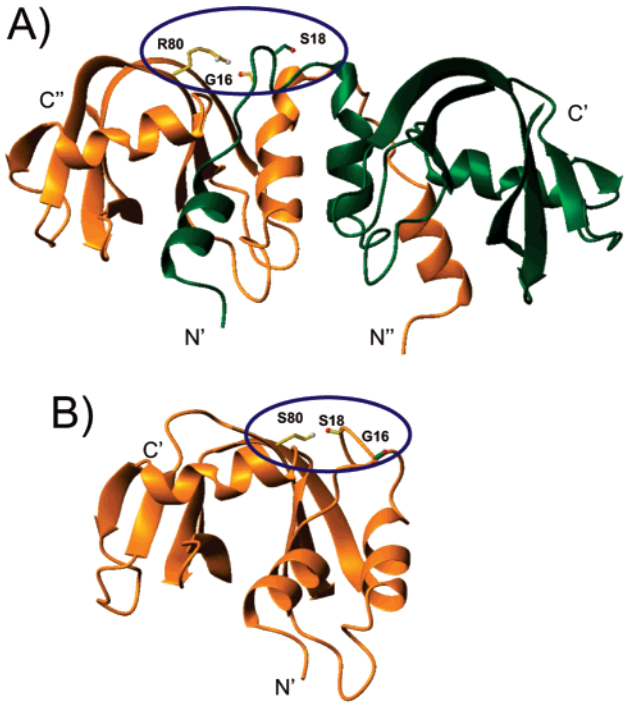

FIGURE 1: 3D structure of BS-RNase (1BSR) (panel A) and RNase A (1KF3) (panel B) showing the interactions between the side chain of residue 80 and the hinge region.

2:1 equilibrium ratio between the M×M and M═M forms. A detailed structural analysis allowed us to explain this result as a subtle balance of enthalpic and entropic contributions but still leaves the question of the role of the hinge loop open.

In this paper we have focused on residues that interact with the hinge loop. In the X-ray structure of the swapped form of BS-RNase (*4*) one of the two hinge conformations (extended) is stabilized by a hydrogen bond involving the backbone oxygen of Gly16 and the side chain of Arg80 of the other subunit (Figure 1A). On the other hand, in RNase A (*9*) residue 80 is a serine, whose side chain makes a hydrogen bond with the backbone oxygen of Ser18 (Figure 1B), thus stabilizing the hinge conformation. The importance of this interaction is proved by the observation that in the variant of RNase A with Arg at position 80 the hinge residues are disordered (*10*). To evaluate the influence of this residue in the swapping process, we have expressed two monomeric mutants by substituting Arg80 with Ser either in BS-RNase or in the mutant containing the hinge loop of RNase A (hA-BS-RNase). Here we compare the structural features of the new mutants with those of the respective parent proteins, i.e., monomeric BS-RNase and hA-BS-RNase. We show also a clear correlation between the flexibility of the hinge regions in monomers and the swapping propensity of the corresponding dimers.

MATERIALS AND METHODS

Protein Samples. The R80S mutation has been introduced in two different pET-22b(+) plasmid cDNA, the former coding for BS-RNase, to obtain the R80S-BS-RNase variant, and the second one coding for the mutant G16S-N17T-P19A-S20A-BS-RNase, to obtain the G16S-N17T-P19A-S20A-R80S-BS-RNase variant. The QuikChange site-directed mutagenesis kit protocol (Stratagene, La Jolla, CA) has been used. To avoid heterogeneity arising from the spontaneous deamidation of Asn67 (*11*, *12*), in all of the plasmids the basic sequence of BS-RNase contains the Asn67Asp substitution.

The unlabeled and ^{15}N-labeled proteins were expressed in *Escherichia coli* cells and purified in monomeric form, with cysteines 31 and 32 linked to two glutathione molecules, as previously described (*13*, *14*). Monomers in the reduced form, obtained by selective reduction of the mixed disulfide bridges, were either carboxamidomethylated with iodoacetamide (*15*), to obtain the monomeric proteins used for CD, or submitted to dialysis against 0.1 M Tris−acetate, pH 8.4, followed by gel filtration on Sephadex G-75, to obtain dimers (*15*). All dimerization steps were performed at 4 °C.

