The Role of the Hinge Loop in Domain Swapping

THE SPECIAL CASE OF BOVINE SEMINAL RIBONUCLEASE*

Received for publication, November 22, 2004, and in revised form, December 17, 2004 Published, JBC Papers in Press, January 12, 2005, DOI 10.1074/jbc.M413157200

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Domain swapping, a process by which two or more protein molecules exchange identical structural elements to form dimers or higher oligomers (1), has been observed in an increasing number of proteins. More than 50 crystal structures of domain-swapped proteins have been deposited in PDB1 since the first x-ray structure of a protein showing the interchange of N-terminal regions between the two polypeptide chains was reported (2). However, the possible physiological significance of this phenomenon is still unclear (3). It has been proposed that a large number of proteins may undergo this process under physiological or pathological conditions; thus it could represent a mechanism to regulate function, or even an evolutionary strategy to increase protein complexity.

In the panorama of the swapping proteins a special case is represented by bovine seminal ribonuclease (BS-RNase), a homodimeric protein in which the two subunits are covalently linked through two disulfide bridges between cysteines 31 and 32 of one subunit with cysteines 32 and 31 of the partner subunit, respectively (4). In this protein the swapping process involves two dimers, in which the two subunits change their tertiary structure within a basically invariant quaternary assembly imposed by the two interchain disulfides: in the dimer-dubbed MxM the N-terminal arms (residues 1–15) are exchanged, or swapped, between the two subunits, whereas in the dimer indicated as M=M no swapping occurs. Thus, in this particular system the swapping phenomenon does not depend on the overall concentration of the protein. Furthermore, in the quaternary structure of MxM, the acceptable values of the end-to-end distance, spanned by the hinge peptide, are almost sharply restricted as they are within the tertiary structure of the unswapped dimer. This finding is at variance with what is usually observed in the swapping process, where a monomer to dimer (M/D) transition is commonly observed and the swapped dimer often presents a considerable degree of flexibility and, therefore, a certain degree of variability of the end-to-end distance of the hinge peptide. In the latter case, the swapped state is expected to become statistically more favored as the rigidity of the hinge is increased. Indeed, this argument has been used to explain the elevated frequency of proline in the hinge peptide sequence of proteins prone to swap (5). Furthermore, in an M/D transformation other parameters may influence significantly the process, such as the nature and the extent of “O-interface,” i.e., the additional interface formed in the dimer and exposed to the solvent in the monomer (1). In the MxM=M equilibrium of BS-RNase, the quaternary struc-

1 The abbreviations used are: PDB, Protein Data Bank; BS-RNase, bovine seminal ribonuclease; CD, circular dichroism spectroscopy; mBS, monomeric N67D variant of BS-RNase with cysteines 31 and 32 linked to glutathione moieties; RMSD, root-mean-square deviation; RNase A, bovine pancreatic ribonuclease; Ala22-mBS, P19A variant of mBS; Ser17-Thr18-Ala19-Ala20-mBS, G16S/N17T/P19A/S20A variant of mBS; Ala19-BS-RNase, P19A variant of BS-RNase; Ser17-Thr18-Ala19-Ala20-BS-RNase, G16S/N17T/P19A/S20A variant of BS-RNase; TOCSY, total correlation spectroscopy; PEG, polyethylene glycol.

* This work was supported by the Ministero dell’Istruzione dell’Università e della Ricerca (Prin 2002 and Regione Campania (L.R.n.5)). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (codes 1Y92 and 1Y94) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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ture is highly preserved and the O-interface varies only for the different conformation adopted by the hinge peptide in the two dimers (6). This feature offers the unique opportunity to isolate the effects of the hinge sequence on the equilibrium ratio between MxM and M=M. In the native protein the relative amount of the two dimers is about 70:30 (7). Interestingly, the selective reduction of the interchain disulfides produces the species which could be involved into a M/D swapping transformation, as MxM gives rise to a non-covalent swapped dimer (NCD), whereas M=M readily dissociates into monomers.

BS-RNase sequence is 81% identical to that of bovine pancreatic ribonuclease (RNase A), the first protein that was shown to dimerize by virtue of the N termini interchange between the two intervening chains, upon lyophilization in acetic acid (8). For this protein, however, more recent experiments have demonstrated that only a minor fraction of the dimers is swapped at the N terminus, whereas a major fraction is swapped at the C terminus (9–11). In the former dimer the contacts at the so-called “interface,” i.e. the interface between the swapped domain (residues 1–15) and the major domain (residues 23–124), are identical to those found in the covalent dimers of BS-RNase. The sequence alignment of the two proteins shows that four substitutions out of a total of 23 are located in the 16–22-hinge region. In order to clarify the actual role of the hinge sequence in the swapping process of BS-RNase, a homologue-scanning mutagenesis approach has been followed, using as reference the sequence of RNase A. We have prepared two mutants, Ala19-BS-RNase and Ser16-Thr17-Ala19-Ala20-BS-RNase, in which either Pro19 or all four residues of the BS-RNase sequence have been substituted with the corresponding ones of the pancreatic enzyme. Here we report the x-ray structures of the MxM form of the two mutants and discuss the results on the basis of the MxM/M=M equilibrium data measured in solution for Ser16-Thr17-Ala19-Ala20-BS-RNase and those previously published for Ala19-BS-RNase and for the parent BS-RNase (12).

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutagenesis was performed using a megaprim polymerase chain reaction method (13) to produce the mutants coding for G16SN17TP19A BS-RNase and G165/N177P19A BS-RNase, and G165/N177P19A/S20A BS-RNase, starting from the pET-22b (+) plasmid cDNA coding for the P19A BS-RNase (12).

PCR amplification was performed with an Eppendorf Mastercycler amplifier as previously described (12). For the two mutant constructs mutagenic primers 5′-TACGAGCTCGTCTGTC-3′ and 5′-AAAGCTACAGCAGAC-3′ were used in succession (nucleotides that represent mutations are underlined). The amplified, mutated genes were separated, excised, and purified from the agarose gel followed by cloning into the pET-22b (+) plasmid between HindIII and NdeI sites. Mutations were confirmed by DNA sequencing.

To avoid heterogeneity (see “Results and Discussion”), the basic sequence of BS-RNase contains the substitution of Asn67 with an aspartic residue. This modified sequence, together with the N-terminal Met, constitutes the parent protein (henceforth referred to as mBS for monomeric residue). This modified sequence, together with the N-terminal Met, constitutes the parent protein (henceforth referred to as mBS for monomeric residue). The selective reduction of the interchain disulfides produces the species which could be involved into a M/D swapping transformation, as MxM gives rise to a non-covalent swapped dimer (NCD), whereas M=M readily dissociates into monomers.

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Here an extended use of this term, with reference to a monomeric species prior to its conversion to the unswapped (M=M) or to the swapped (MxM) species.
refinement with manual building by using O (23). The missing hinge peptide residues were built by fitting the electron density from difference Fourier maps. Water molecules were then added to the models through the automatic protocol of CNS together with the contribution of the disordered solvent.

Reflections with intensity greater than 2σ(I) in all resolution range (20.00–2.20 Å for both mutants) were included in the minimization procedure. The final models of the two proteins have R_{factor}/R_{free} indexes of 0.171/0.202 and 0.208/0.259, respectively. The models have good geometry as evaluated with WHATCHECK (24). The full lists of refinement statistics are reported in Table II.

Superimposition of the mutants with the wild-type protein was achieved by using the Cα carbon atoms of residues 2–15 and 23–124 of the two chains. Because of the swapping, the quaternary structure was compared by first superimposing one structural unit (residues 2–15 of one chain and 23–124 of the second chain) and, successively, computing the angle needed to superimpose the second structural unit.

RESULTS AND DISCUSSION

Characterization of the Monomers—All the plasmids were coding for Asp at position 67 in order to avoid heterogeneity arising from the spontaneous deamidation of Asn67, which characterizes the native enzyme (25). Asn67 is located in a disulfide-linked octapeptide loop (65–72) exposed to the solvent and on the most far site with respect to the swapping domain and to the O-interface. This substitution in RNase A has been shown not to influence the correct folding of the chain and its thermal stability (26). mBS shows a similar behavior, and, in addition, the extent of the swapping phenomenon in the dimeric species is not altered (12).