CD Spectra Measurements. The CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature control system (Model PTC-348WI). A protein concentration of about 0.3 mg mL^{-1} in 10 mM sodium acetate buffer, pH 5.0, was used. Thermal unfolding curves were recorded in the temperature scan mode at 222 nm from 25 to 85 °C with a scan rate of 1.0 °C min^{-1}. For each protein sample, T_m was calculated by nonlinear regression analysis of the experimental data as the value corresponding to the midpoint of the denaturation curve. The full reversibility of thermal transition was assessed by CD for all proteins.

NMR Spectra Measurements. NMR measurements were performed at 27 °C on Bruker DRX600 and Varian INOVA 700 spectrometers, using standard pulse sequence libraries. Protein concentration was 1 mM in 95% H_2O−5% D_2O, pH 5.65, in all experiments. ^{1}H chemical shifts were relative to the water signal at 4.70 ppm; ^{15}N chemical shifts were indirectly referenced to the ^{1}H chemical shifts according to magnetogyric ratios (*16*).

The heteronuclear-edited 3D experiments used in the assignment procedure were ^{1}H−^{15}N NOESY-HSQC (*17*) and ^{1}H−^{15}N TOCSY-HSQC (*18*). Heteronuclear ^{1}H−^{15}N NOEs were measured at 600 MHz ^{1}H frequency using standard methods (*19*).

Spectra were processed with NMRPipe (*20*) and analyzed with NMRVIEW (*21*) version 4 programs.

Extent of N-Terminal Swapping in Dimers. Kinetic analysis of the swapping was performed by incubation of the dimers at 37 °C followed by selective reduction of the interchain disulfide bridges and gel filtration chromatography (*1*) on an analytical Superdex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden). The amount of M×M and M═M was quantitatively evaluated by integration of the peaks of dimer and monomer, respectively.

The swapping extent in the equilibrium mixtures was also assessed by cross-linking experiments with divinyl sulfone (DVS) followed by SDS−PAGE under reducing conditions, as previously reported (*22*). Semiquantitative estimation of the cross-linked dimer to monomer ratio, corresponding to M×M and M═M forms, respectively, was done by Coomassie blue staining.

RESULTS

Protein Expression and Conformational Analysis. The correct folding of the monomeric R80S-BS-RNase (henceforth called S_{80}-mBS), G16S/N17T/P19A/S20A-BS-RNase

(henceforth called hA-mBS), and R80S/G16S/N17T/P19A/S20A-BS-RNase (henceforth called S_{80}-hA-mBS) was checked by comparing their CD and ^{1}H NMR spectra with those of the parent monomeric BS-RNase (henceforth called mBS;, data not shown). In addition, their enzymatic activity on yeast RNA (*23*) was equivalent to that of native monomeric BS-RNase, thus confirming also the correct fold of the active site. However, the comparison of the natural abundance ^{15}N−^{1}H HSQC spectra of the mutants with that of mBS indicated that several amide resonances other than those of the mutated residues were shifted, suggesting that some local conformational differences were occurring. Indeed, the overlay of the ^{15}N−^{1}H HSQC spectra of S_{80}-hA-mBS and hA-mBS (data not shown), which differ by only one residue at position 80, indicated that almost all resonances belonging to residues of the 16−22 hinge regions were shifted. To evaluate the effects of the mutations on this region, we undertook a comparison of the backbone resonances as well as of ^{1}H−^{15}N heteronuclear NOEs of S_{80}-hA-mBS and hA-mBS with those previously reported for mBS and RNase A (*8*, *13*).