The first products of our purification procedure were about 15 mg/liter of recombinant BS-RNase or its variants in monomeric form, with cysteines 31 and 32 linked to two glutathione molecules. The thermal stability of the two mutant proteins was monitored by CD spectroscopy. Fig. 1 reports the folded fraction (fF), calculated as (θ_{measured} - θ_{unfolded})/(θ_{folded} - θ_{unfolded}) at 222 nm, as a function of the temperature for mBS, Ala19-mBS, and Ser16-Thr17-Ala19-Ala20-mBS in comparison with that of recombinant RNase A. The values of T_m, calculated by linear regression analysis of the experimental data, indicate that both mutants display only a very small difference with respect to the parent form (T_m = 53 °C). On the other hand, the T_m of Ala19-mBS (55 °C) is 6 °C lower than that of RNase A; the difference is even higher in the mutant containing four substitutions in the hinge region (T_m = 53.5 °C), despite the greater similarity of its sequence to that of RNase A. In both cases the effect is significantly greater than that produced by the sole substitution of Asn67 (27).

For a more accurate evaluation of the effect of the mutations on the solution structure of the monomeric proteins we resorted to two-dimensional NMR spectra. The overlay of TOCSY spectra of Ser16-Thr17-Ala19-Ala20-mBS and mBS indicated that
most resonances were coincident, thus confirming that the two monomers have a very similar conformation. In the expanded regions reported in Fig. 2 it is evident the presence of three new NH-CH₃ connectivities in the spectrum of Ser₁₆-Thr₁₇-Ala₁₉-Ala₂₀-mBS (black), tentatively assigned to Thr₁₇, Ala₁₉, and Ala₂₀.

The analysis of sequential contacts in the NOESY spectrum allowed the proton assignment of all the spin systems of the hinge residues, which is reported in Table I. The table also reports the proton resonances of the 16–22 region for mBS and for RNase A (28) for comparison. Despite the sequence identity, the chemical shift values of Ser₁₆-Thr₁₇-Ala₁₉-Ala₂₀-mBS show a striking difference with those of RNase A, suggesting that the hinge loop has a different local environment in the two proteins.

Although the hinge peptide was found to be flexible in RNase A too (28), its greatly enhanced mobility in Ser₁₆-Thr₁₇-Ala₁₉-Ala₂₀-mBS clearly indicates that more substitutions external to the hinge region, such as Ser₂₀ replaced by Arg in BS-RNase, play a role in fixing this peptide in RNase A. In conclusion, the disorder, observed for the hinge region of the monomeric derivative of BS-RNase in the solid state (29) and in solution (28), appears to be a feature of the mutants. Moreover, it seems reasonable to assume that similar disorder characterizes the corresponding unswapped dimers, where each subunit shares the global fold of the monomeric derivatives.

Extent of the Swapping—

BS-RNase and its variants in their monomeric form, with cysteines 31 and 32 linked to two glutathione molecules, can be easily converted into dimers by selective reduction of the disulfide bridges followed by air oxidation of the exposed sulfhydryls. The characterization of the swapping process for Ala₁₉-BS-RNase has been already reported (12). Two independent methods have been used to evaluate the

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**TABLE I**

Proton chemical shift of the 15–21 loop residues for mBS, Ser₁⁶-Thr₁⁷-Ala₁⁹-Ala₂₀-mBS and RNase A at pH 5.65, 300 K

| No. of residue | mBS     | Ser₁⁶-Thr₁⁷-Ala₁⁹-Ala₂₀-mBS | RNase A |
|---------------|---------|-----------------------------|---------|
| 15            | Ser NH  | 8.90 Ser NH                 | 8.94 Ser NH  |
|               | Ser HA  | 4.76 Ser HA                 | 4.24 Ser HA  |
|               | Ser HB1 | 3.79 Ser HB1                | 3.87 Ser HB1  |
|               | Ser HB2 | 3.65 Ser HB2                | 3.74 Ser HB2  |
| 16            | Gly NH  | 8.59 Gly HB1                | 8.18 Gly HA  |
|               | Gly HA1 | 4.09 Gly HA1                | 4.27 Gly HA  |
|               | Gly HA2 | 3.87 Gly HB1                | 3.76 Gly HB1  |
| 17            | Asn NH  | 7.98 Asn HA1                | 8.13 Asn HB1  |
|               | Asn HA1 | 4.63 Asn HB1                | 4.20 Asn HB1  |
|               | Asn HB1 | 2.64 Asn HB1                | 4.20 Asn HB1  |
| 18            | Ser NH  | 8.29 Ser NH                 | 8.75 Ser NH  |
|               | Ser HA  | 4.17 Ser HA                 | 4.38 Ser HA  |
|               | Ser HB1 | 3.63 Ser HB1                | 4.05 Ser HB1  |
|               | Ser HB2 | 3.58 Ser HB2                | 4.00 Ser HB2  |
| 19            | Pro HA  | 4.18 Ala NH                 | 8.06 Ala NH  |
|               | Ser NH  | 8.28 Ala HA1                | 7.75 Ala HB1  |
|               | Ser HA  | 4.32 Ala HB1                | 3.88 Ala HA  |
|               | Ser HB1 | 3.78 Ala MB                 | 1.00 Ala MB  |
|               | Ser HB2 | 3.62 Ala MB                 | 1.00 Ala MB  |
| 20            | Ser NH  | 8.41 Ser NH                 | 8.30 Ser NH  |
|               | Ser HA  | 4.36 Ser HA                 | 4.47 Ser HA  |
|               | Ser HB1 | 3.90 Ser HB1                | 3.91 Ser HB1  |
|               | Ser HB2 | ND Ser HB2                  | 3.78 Ser HB2  |
| 21            | Ser NH  | 8.41 Ser NH                 | 8.30 Ser NH  |
|               | Ser HA  | 4.36 Ser HA                 | 4.47 Ser HA  |
|               | Ser HB1 | 3.90 Ser HB1                | 3.91 Ser HB1  |
|               | Ser HB2 | ND Ser HB2                  | 3.78 Ser HB2  |

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**Fig. 3.** Kinetic analysis of the interconversion of BS-RNase and Ser₁⁶-Thr₁⁷-Ala₁⁹-Ala₂₀-BS-RNase. The percentage of M=M form as a function of the incubation time at 37 °C for each dimeric protein as follows: Ser₁⁶-Thr₁⁷-Ala₁⁹-Ala₂₀-BS-RNase, M=M to M=H₁₁₀₀₅ M ( ), Ser₁⁶-Thr₁⁷-Ala₁⁹-Ala₂₀-BS-RNase, M=H₁₁₀₀₅ to M=M ( ), and BS-RNase, M=M to M=H₁₁₀₀₅ ( ). The inset shows the kinetic results up to 600 h.

**Fig. 4.** SDS-PAGE analysis of the divinyl sulfone cross-linking reaction of BS-RNase (lane 1) and Ser₁⁶-Thr₁⁷-Ala₁⁹-Ala₂₀-BS-RNase (lane 2).

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a ND, not determined.
b Overlapped.
swapping tendency of the other mutant.

The first protocol is based on the higher reactivity of the interchain disulfide bridges with respect to the intrachain ones (7). As in the case of the parent protein and of Ala19-BS-RNase, Ser16-Thr17-Ala19-Ala20-BS-RNase folds mainly in the un-swapped form, and in freshly prepared samples the MxM content is about 15%. Fig. 3 displays the course of the M–M to MxM conversion as a function of the incubation time at 37 °C. The process in Ser16-Thr17-Ala19-Ala20-BS-RNase is very similar to that reported for BS-RNase, as it reaches the same 70:30 equilibrium ratio previously found. Both reactions were essentially complete within 2 days, whereas at 4 °C the interconversion was effectively blocked (data not shown).

The extent of swapping on the equilibrium mixtures was also investigated by cross-linking experiments with divinyl sulfone (DVS), followed by SDS-PAGE analysis under reducing conditions. DVS joins covalently the two histidines of the active site (His12 and His119), which belong to the same subunit in M–M and to different subunits in MxM. Under reducing and denaturing conditions, either a product of mass 27,000 Da (from MxM), or a product of mass 13,500 Da (from M–M) is obtained on gel electrophoresis (19). The reaction requires more than 24 h to be completed, thus this method is not suitable to follow kinetically the interconversion. However, it gives for the equilibrium ratio between MxM and M–M a semiquantitative indication, which does not depend on the reactivity of the disulfide bridges. Fig. 4 illustrates the time-dependent course of this reaction for Ser16-Thr17-Ala19-Ala20-BS-RNase in comparison with that of the parent BS-RNase. At any time, the two proteins show the same relative amount of exchanging and non-exchanging forms, thus indicating a very similar equilibrium composition.