A de novo assignment of the ^{15}N and ^{1}H chemical shifts of the new mutants was achieved via a standard set of 3D spectra, which were acquired on the ^{15}N-labeled protein samples. As already observed when comparing hA-mBS and RNase A (*8*, *13*), despite the sequence identity the proton chemical shifts of the hinge residues of hA-mBS and RNase A are completely different. However, the introduction of Ser at position 80 shifted the resonances of the hinge residues that in S_{80}-hA-mBS become almost coincident with those of RNase A. The ^{15}N and ^{1}H resonances of the 16−21 residues of both mutants and those of mBS and RNase A, taken from Avitabile et al. (*13*), are reported as Supporting Information (Table 1S). The effect produced by Ser80 on the hinge residues is evidenced for Ala19 in Figure 2A, showing the overlay of the ^{1}H−^{15}N HSQC cross-peak for hA-mBS (red), S_{80}-hA-mBS (green), and RNase A (blue). A further comparison of the structural differences among the monomeric proteins was performed by measuring steady-state ^{1}H−^{15}N heteronuclear NOEs. Figure 2B reports the patterns of S_{80}-hA-mBS (green) and hA-mBS (red) together with those of mBS (black) and RNase A (blue). Despite the absence of several resonances, very similar NOE values were found for all regions with regular secondary structure, whereas some differences were observed in the loop regions. The most interesting result regards the 16−22 hinge region which, in the picosecond time scale, is the most flexible region in all proteins. Although three out of four proteins (both S_{80}-hA-mBS and hA-mBS mutants and RNase A) share the same sequence in this region, the heteronuclear NOE pattern indicates very clearly that the flexibility of the hinge in hA-mBS is substantially identical to that of mBS and much higher than in RNase A. On the other hand, the introduction of Ser80 reduced consistently the flexibility of the 16−22 region, whose pattern in S_{80}-hA-mBS is almost identical to that of RNase A.

Thermal Stabilities of Monomers. The thermal stabilities of S_{80}-mBS, S_{80}-hA-mBS, and hA-mBS were compared to that of mBS by measuring the CD denaturation curves at 222 nm. Figure 3 reports the folded fraction, corresponding to $(\theta_{measured} - \theta_{unfolded})/(\theta_{folded} - \theta_{unfolded})$, as a function of the temperature, for all proteins. The inset in the same figure contains the values of the melting temperature (T_m), i.e., the

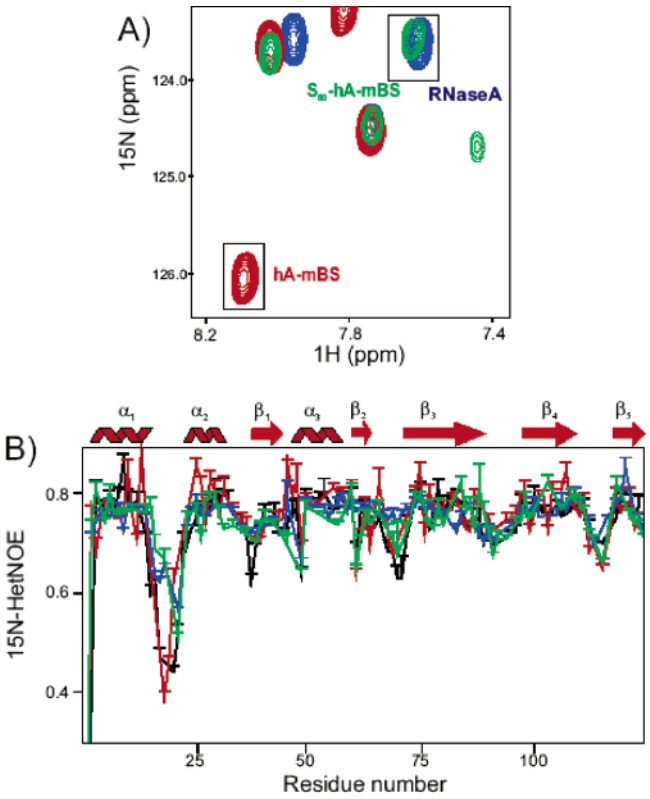

FIGURE 2: Panel A: Overlay of the 600 MHz ^{1}H−^{15}N HSQC peaks of Ala19 for hA-mBS (red), S_{80}-hA-mBS (green), and RNase A (blue). Panel B: ^{1}H−^{15}N heteronuclear NOEs for S_{80}-hA-mBS (green), hA-mBS (red), mBS (black), and RNase A (blue). On the top of the graph are indicated secondary structure elements, spirals for helices and arrows for sheets. In particular, they correspond to residues 4−12 (α1), 21−34 (α2), 50−59 (α3), 43−47 (*β*1), 61−64 (*β*2), 71−74 (*β*3), 79−85 (*β*4), and 98−111 (*β*5).