**Overall Structures**—For comparison with the native MxM forms of the enzyme, data collection for both mutants was initially carried out at room temperature. Since crystals of Ser16-Thr17-Ala19-Ala20-BS-RNase diffracted at lower resolution (2.5 Å) and the quality of the diffraction data was considerably lower with respect to the other mutant, the data were recollected at 100 K. The final models of Ala19-BS-RNase and Ser16-Thr17-Ala19-Ala20-BS-RNase, containing 96 and 102 solvent molecules respectively, have been refined to a root mean square deviation of 0.171/0.202 and of 0.208/0.259 using diffraction data up to 2.20-Å resolution. A full list of refinement statistics of the two proteins is reported in Table II. In both cases the quality of the electron density maps allowed a detailed description of nearly the whole molecule, with the exclusion of the hinge peptide of one chain of Ala19-BS-RNase.

The overall structure of the two mutants is very close to that of the MxM isomer of the wild-type enzyme (PDB code 1R5D). Fig. 5 illustrates the time-dependent course of this reaction for Ser16-Thr17-Ala19-Ala20-BS-RNase in comparison with that of the parent BS-RNase. At any time, the two proteins show the same relative amount of exchanging and non-exchanging forms, thus indicating a very similar equilibrium composition.

**Table II**

| Crystal data | Ala19-BS-RNase | Ser16Thr17-Ala19-Ala20-BS-RNase |
|--------------|---------------|-------------------------------|
| Space group  | P2₁,2,2       | P2₁,2,2                       |
| Cell parameters |               |                               |
| a (Å)        | 49.50         | 48.96                         |
| b (Å)        | 62.43         | 61.10                         |
| c (Å)        | 51.60         | 51.22                         |
| Data collection |             |                               |
| Resolution limits (Å) | 20.00–2.20 | 20.00–2.20                    |
| Highest resolution shell (Å) | 2.24–2.20 | 2.25–2.20                    |
| No. of observations | 58,421     | 68,829                        |
| No. of unique reflections | 12,492      | 12,548                        |
| Completeness (%) | 93.4 (93.5) | 98.2 (99.5)                   |
| R(f) (%)     | 18 (5)        | 25 (7)                        |
| Average multiplicity | 5           | 5                              |
| Rwork (%)   | 7.2 (24.7)   | 5.7 (24.6)                    |
| Mosaicity   | 0.18          | 0.32                          |
| Refinement results |         |                               |
| Molecules for asymmetric unit | 1           | 1                              |
| Resolution limits (Å) | 20.00–2.20 | 20.00–2.20                    |
| Number of reflections with F>2σ(F) | 9,456 | 11,641                        |
| No. of reflections in working set | 11,167 | 10,439                        |
| No. of reflections in test set | 1,150 | 1,202                         |
| Rwork (%)   | 17.1          | 20.8                          |
| Rfree (%)   | 20.2          | 25.9                          |
| No. of protein atoms | 1,853     | 1,878                         |
| No. of water molecules | 96         | 102                           |
| RMSD from ideal values |         |                               |
| Bond lengths (Å) | 0.006    | 0.008                         |
| Bond angles (*) | 1.24       | 1.57                          |
| Dihedral angles (*) | 24.80    | 25.52                         |
| Improper angles (*) | 0.75      | 0.91                          |
| Average B-factors (Å²) |         |                               |
| Protein, overall | 30.33     | 28.66                         |
| Main chain   | 28.80        | 28.24                         |
| Side chain   | 31.25        | 28.81                         |
| Solvent atoms | 37.45     | 31.48                         |
| Ramachandran plot statistics |         |                               |
| Most favored regions (%) | 90.0     | 85.0                          |
| Additional allowed regions (%) | 10.0   | 14.6                          |
| Generously allowed regions (%) | 10.0    | 0.0                           |
| Disallowed regions (%) | 0.0        | 0.4                           |

Numbers in parentheses indicate values for the highest resolution shell.
RNase and 0.28 Å for Ser^{16}-Thr^{17}-Ala^{19}-Ala^{20}-BS-RNase.

With respect to the native enzyme the RMSD is 0.73 Å and 0.63 Å, respectively. When the comparison is limited to a single structural unit (residues 1–15 of one chain and 23–124 of the partner chain), the RMSD is halved as a result of a small difference in the relative orientation of the two structural units, which is less than 5° in both mutants. These results indicate that the substitutions at the hinge peptide only marginally perturb the quaternary assembly, which is highly constrained by the interchain disulfides and by the swapping. Indeed, even in the absence of the swapping, the quaternary association of the native enzyme, as shown by the structure of native MxM (6), is hardly affected, and this is also expected to hold for the M–M form of the mutants.

In both mutants, the substitution of Asp^{67} of the native sequence with Asp does not modify the structure of the loop in which the residue is inserted, neither the hydrogen bonding network in which the asparagine side chain is involved. Even the nearby side chains of the catalytically important Asp^{121} and His^{119} are not significantly perturbed by the presence of the additional negative charge carried by Asp^{67}.

**Hinge Peptide**—The electron density associated with the hinge region (residues 16–22) of both mutants is significantly less well defined than in the remaining part of the molecules. In the native MxM forms, studied in two different crystal environments (2, 20), and in the complexes with dinucleotides (20, 30), the hinge peptide of the two chains adopts different structures (F and E), both characterized by the presence of a 1–4 β-bend with Pro^{19} in the second position of the turn. The switch between the two structures occurs at Gly^{16}, which adopts either a folded (F) or an extended (E) conformation (Fig. 6). In the F structure, Pro^{19} side chain of one subunit is well placed in a niche lined by the side chains of Tyr^{25} and Gln^{101} of the other subunit. In the E structure the extended conformation of Gly^{16} determines a different orientation of the β-bend and displaces the proline side chain out of the pocket. In this case the turn is somewhat distorted and the distance between the carbonyl of Ser^{18} and the amide nitrogen of Ala^{21} is slightly too long for a good hydrogen bonding interaction. In the crystal form of the wild-type enzyme, isomorphous to that of the present mutants (20), the F conformation is also stabilized by a number of hydrogen bonding interactions with a neighboring molecule, whereas the hinge peptide in the E conformation is relatively free from packing interactions.

As for the present mutants, the hinge peptide of Ala^{19}-BS-RNase corresponding to the E conformation is fully disordered, whereas the second peptide can be confidently traced in the electron density map (Fig. 7a), and its structure approximately resembles that of the F conformation.

Because of the uncertainty in the orientation of the peptide groups, a comparison with the native structure is better grasped in terms of the virtual C^α-C^α+1 bond representation of the chain, which yields an almost unequivocal description of the backbone conformation. Indeed the torsion angles about the virtual bonds, given in Table III, clearly show the similarity of the peptide conformation with the F conformation of the native enzyme. For comparison, the values of τ angles for an unwrapped conformation of the hinge peptide is also given; since this peptide is disordered in both M=M (6) and in the monomeric derivative of BS-RNase (29), the conformational parameters presented in Table III are those of RNase A refined at atomic resolution at pH 5.9 (PDB code 1KF3) (31). The hinge peptide of this protein is also shown in Fig. 6 as a model of the unwrapped conformation.

With respect to native MxM, the 1–4 β-bend, encompassing residues 18–21, is partially disrupted, and the cavity, hosting the wild-type proline side chain, is occupied in the mutant by two water molecules, which bridge through hydrogen bonds the OG of Ser^{18} to OG of Ser^{20} and to OE1 of Gln^{101} of the partner chain. The peptide forms a number of hydrogen bonds with a neighboring molecule, as for the native enzyme, although the specific pattern is slightly modified. The results, therefore, indicate that the replacement of Pro^{19} by Ala considerably increases the flexibility of the hinge peptide, which is fully disordered in one chain and is more ordered in the partner chain, likely because of the stabilizing interactions due to crystal packing.
Surprisingly, in the case of the tetramutant, both peptides present a better defined structure (Fig. 7b) with respect to Ala19-BS-RNase. It should be stressed, however, that in this case the diffraction data were collected at T = 100 K, and it is conceivable that the low temperature may push each peptide to adopt a single conformation as it occurs in the native protein, despite its intrinsic greater flexibility. Also in this case, the conformation of the hinge peptide is different in the two chains and closely resemble the F and E conformations, respectively, of the native enzyme (Table III), showing that the replacement of Gly16 by Ser does not prevent the peptide to assume the E conformation.