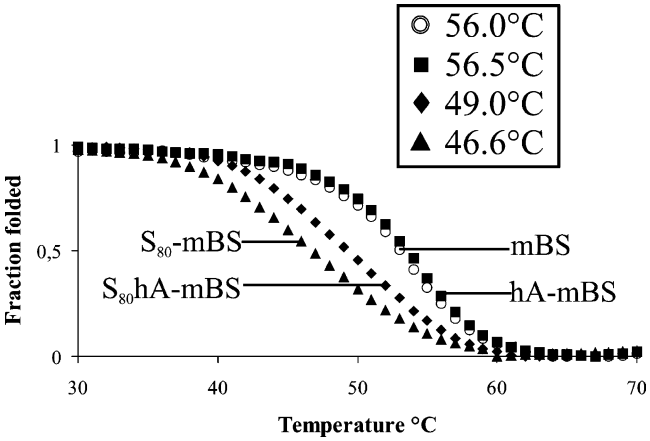

FIGURE 3: CD thermal unfolding curves at 222 nm for mBS, hA-mBS, S_{80}-mBS, and S_{80}-hA-mBS. The inset contains the values of T_m, corresponding to the midpoint of the denaturation process. The estimate of uncertainty is ±0.1 °C.

temperature value corresponding to the 0.50 folded fraction, calculated by nonlinear regression analysis of the experimental data. The substitution of Arg80 by Ser decreases significantly the thermal stability of both S_{80}-mBS and S_{80}-hA-mBS variants with respect to the corresponding parent proteins, i.e., mBS and hA-mBS, both characterized by the same T_m value of about 56 °C. On the other hand, the comparison between S_{80}-mBS and S_{80}-hA-mBS indicates that when the Ser80 residue is combined with the presence of the 16−22 hinge loop of RNase A, the effect is less

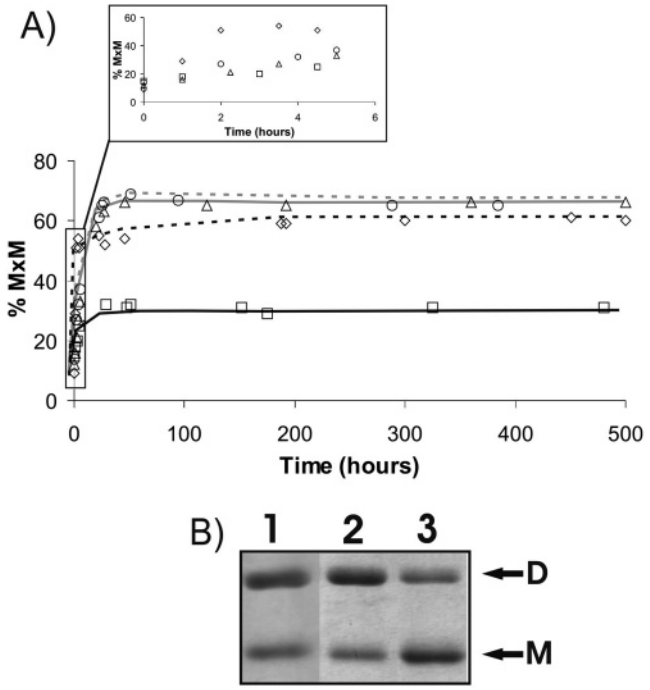

FIGURE 4: Swapping tendency of dimers. Panel A: Percentage of M×M form at 37 °C as a function of the incubation time from 0 to 500 h (lower panel) and from 0 to 6 h (upper panel) for BS-RNase (dotted gray line), hA-BS-RNase (solid gray line), S_{80}-BS-RNase (dotted black line), and S_{80}-hA-BS-RNase (solid black line). Panel B: SDS−PAGE analysis of the divinyl sulfone cross-linking reaction for BS-RNase (lane 1), S_{80}-BS-RNase (lane 2), and S_{80}-hA-BS-RNase (lane 3).