As for the hinge peptide, the x-ray data show that this region is more mobile in the MxM dimer of the mutants than in the native enzyme. Therefore, it is reasonable to conclude that the substitutions increase the flexibility of the swapped hinge peptide, thus reducing, with respect to the native protein, the entropy loss which accompanies the M=M to MxM transformation. On the other hand, in the MxM form of the mutants, the disruption of good contacts between the hinge peptide and the protein matrix, and in particular of the stabilizing interactions of the proline side chain within the cavity formed by the side chains of Tyr25 and Gln101, also reduces the enthalpic gain in the same transformation. Thus, the insensitivity of the swapping equilibrium to the substitutions in the hinge region appears to be the result of a subtle balance of enthalpic and entropic effects.

From an evolutionary point of view, the present results clearly contrast the general expectation that the substitutions in the hinge region of the seminal enzyme with respect to RNase A could be related to a greater efficiency of the swapping in the seminal enzyme. Vice versa, they indirectly lend new credit to a recently published hypothesis (32) regarding the role of these substitutions and in particular that of proline in position 19 of the seminal sequence. According to this hypothesis, their major role is associated to the stabilization of a quaternary structure of the swapped non-covalent dimer NCD, i.e. the dimer with the interchain disulfides broken, which is capable to evade the ribonuclease protein inhibitor and is considered responsible for the antitumor action of the enzyme.

Acknowledgments—We thank Dr. Teodoro Tancredi for help and assistance with NMR measurements and M. Amendola and G. Sorrentino for their skillful technical assistance with x-ray measurements. We thank Prof. Alberto Di Donato for the kind use of some laboratory equipments. We thank the CICMF of the University Federico II of Naples for mass spectra analysis.

REFERENCES

1. Schlunegger, M. P., Bennett, M. J., and Eisenberg, D. (1997) Adv. Prot. Chem. 50, 61–122
2. Mazzarella, L., Capasso, S., Demasi, D., Di Lorenzo, G., Mattia, C. A., and Zagari, A. (1993) Acta Crystallogr. Sect. D 49, 389–402
3. Rousseau, F., Schymkowitz, J. W. H., and Itakai, L. S. (2003) Structure 11, 243–251
4. D’Alessio, G., Di Donato, A., Mazzarella, L., and Piccoli, R. (1997) in Ribonucleases: Structures and Functions (D’Alessio, G., and Riordan, J. F., eds) pp. 383–423, Academic Press, New York.
5. Bergolzi, M., Remy, M. H., Cagnon, C., Masson, J. M., and Dumas, P. (1997) Structure 5, 391–401
6. Berusii, R., Sica, F., De Lorenzo, C., Di Fiore, A., Piccoli, R., Zagari, A., and Mazzarella, L. (2003) FEBS Lett. 554, 165–170
7. Piccoli, R., Tamburrini, M., Piccialli, G., Di Donato, A., Parente, A., and D’Alessio, G. (1992) Proc. Natl. Acad. Sci. 89, 1770–1784
8. Crestfield, A. M., Stein, W. H., and Moore, S. (1962) Arch. Biochem. Biophys. 1, 217–222
9. Gotte, G., Bertoldi, M., and Libonati, M. (1999) Eur. J. Biochem. 265, 680–687
10. Liu, Y., Hart, P. J., Schlunegger, M. P., and Eisenberg, D. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 3437–3442
11. Liu, Y., Gotte, G., Libonati, M., and Eisenberg, D. (2001) Nat. Struct. Biol. 8, 211–214
12. Kreidle, C., Avitabile, F. del Vecchio, P., Crescenzi, O., Tancredi, T., and Picone, D. (2003) J. Biol. Chem. 278, 4729–4735
13. Ke, S., and Madison, R. (1997) Nucleic Acids Res. 25, 3371–3372
14. Crescenzi, O., Carotenuto, A., D’Ursi, M. A., Tancredi, T., D’Alessio, G., Avitabile, F., and Picone, D. (2001) J. Biol. Chem. 276, 289–290
15. D’Alessio, G., Malorni, M. C., and Parente, A. (1975) Biochemistry 14, 1116–1122
16. Kunitz, M. (1946) J. Biol. Chem. 164, 563–568
17. Delaglio, F., Grzesiek, S., Vuister, G. Z., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biol. Chem. 270, 727–273
18. Johnson, B. A., and Blevins, R. A. (1994) J. Biol. Chem. 269, 603–614
19. Ciglic, M. I., Jackson, P. J., Raillard, S., I. Haugg, M., Jermann, F., Opitz, J. G., Trabesinger-Ruf, N., and Benner, S. A. (1998) Biochemistry 37, 4008–4022
20. Merlino, A., Vitagliano, L., Sica, F., Zagari, A., and Mazzarella, L. (2004)