pronounced. The reduced thermal stability of the two mutants containing Ser at position 80 with respect to the parent proteins can be explained by considering two different factors: first, the loss of the favorable electrostatic interactions involving the side chain of Arg80 and those of Glu49 and Glu103, which stabilize the monomeric form of BS-RNase (*24*), and, second, a large unfavorable desolvation energy directly connected to the removal of a charged residue from the water-exposed surface of the protein (*25*). On the other hand, the increased thermal stability of S_{80}-hA-mBS with respect to S_{80}-mBS suggests the presence of a specific interaction in the first protein, probably due to the reconstitution of the same hydrogen bond between the side-chain −OH proton of Ser80 and the backbone oxygen of Ser18, already observed in RNase A. The similarity of T_m for mBS and hA-mBS in fact clearly indicates that the intrinsic conformational tendencies of the hinge residues do not affect significantly their thermal stability.

Swapping Propensity of Dimers. Monomeric forms of BS-RNase and its variants were converted into dimers by selective reduction of the disulfide bridges linking cysteines 31 and 32 to glutathione moieties, followed by gentle air oxidation. Upon gel filtration on Sephadex G-75, we obtained 80% dimer (and 20% monomer) for all of the proteins purified, indicating that the mutations do not affect the dimerization process. As in the case of the parent BS-RNase, freshly prepared dimeric samples at 4 °C were constituted mainly by the nonswapped form, the extent of the exchange of N-terminal ends being initially about 15% for all of the variants (time = 0 in Figure 4A). The proteins were then incubated at 37 °C to allow for the exchange of N-terminal arms. Aliquots were withdrawn at different times, and the relative amount of M×M and M=M forms was evaluated by selective reduction followed by gel filtration, as described in detail by Piccoli et al. (*1*). The time course of the M=M to M×M conversion for S_{80}-BS-RNase and S_{80}-hA-BS-RNase is reported in Figure 4A, showing for comparison also the curves of BS-RNase and hA-BS-RNase (*8*). The found swapping tendency of S_{80}-BS-RNase was very similar to that reported for the wild-type enzyme, the M×M form representing about 60% of the equilibrium mixture, whereas for the S_{80}-hA-BS-RNase mutant the dislocation of N-terminal arms was significantly decreased, the swapped form representing only 30% of the equilibrium mixture (Table 1).

Table 1: Swapping Tendency of Four Dimeric Ribonucleases Differing for the Hinge Region and/or Residue 80 at 37 °C

	hinge sequence							residue	%
protein	16	17	18	19	20	21	22	80	exchange[a]
S_{80}-hA-BS-RNase	S	T	S	A	A	S	S	S	30
hA-BS-RNase	S	T	S	A	A	S	S	R	70
BS-RNase	G	N	S	P	S	S	S	R	70
S_{80}-BS-RNase	G	N	S	P	S	S	S	S	60

[a] Amount of swapped form at equilibrium, corresponding to the protein eluted as dimer on gel filtration after selective reduction of the interchain disulfide bridges (see Materials and Methods for more details).

A similar result was obtained in the experiment based on cross-linking of the N- and C-terminal ends with divinyl sulfone (DVS) followed by SDS−PAGE under reducing conditions. Figure 4B demonstrates that at the equilibrium the M×M:M=M ratio is about 2:1 for S_{80}-BS-RNase and 1:2 for S_{80}-hA-BS-RNase, respectively.

DISCUSSION

Three-dimensional domain swapping has been the object of a renewed interest in the past few years, since the number of proteins with exchange of structural elements between the subunits is continuously increasing. Yet, neither the structural grounds underlying this process nor its biological implications have been completely understood.