**TABLE III**

| Torsion angles (°) about the virtual bond between consecutive Cα atoms of the hinge peptide | Swapped E | Unswapped RNase A |
|---|---|---|
| N1⁰ | N2⁰ | STAA⁰ | N1 | N2 | P19A⁰ | STAA |
| Conformation Cα₁, Cα₃ | | | | |
| 13–16 | −164 | −165 | −168 | −173 | 158 | 131 | 131 | 157 |
| 14–17 | 103 | 94 | 125 | 10 | 31 | 58 | 66 | 76 |
| 15–18 | −116 | −111 | −123 | 96 | 85 | 82 | 70 | 41 |
| 16–19 | 25 | 23 | 5 | −160 | −150 | −118 | −112 | −144 |
| 17–20 | 170 | 150 | 168 | −118 | −142 | −120 | −156 | −15 |
| 18–21 | 43 | 56 | 57 | 57 | 96 | 62 | 72 | 12 |
| 19–22 | 138 | 130 | 136 | 110 | 112 | −159 | 120 | −167 |
| 20–23 | −118 | −132 | −132 | 178 | −172 | 64 | −121 | 18 |
| 21–24 | 70 | 67 | 71 | 67 | 68 | 37 | 69 | −84 |

a N1 is the native BS-RNase crystallized from (NH₄)₂SO₄ (2).

b N2 is the native BS-RNase crystallized from PEG (20).

c STAA stands for Ser¹⁹-Thr¹⁹-Ala¹⁹-Ala¹⁹-BS-RNase.

d P19A stands for Ala¹⁹-BS-RNase. The hinge peptide of one subunit is disordered and could not be modeled.
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21. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
22. Brunger, A. T. (1996) Department of Molecular Biophysics and Biochemistry, Yale University Press, New Haven, CT
23. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
24. Hoof, R. W., Vriend, G., Sander, C., and Abola, E. E. (1996) Nature 381, 272
25. Di Donato, A., and D'Alessio, G. (1981) Biochemistry 20, 7232–7237
26. Catanzano, F., Graziano, G., Capasso, S., and Barone, G. (1997) Protein Sci. 6, 1682–1693
27. Catanzano, F., Graziano, G., Cafare, V., D'Alessio, G., Di Donato, A., and Barone, G. (1997) Biochemistry 36, 14403–14408
28. Avitabile, F., Alfano, C., Spadaccini, R., Crescenzi, O., D'Ursi, A. M., D'Alessio, G., Tancredi, T., and Picone, D. (2003) Biochemistry 42, 8704–8711
29. Sica, F., Di Fiore, A., Zagari, A., and Mazzarella, L. (2003) Proteins 52, 263–271
30. Vitagliano, L., Adinolfi, S., Sica, F., Merlino, A., Zagari, A., and Mazzarella, L. (1999) J. Mol. Biol. 293, 569–577
31. Berisio, R., Sica, F., Lamzin, V. S., Wilson, K. S., Zagari, A., and Mazzarella, L. (2002) Acta Crystallogr. Sect. D 58, 441–450
32. Sica, F., Di Fiore, A., Merlino, A., and Mazzarella, L. (2004) J. Biol. Chem. 279, 36753–36760
33. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
34. Esnouf, R. M. (1997) J. Mol. Graphics 15, 132–134