Among the domain-swapped proteins, BS-RNase and its mutants represent a unique case because only for these proteins the swapping involves an equilibrium process among two forms with the same quaternary structure, guaranteed by the two interchain disulfide bridges. Therefore, it is possible to study the swapping without additional complicating factors such as temperature, protein concentration, and relative orientation of the monomers that, in general, make the intrinsic swapping propensity very difficult to predict (*26*). We have already shown in a previous study, based on NMR and X-ray data, that the primary structure of the hinge region does not affect directly the swapping of BS-RNase, since neither the M×M:M=M equilibrium ratio nor the kinetics of the process change upon substitution of Pro19 with Ala or even of the whole 16−22 hinge region with the corresponding residues of RNase A (*8*). This unexpected behavior has been explained as the result of a subtle balancing of entropic and enthalpic contributions (*8*): the substitutions that increase the flexibility of the swapped hinge peptide produce an entropic advantage in the M=M to M×M transformation but at the same time are accompanied by the disruption of favorable contacts between the hinge peptide

and the protein matrix, which contribute to the enthalpic advantage of the same process.

The insensitivity of the swapping of BS-RNase to a specific hinge sequence emphasizes the role of the region other than the hinge itself in determinining the singular features of this protein. Besides the above-mentioned cysteines 31 and 32, that impose the correct orientation of the subunits, the experimental results reported here highlight the importance of Arg80. We have found indeed that if we replace Arg80 by Ser, in the mutant containing already the hinge loop of RNase A, the conformation of the hinge loop in the monomer is switched to RNase A-like status, as suggested by the chemical shift and heteronuclear NOE data. Furthermore, the increased thermal stability of S_{80}-hA-mBS with respect to S_{80}-mBS suggests the presence of a specific interaction between Ser80 and the hinge region, possibly due to the reconstitution of the same H-bond between Ser18 and Ser80 which locks the hinge conformation in RNase A. Although the experimental data have been obtained only for the monomeric derivatives, the structural features are actually very similar to those of the M=M dimers. Accordingly, we have found a clear correlation between the flexibility of the hinge loop of the monomers and the swapping propensity of the corresponding dimers. Thus it seems reasonable to assume that the H-bond between Ser80 and the hinge region in S_{80}-hA-BS-RNase leads to the stabilization of the M=M form and consequently shifts the swapping equilibrium on its side. On the other hand, the same Arg80 to Ser substitution in BS-RNase produces only a small decrease of the swapping tendency, possibly due to the absence of the stabilizing interaction between the Arg80 side chain of one subunit and the Gly16 backbone oxygen of the other subunit observed in the wild-type enzyme and evidenced in Figure 1A.

By analyzing the experimental data reported here in a hypothetical evolutionary pathway starting from RNase A and leading to BS-RNase, it is well evident that the same results, in terms of both hinge flexibility and swapping amount, can be achieved by the single substitution of Ser80 by Arg instead of the costly substitution of the whole 16−22 region. No further differences are produced by the four substitutions (S16G/T17N/A19P/A20S) needed to make the hinge region of BS-RNase. These considerations call for an additional role of the hinge loop of BS-RNase, not restricted simply to the swapping process. It has been recently proposed that this region could be responsible for the stability of the dimer under mild reducing conditions as it is in the cytosol, the so-called noncovalent dimer of BS-RNase (NCD), which could represent the active form of the enzyme (*27*). This hypothesis is based on solid-state structures of BS-RNase with (*4*) and without (*5*) the interchain disulfide bridges, which clearly indicates that one of the Pro19 side chains makes an hydrophobic interaction with the Tyr25 and Gln101 side chains of the other subunits. Accordingly, preliminary data obtained in our laboratory indicate that the dissociation rate of BS-RNase is lower than that of hA-BS-RNase, S_{80}-BS-RNase, and also S_{80}-hA-BS-RNase mutants and confirm that the replacement of Ser80 by Arg represents a crucial step in a hypothetical evolutionary transition from monomeric to dimeric ribonucleases, fully stable even when the covalent constraints imposed by the disulfide bridges are released.

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SUPPORTING INFORMATION AVAILABLE

Table 1S listing proton chemical shifts of the 16−21 loop residues for mBS, hA-mBS, S_{80}-hA-mBS, and RNase A at pH 5.65 and 300 K. This material is available free of charge via the Internet at http://pubs.acs.org.

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